PROTEIN EXPRESSION AND LOCALIZATION OF PIGMENT EPITHELIUM DERIVED FACTOR IN THE CENTRAL NERVOUS SYSTEM OF THE RAT DURING POSTNATAL DEVELOPMENT

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER NATURWISSENSCHAFTLICHEN FAKULTÄT III-BIOLOGIE UND VORKLINISCHE MEDIZIN- DER UNIVERSITÄT REGENSBURG

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Abbreviations

AMD	Age related macular degeneration
^{o}C	Centigrade
CC	Choroid
cDNA	Complementary DNA
CGN	Cerebellar granule neurons
CNS	Central nervous system
CSF	Cerebrospinal fluid
kDa	kilo Dalton
DAB	3,3'diaminobenzidinetetrahydrochlorid
DNA	Deoxyribonucleic acid
E	Embryonic day
ED1	Ectodysplasin A
g	Gram (10^{-3})
GAPDH	Glycerinaldehyde-3-Phosphate-Dehydrogenase
GFAP	Glial acidic fibrillary protein
Glut-1	Glucose transporter 1
H_2O_{dist}	distilled water
IκB	Inhibitor of NFκB
IgG	Immunglobulin G
INL	Inner nuclear layer
ISL	Inner synaptic layer
l	Liter
MBP	Myelin basic protein
mg	milligram (10^{-6})
ml	milliliter (10^{-6})
mRNA	messenger RNA
NBCL	Neuroblastic cell layer
NeuN	Neuronal Nuclei
NFκB	Nuclear factor κB
µ	micro (10^{-6})

ng	nanogram (10^{-9})
ONL	Outer nuclear layer
OSL	Outer synaptic layer
Ox 42	Macrophage complement receptor-3
P	Postnatal day
PBS	Phosphate buffered NaCl-solution
PCR	Polymerase chain reaction
pH	Negative Logarithm of the H^+-Ion concentration
PEDF	Pigment Epithelium Derived Factor
PFA	Paraform aldehyde
pg	picogram (10^{-12})
PhR	Photoreceptor
rcf	Relative centrifugal force
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
SCL	Sclera
SD	Standard deviation
SN	Supernatant
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

I. Introduction: Pigment Epithelium Derived Factor (PEDF)

PEDF is a multifunctional protein with relevant clinical potential. To date, the protein has been described to have antiangiogenic, neuroprotective and antivasopermeability properties. It has also been proven to have differentiating activity for certain neurons, as well as tumor cells.

I.1. The gene

PEDF is a member of the serine protease inhibitor (SERPIN) super gene family (Steele et al., 1993). Southern blot analysis indicates that there is only one splicing form for the mouse and human gene (Singh et al., 1998; Tombran-Tink et al., 1996). The gene is particularly conserved among primate species, but PEDF sequences have been identified in mammals, birds, amphibians and fish, while no hybridization signals could be detected in lower species such as Drosophila and Caenorhabditis elegans (Tombran-Tink et al., 1996). The human gene spans about 16kb and has 8 exons and 7 introns (see Fig. I.1.1). While the intron-exon organization of PEDF is highly conserved in all investigated species, the intron size considerably varies even within mammals (Barnstable and Tombran-Tink, 2004). The genomic structure of the PEDF gene suggests that PEDF is part of the ovalbumin family of serpins to which ovalbumin and plasminogen activator inhibitor also belong. Like most other serpins, the first exon is non-coding. Structurally, the PEDF gene is rather different from glia-derived nexin/protease nexin-1, the only other serpin with known neurotrophic activity (Tombran-Tink et al., 1996).

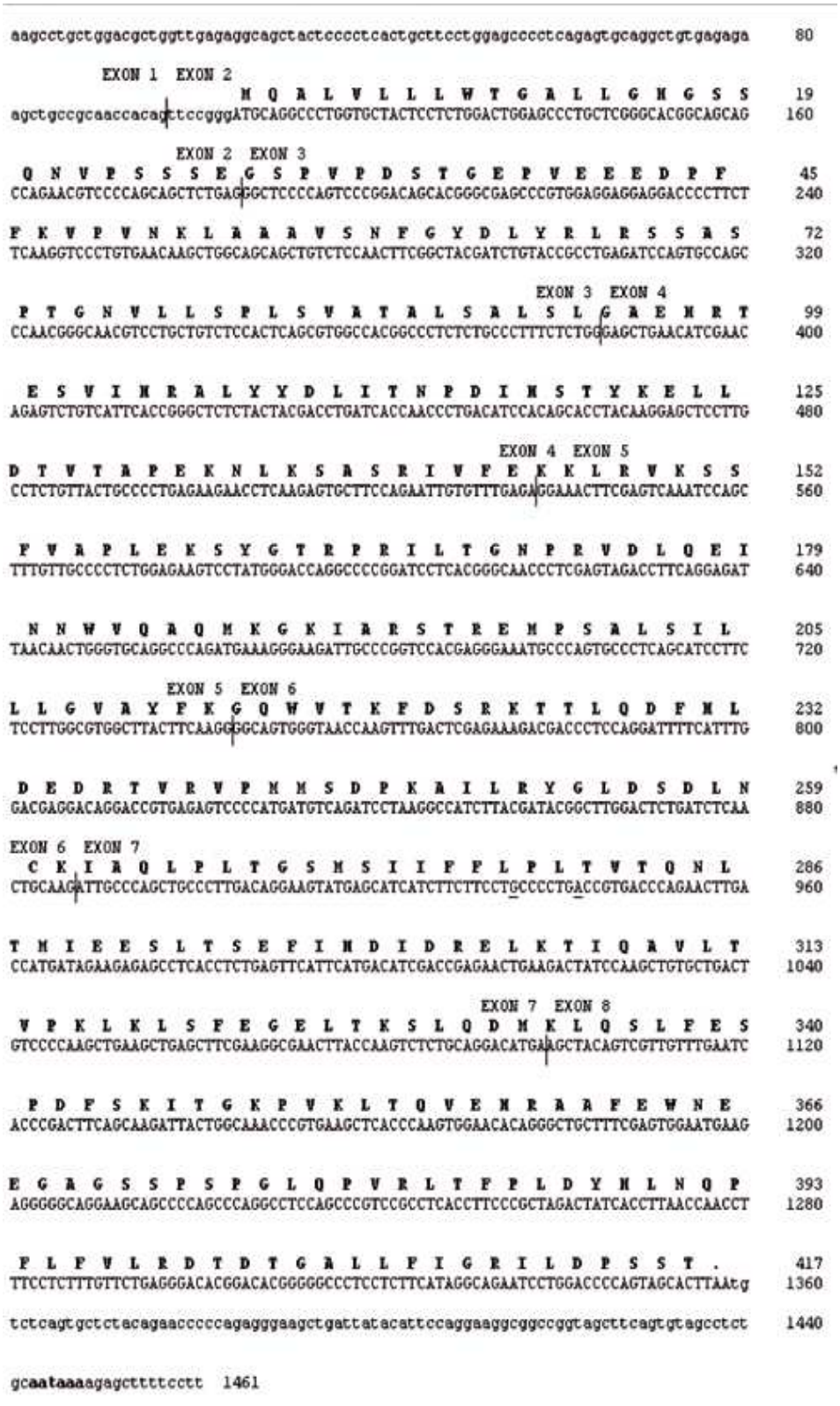

aagcctgctggacgctggttgagaggcagctactcccctcactgcttcctggagccccctcagagtgcaggctgtgagaga 80
EXON 1 EXON 2
M Q A L V L L L W T G A L L G M G S S 19
agctgccgcaaccacagttccgggATGCAGGCCCTGGTGCTACTCCTCTGGACTGGAGCCCTGCTCGGGCACGGCAGCAG 160
EXON 2 EXON 3
Q N V P S S S E G S P V P D S T G E P V E E E D P F 45
CCAGAACGTCCCCAGCAGCTCTGAGGGCTCCCCAGTCCCGGACAGCACGGGCGAGCCCGTGGAGGAGGAGGACCCCTTCT 240
F K V P V N K L A A A V S N F G Y D L Y R L R S S A S 72
TCAAGGTCCCTGTGAACAAGCTGGCAGCAGCTGTCTCCAACTTCGGCTACGATCTGTACCGCCTGAGATCCAGTGCCAGC 320
EXON 3 EXON 4
P T G N V L L S P L S V A T A L S A L S L G A E N R T 99
CCAACGGGCAACGTCCTGCTGTCTCCACTCAGCGTGGCCACGGCCCTCTCTGCCCTTTCTCTGGGAGCTGAACATCGAAC 400
E S V I N R A L Y Y D L I T N P D I N S T Y K E L L 125
AGAGTCTGTCATTCACCGGGCTCTCTACTACGACCTGATCACCAACCCTGACATCCACAGCACCTACAAGGAGCTCCTTG 480
EXON 4 EXON 5
D T V T A P E K N L K S A S R I V F E K K L R V K S S 152
CCTCTGTTACTGCCCCTGAGAAGAACCTCAAGAGTGCTTCCAGAATTGTGTTTGAGAGGAAACTTCGAGTCAAATCCAGC 560
F V A P L E K S Y G T R P R I L T G N P R V D L Q E I 179
TTTGTTGCCCCTCTGGAGAAGTCCTATGGGACCAGGCCCCGGATCCTCACGGGCAACCCTCGAGTAGACCTTCAGGAGAT 640
N N W V Q A Q M K G K I A R S T R E M P S A L S I L 205
TAACAACTGGGTGCAGGCCCAGATGAAAGGGAAGATTGCCCGGTCCACGAGGGAAATGCCCAGTGCCCTCAGCATCCTTC 720
EXON 5 EXON 6
L L G V A Y F K G Q W V T K F D S R K T T L Q D F M L 232
TCCTTGGCGTGGCTTACTTCAAGGGGCAGTGGGTAACCAAGTTTGACTCGAGAAAGACGACCCTCCAGGATTTTCATTTG 800
D E D R T V R V P M M S D P K A I L R Y G L D S D L N 259
GACGAGGACAGGACCGTGAGAGTCCCCATGATGTCAGATCCTAAGGCCATCTTACGATACGGCTTGGACTCTGATCTCAA 880
EXON 6 EXON 7
C K I A Q L P L T G S M S I I F F L P L T V T Q N L 286
CTGCAAGATTGCCCAGCTGCCCTTGACAGGAAGTATGAGCATCATCTTCTTCCTGCCCCTGACCGTGACCCAGAACTTGA 960
T M I E E S L T S E F I N D I D R E L K T I Q A V L T 313
CCATGATAGAAGAGAGCCTCACCTCTGAGTTCATTCATGACATCGACCGAGAACTGAAGACTATCCAAGCTGTGCTGACT 1040
EXON 7 EXON 8
V P K L K L S F E G E L T K S L Q D M K L Q S L F E S 340
GTCCCCAAGCTGAAGCTGAGCTTCGAAGGCGAACTTACCAAGTCTCTGCAGGACATGAAGCTACAGTCGTTGTTTGAATC 1120
P D F S K I T G K P V K L T Q V E N R A A F E W N E 366
ACCCGACTTCAGCAAGATTACTGGCAAACCCGTGAAGCTCACCCAAGTGGAACACAGGGCTGCTTTCGAGTGGAATGAAG 1200
E G A G S S P S P G L Q P V R L T F P L D Y N L N Q P 393
AGGGGGCAGGAAGCAGCCCCAGCCCAGGCCTCCAGCCCGTCCGCCTCACCTTCCCGCTAGACTATCACCTTAACCAACCT 1280
F L F V L R D T D T G A L L F I G R I L D P S S T . 417
TTCCTCTTTGTTCTGAGGGACACGGACACGGGGGCCCTCCTCTTCATAGGCAGAATCCTGGACCCCAGTAGCACTTAAtg 1360
tctcagtgctctacagaacccccagagggaagctgattatacattccaggaaggcggccggtagcttcagtgtagcctct 1440
gcaataaaagagcttttcctt 1461

Fig. I.1.1. Full length sequence of the mouse PEDF cDNA as shown by Singh (1998).

The sequence was obtained from a mouse liver cDNA clone. Exons are labeled and untranslated sequences are shown in lower case while coding regions are in upper case letters. The amino acids are shown in single letter code above the second base of the codon. GenBank accession number AF017057.

The gene has been mapped to the short arm of human chromosome 17p13.3 (Tombran-Tink et al., 1994; Goliath et al., 1996) and is tightly and probably also functionally linked to the locus RP13 for Retinitis Pigmentosa, an inherited retinal degeneration (Goliath et al., 1996; Cayouette et al., 1999). Although deletion of 17p is the most frequent abnormality observed in primitive neuroectodermal tumors of the central nervous system (CNS), no mutations of the PEDF gene have been associated with these tumors (Slavc et al., 1997). However, complete or allelic loss of PEDF in knockout animals was found to be associated with aggressive metastatic tumors (Doll et al., 2003; Guan et al., 2004).

Contrary to the strong conservation of the coding sequences of the gene among species, available data indicate that there is only limited homology among species in their promotor regions (Singh et al., 1998). The PEDF promoter contains a functional retinoic acid receptor element and PEDF gene expression has been shown to be controlled by all-trans-retinoic acid in cell types of various origin (Tombran-Tink et al., 2004). The PEDF gene is strongly expressed in differentiated retinal pigment epithelial (RPE) cells of the retina and barely detectable in undifferentiated RPE cells (Ohno-Matsui et al., 2001). Similarly, a loss of PEDF expression has been found in glioma progression and is associated

with the degree of differentiation of glioblastoma and neuroblastoma (Guan et al., 2003; Crawford et al., 2001). Furthermore, PEDF gene expression is high in quiescent (G0) and actively dividing young cells (Pignolo et al., 1993 and 2003; Tombran-Tink et al., 1995), but low in senescent cells of various origins (Pignolo et al., 1993 and 2003; Tombran-Tink et al., 1995), so that PEDF has also been called early population doubling cDNA-1 (EPC-1). PEDF has been therefore suggested to play a role in the entry of young cells into the G0 state or the maintenance of this state once reached. There is some evidence that the decline of PEDF gene expression in senescent cells involves a change in stability of the PEDF RNA and that this occurs at the posttranslational level (Coljee et al., 2000).

I.2.The protein

Serpins are a protein family with similar, basic globular structure, but wide functional diversity. Human PEDF is a 46-50 kDa monomeric glycoprotein of 418 amino acids (see also Fig. I.1.1.) that was first purified from the conditioned medium of human retinal pigment epithelial (RPE) cells as a factor that induces neuronal differentiation of cultured Y-79 retinoblastoma cells (Tombran-Tink and Johnson, 1989; Tombran-Tink et al., 1991). Although PEDF belongs to the serine protease inhibitor (SERPIN) super gene family (Steele et al., 1993), no inhibitory effect on serine proteases has been found (Becerra et al., 1995; Becerra, 1997). Furthermore, residue position 78-121 (44-mer) that lacks the homologous serpin-reactive loop retains its neuronal differentiation, survival, anti-angiogenic and anti-vasopermeability activities (Becerra et al., 1995; Araki et al., 1998; Dawson

et al., 1999; Liu et al., 2004). Analysis of the crystal structure of the PEDF protein has shown that the distribution of the surface charge is of striking asymmetric distribution and is thought to be of physiological relevance (Fig. I.2.1) (Simonovic et al., 2001). Interestingly, the N-terminal of the bioreactive 44-mer is buried in the protein and the C-terminal forms an exposed loop with a cleft (Barnstable and Tombran-Tink 2004).

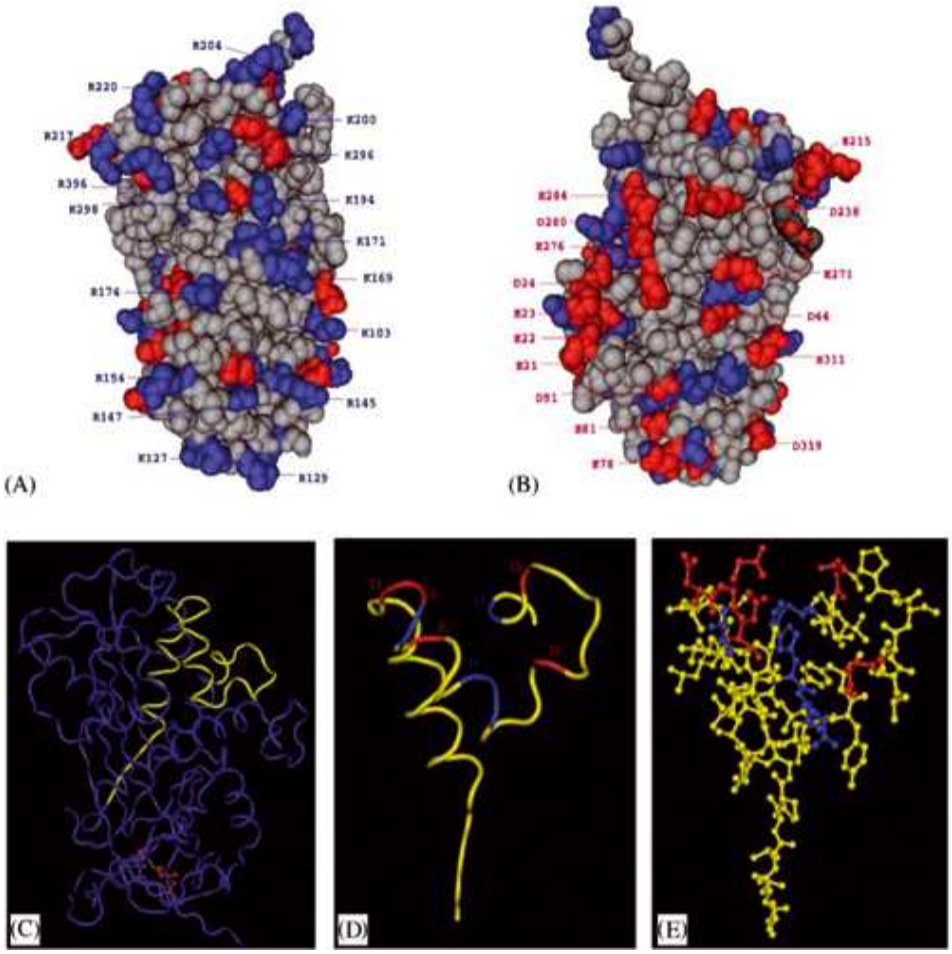


Fig.I.2.1 Crystal structure of PEDF as shown in Barnstable and Tombran-Tink (2004).
(A) A space filling model of human PEDF showing a cluster of positive charges (blue) on one side of the molecule and a cluster of negative charges (red) on the other side of the molecule (B). These charged groups are thought to interact with extacellular matrix molecules. (C-E) The 44-mer peptide shown within the whole PEDF molecule (C), its polypeptide backbone (D) and with the amino acid side chains (E).

Thus far, there are several posttranslational modifications of PEDF, one of which has been shown to change the function of the protein (Fig. I.2.2). Extracellular phosphorylation of PEDF at Ser 24 and 114 by protein kinase CK2 abolishes its neurotrophic activity and enhances the antiangiogenic effect, while intracellular phosphorylation at Ser 227 by protein kinase A reduces its antiangiogenic activity (Maik-Rachline et al., 2004). Furthermore, PEDF has been reported to carry one N-linked glycosylation at Asn 266. Depending on the type of tissue in which it is produced, the N-terminal can be modified to pyroglutamate and is therefore blocked to Edman degradation (Wu et al., 1995; Wu and Becerra, 1996; Petersen et al., 2003).

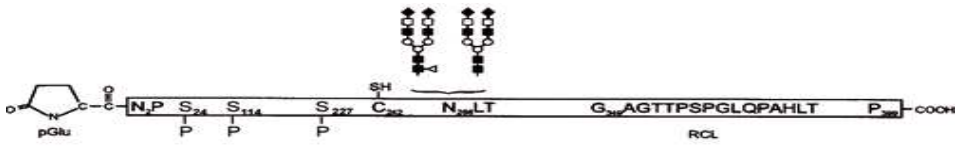


Fig.I.2.2 Adjusted schema from Petersen et al. (2003), depicting the post-translational modifications of PEDF.
The open bar represents mature PEDF. The reactive center loop (RCL) of the serpin structure is indicated. The N-terminal can be modified to a pGlu and Asn 266 can be glycosylated with or without a core fucosilation. In the blood, the sulfhydryl group Cys-242 is free. Ser 24 and 114 can be phosphorylated by protein kinase CK2, while protein kinase A phosphorylates Ser 227.

I.3. Binding of PEDF

To date, no distinct receptor for PEDF has been found and it is not well understood how PEDF exerts its various effects. There are indications that the different functions of PEDF as angiogenic inhibitor and neuroprotective agent may be mediated by PEDF binding molecules/receptors of different size. Thus far, a PEDF-binding molecule of about 80-85kDa has been shown in retinoblastoma Y79 and cerebellar granule cells (Alberdi et al., 1999). The binding of PEDF to Y79 (Kd=3nM) and cerebellar granule cells (Kd=4.5nM) was saturable and specific to a single class of binding molecules with similar affinities. A putative receptor binding site of PEDF protein has been predicted to include the exposed parts of helices C and D and loop 90 (Fig. I.3.1; Simonovic et al., 2001). PEDF was also found to bind to motor neurons (Kd=2.4-18.9nM) and bovine retinal cells (Kd=6.5nM) with similar binding affinity (Bilak et al., 2002; Aymerich et al., 2001), suggesting a homologous protein for the PEDF receptor in these neuronal systems. Pericytes surrounding the vasculature were however shown to have a 62 kDa PEDF binding protein with a dissociation constant of 7.46 nM, which also suggests an interaction with a cellular receptor (Yamagishi et al., 2002). However, it is still not clear whether these proteins are a receptor or a regulatory binding protein that controls the function of PEDF.

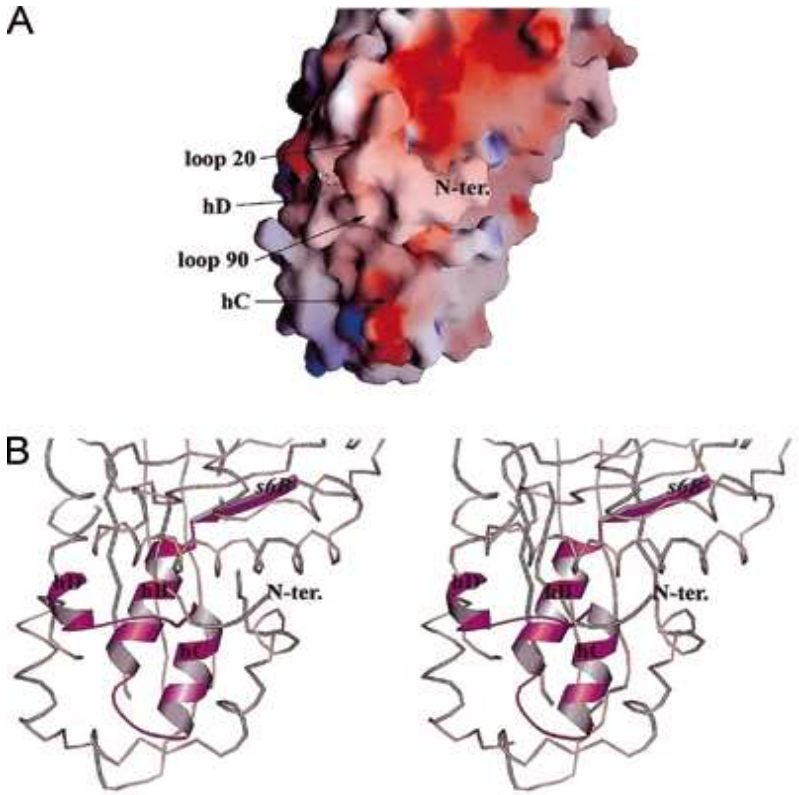


Fig. I.3.1. Crystal structure of the putative receptor binding site of PEDF as shown by Simonovic (2001).
A) A GRASP representation with basic (blue) and acidic (red) side chains and B) stereo view. The binding site based on peptide studies is in magenta with its secondary structure elements displayed as ribbons.

The secreted protein has also been found to bind to extracellular matrix components such as collagen type I and III, heparin, chondroitin sulfates and dextran sulfates in an ionic fashion (Alberdi et al., 1998; Meyer et al., 2002; Kozaki et al., 1998). There is evidence that binding of PEDF to collagen is critical for its antiangiogenic activity (Hosomichi et al., 2005). It was suggested that the heparin and proteoglycan-binding sites are located at the basic surface of PEDF (see Fig. I.2.1). The bioreactive peptide within the protein that confers the neurotrophic function of PEDF is distinct and nonoverlapping from the serpin

exposed loop and the glycosaminoglycan binding site, which are located on opposite sides of the protein (Alberdi et al., 1998).

I.4. Localization and protein expression

PEDF protein has been originally localized in the interphotoreceptor matrix of eyes of various species (Becerra et al., 2004; Behling et al., 2002, Karakousis et al., 2001; Ogata et al., 2002, Ortego et al., 1996; Wu et al., 1995) and exists in basically all compartments of the eye. Apart from the eye, PEDF protein is present in various other organs, biological fluids (vitreous, serum and cerebrospinal fluid (CSF)) and the CNS. However, due to the fast secretion of PEDF, it has been difficult to localize the protein in certain conditions (Kozaki et al., 1998). PEDF mRNA has been found in nearly all adult human organs including the eye, liver, bone marrow, kidney, pancreas, lung, appendix, heart, muscles, placenta, small intestine, colon, stomach, ovary, testis, various brain regions (Tombran-Tink et al., 1996) and several types of blood cells (unpublished data from our laboratory; see Fig. V.1.1.). However, it is not known if cells of these organs in fact produce the PEDF protein or just bind PEDF and which cells of these organs exactly produce and respond to PEDF.

The protein has been also detected in tissues of the embryonic mouse that are rich in collagen I and III, such as teeth, bone, skin and smooth muscle layers of aorta, esophagus and so forth, but not in embryonic brain (Kozaki et al., 1998). There are also conflicting reports about the immunohistochemical detection of PEDF in embryonic mouse liver (Kozaki et al., 1998; Sawant et al.,

2004). In the CNS, thus far PEDF immunoreactivity has been reported only in motor neurons of the ventral horn in the adult human, monkey and rat spinal cord, as well as in ependymal cells (Bilak et al., 1999 and 2002).

I.5. Biological functions

PEDF has been described to have multiple functions. There is plenty of evidence that PEDF serves as a neuroprotective factor for various cells of the CNS (Taniwaki et al., 1995 and 1997; Araki et al., 1998; DeCoster et al., 1999; Houenou et al., 1999, Bilak et al., 1999 and 2002) which will be described in more detail later on. PEDF has been also reported to have differentiating activity for several tumor cells, but also some neuronal cells (Houenou et al., 1999; Crawford et al., 2001; Tombran-Tink and Johnson, 1989). Furthermore, PEDF is a potent antiangiogenic factor that controls the growth of new blood vessels in normal and pathological conditions which will also be discussed in more detail in chapter IV (Tombran-Tink and Barnstable, 2003; Dawson et al., 1999; Mori et al., 2001; Stellmach et al., 2001; Duh et al., 2002). As an antiangiogenic agent, PEDF has been proven to inhibit tumor growth by suppressing tumor vascularisation of various organs (Abe et al., 2004; Mahtabifard et al., 2003; Wang et al., 2003, Abramson et al., 2003). There are also indications that PEDF may have antivasopermeability function (Liu et al., 2004). It has been even speculated that PEDF may play a role in wound healing (Peterson et al., 2003), as well as in organogenesis or morphogenesis (Kozaki et al., 1998).

I.5.1. PEDF in the eye

PEDF protein is located in various cell types of the human, monkey, bovine, mouse and rat retina, (Behling et al., 2002, Karakousis et al., 2001; Ogata et al., 2002, Ortego et al., 1996; Wu et al., 1995) and was shown to exist in basically all compartments of the eye, including the vitreous, RPE, retina, choroid, ciliary body, lens and cornea (Fig. I.5.1.1). Lens and cornea are thought to be the source of PEDF secretion into the vitreous and aqueous humor (Ogata et al., 2002).

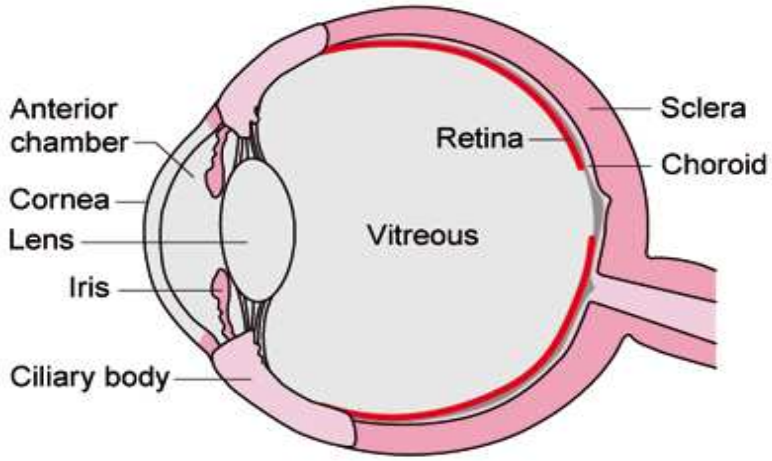


Fig. I.5.1.1 Schematic diagram of the eye as shown in Bouck, 2002.
Avascular tissues are shown in gray and well vascularised tissues in pink/red.

It has been shown that PEDF has differentiating effects on various retinal cell types (Jablonski et al., 2000 and 2001) including the differentiation of albino RPE cells from Wistar rats and the maturation of their pigment granules (Malchiodi-Albedi et al., 1998). There is also evidence that PEDF protects various cell types of the rat retina from different toxic conditions including

phototoxicity, hydrogen peroxide toxicity and ischemic conditions (Cao et al., 1999 and 2001; Ogata et al., 2001a).

Besides its neuroprotective effect, PEDF is one of the most potent natural inhibitors of angiogenesis in the eye (Dawson et al., 1999; Tombran-Tink and Barnstable, 2003). The protein inhibits the migration of retinal endothelial cells *in vitro* (Duh et al., 2002) and induces apoptosis of endothelial cells that are participating in neovascularisation, but does not harm existing vessels (Stellmach et al., 2001). PEDF is probably responsible for the normal avascularity of several ocular compartments and loss of its expression leads to increased ischemia-induced retinal neovascularisation (Dawson et al., 1999). Treatment of mice with systemic or intra-ocular injections of recombinant PEDF results in a significant decrease of choroidal and retinal neovascularisation (Mori et al., 2001; Stellmach et al., 2001; Duh et al., 2002).

PEDF has been shown to play a role in many eye diseases. Thus, the levels of PEDF in the aqueous humor or vitreous of patients with various degenerative eye diseases have been shown to be decreased, such as in choroidal neovascularisation in age related macular degeneration (AMD) (Holekamp et al., 2002), in proliferative diabetic retinopathy and in Rubeosis (Spranger et al., 2001; Boehm et al., 2003a and b), as well as in retinis pigmentosa and glaucoma (Ogata et al., 2001b). PEDF was found to transiently delay the death of photoreceptors cells in the rd/rd and rds/rds mouse models of retinitis pigmentosa (Cayouette et al., 1999), furthermore indicating its

association with this disease. Increased PEDF concentrations have only been found in rhegmatogeneous retinal detachment, where PEDF was speculated to act as a neuroprotective agent for the detached retina (Ogata et al., 2001b and 2002).

I.5.2. PEDF/VEGF relation in the eye

Vascular Endothelial Growth Factor (VEGF) and PEDF share many similar activities. Both growth factors are neurotrophic and neuroprotective, and control vascular permeability and angiogenesis. In the healthy adult eye and most other healthy tissues, no new vessels are being formed due to a finely tuned balance of pro- and antiangiogenic factors. In conditions such as ischemia and oncogenesis this balance is disturbed and leads to neovascularisation.

VEGF is a major mediator of vascularisation in the eye and other tissues and plays an essential role in the development and maintenance of neuronal tissue in the retina (Robinson et al., 2001). But VEGF has been also shown to be a key angiogenic factor in various pathogenic conditions, such as diabetic retinopathy, retinopathy of prematurity and cancer, and initiates angiogenesis by promoting endothelial cell proliferation (Alon et al., 1995).

Most ocular angiogenesis is caused by low oxygen levels resulting into ischemia which have been shown to stimulate the production of angiogenic inducers including VEGF (Keshet, 2001; Gao et al., 2001, Ohno-Matsui et al., 2001). Hypoxia stemming from prolonged diabetes causes retinal vessels to grow into the vitreous and eventually threaten sight (Klein et al., 1994). In older

adults with AMD, vessels in the choroid can grow into and under the retina where they can cause vision loss (Fine et al., 2000). Recently, it has been shown that oxygen levels also control the production of PEDF. When oxygen levels are high, PEDF production rises in cultured cells and retinal tissue, and when oxygen levels are low, PEDF production decreases (Dawson, 1999; Gao et al., 2001, Ohno-Matsui et al., 2001). Although it is clear, that there are various factors involved in retinal neovascularisation, it has been suggested that an unbalanced ratio of VEGF to PEDF is associated with different conditions leading to pathological neovascularisation (Gao et al., 2001; Tombran-Tink and Barnstable, 2003), and the reason for the high sensitivity of some rat strains to neovascularisation after hyperoxia (Fig. I.5.2.1; Gao et al., 2002a). However, it is not known how PEDF influences the signaling cascade of VEGF or whether PEDF influences the VEGF binding or its expression.

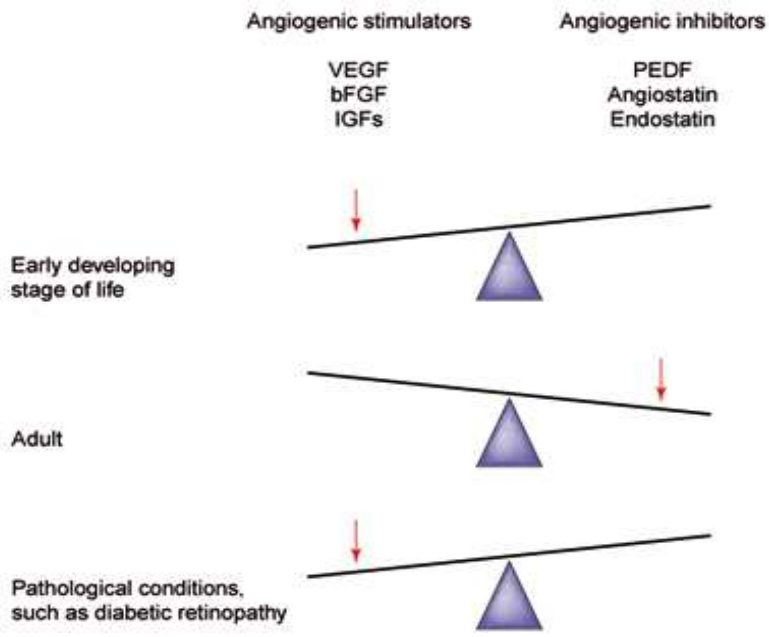


Fig. I.5.2.1. Schematic drawing of different stages of angiogenesis balance as shown in Gao and Ma, 2002. Abbreviations: bFGF, basic fibroblast growth factor; IGF, insuline-like growth factor;

I.5.3. PEDF in the brain

Several studies provide evidence that PEDF does not only have neuroprotective effects on various cell types of the eye, but also on other cells in the CNS (Barnstable and Tombran-Tink 2004). The neurotrophic effect of PEDF on cells of the brain has been demonstrated mainly by *in vitro* experiments by its ability to support neuronal survival, to protect neurons against various neurotoxic effects and in some cases to induce neuronal differentiation. So far, it has been shown that PEDF has survival and/or differentiating effects on primary cultures of rat cerebellar granule neurons (Taniwaki et al., 1995 and 1997; Araki et al., 1998), immature primary rat hippocampal neurons (DeCoster et al., 1999), and

avian and murine spinal motor neurons (Houenou et al., 1999, Bilak et al., 1999 and 2002). Furthermore, the degree of PEDF protein expression has been inversely linked with the malignancy of glioma and neuroblastoma (Guan et al., 2003; Crawford et al., 2001)

To date, there is very little evidence that PEDF protein is produced in cells of the brain, although PEDF mRNA was found in tissue homogenates of various human brain areas (Tombran-Tink et al., 1996). In support of the hypothesis that PEDF may be produced by cells of the CNS is the finding that PEDF protein was proven to exist in CSF (Bilak et al., 1999; Kunck et al., 2002) and is produced by Schwann cells (Crawford et al., 2001). So far, the only immunohistochemical data that might indicate the expression of PEDF protein in the healthy CNS was shown for human, monkey and rat motor neurons and ependymal cells of the spinal cord (Bilak et al., 1999 and 2002).

I.6. Signal transduction of PEDF

To date, there is very little evidence how PEDF exerts its neuroprotective and differentiating effects, as well as its antiangiogenic and antipermeability function. Thus far, three different possible signaling pathways have been identified that are known to regulate pathways that are involved in the control of cell survival, proliferation and cell death (Fig. I.5.3.1). One pathway was discovered in cerebellar granule cells, where the neuroprotective effect of PEDF is mediated by the phosphorylation of IκB, which is an inhibitor of nuclear factor κB (NFκB),

thereby leading to the activation and translocation of NFκB to the nucleus and transcription of anti-apoptotic and neuroprotective genes (Yabe et al., 2001). Interestingly, in neonatal astrocytes PEDF induces the activation of several proinflammatory genes through the activation of NFκB, AP-1-DNA and CREB, indicating that the activation of the same transcription factor can induce a different set of genes in different cell types. A second pathway was found in endothelial cells. PEDF controls the proliferation of these cells only under certain growth conditions and activates the mitogen-activated protein kinase (MAPK) pathway by regulating the phosphorylation of extracellular-signal-regulated kinase (ERK) 1 and 2 (Hutchings et al., 2002). The third PEDF-induced pathway is the Fas-Fas ligand death cascade which was shown to be activated by PEDF in endothelial cells and is associated with the antiangiogenic effect of PEDF (Volpert et al., 2002). However, there are indications that PEDF has additional inhibitory actions in endothelial cells since PEDF still is able to inhibit angiogenesis in Fas or FasL deficient mice (Barreiro et al., 2003).

Thus, many questions remain unanswered about the signaling cascade of PEDF such as if PEDF activates or permits crosstalk with parallel transduction cascades, and if there are different responses to activation of a single pathway, or if there are developmentally regulated PEDF signals and responses (Tombran-Tink and Barnstable, 2003a).

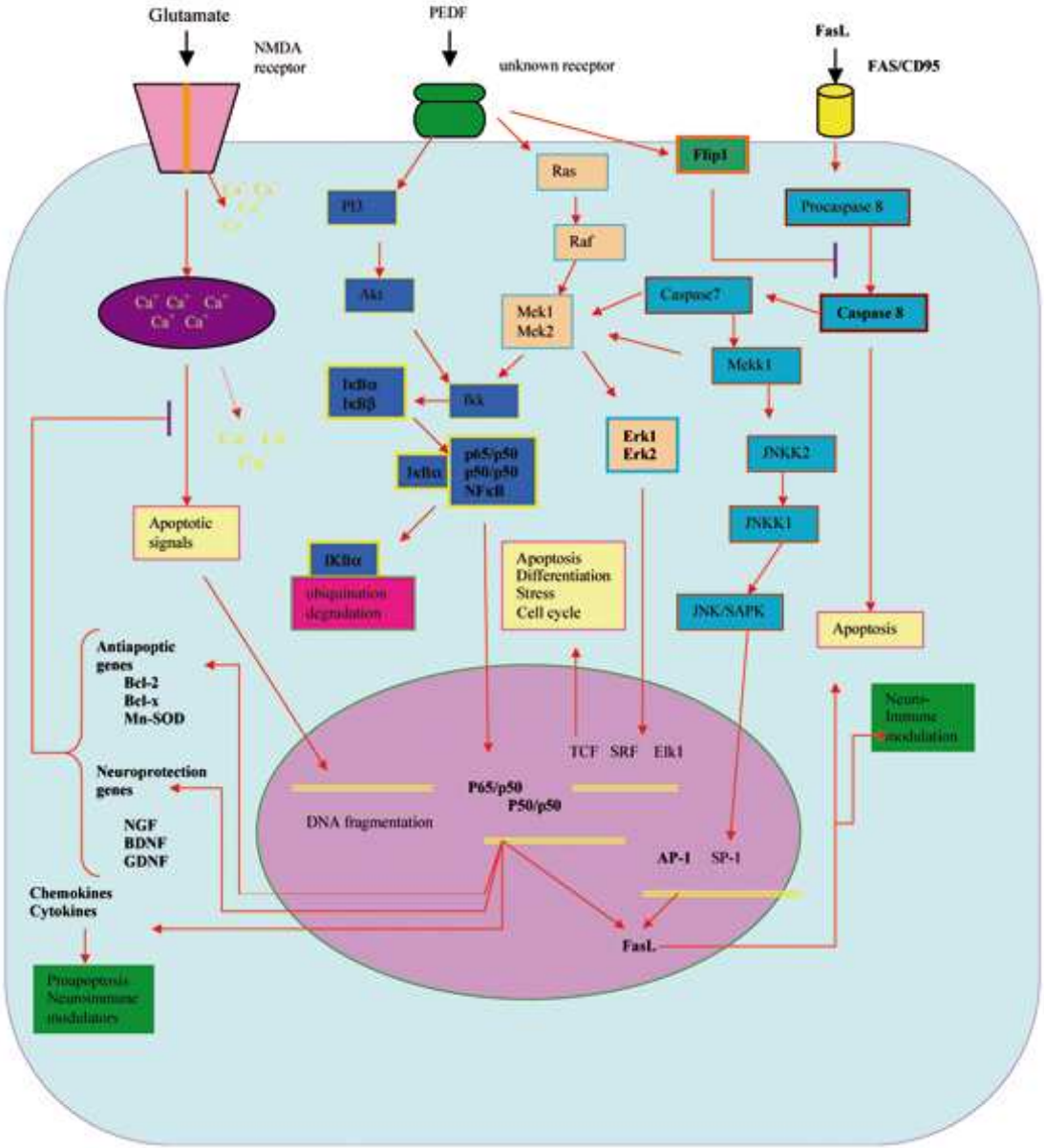


Fig. I.5.3.1 Intracellular transduction pathways activated by PEDF as shown in Barnstable and Tombran-Tink, 2004.

II. Aim of study

II.1. PEDF in the eye

Objective 1:

Since PEDF has been shown to induce the maturation and pigmentation of RPE cells of albino Wistar rats *in vitro,* it will be addressed if albino Wistar and pigmented Long Evans rats differ regarding their PEDF protein content and localization. For that purpose the two rat strains are compared in their PEDF expression and immunolocalization from birth until young adulthood.

Objective 2:

In order to investigate if a change in the PEDF protein expression might be related to the observed retinal degeneration, old albino Wistar rat eyes and retinas with retinal degeneration will be compared to morphologically intact age matched Long Evans eyes in regard to their PEDF content and localization. This question is addressed since old Wistar eyes show signs of retinal degeneration that resemble those of AMD (Sullivan et al., 2002) and choroidal neovascularisation in humans, which are the most common cause for severe visual loss of older patients in developed countries (Fine et al., 2000).

Objective 3:

The relation of PEDF and VEGF protein expression and localization during normal physiological angiogenesis of the rodent eye will be determined from birth until young adulthood. This is to test the hypothesis that during normal

physiological angiogenesis angiogenic stimulators outweigh angiogenic inhibitors similarly as during times of pathological neovascularisation, when the balance between VEGF and PEDF protein expression is disturbed.

Objective 4:

To determine if the VEGF/PEDF ratio changes during postnatal development and during pathological degeneration and neovascularisation of Wistar eyes at old age, old albino Wistar eyes are compared to age matched pigmented Long Evans eyes regarding their VEGF and PEDF content and expression.

II.2. PEDF in the brain

So far, the main research has focused on the antiangiogenic effect of PEDF in the eye where PEDF was originally found. However, very little is known about the localization of PEDF in the brain.

Objective 1:

In order to find out whether PEDF protein is produced by, or bound to particular cells in the brain, the question is addressed if PEDF can be located in the adult rat brain by immunohistochemical means.

Objective 2:

The question to be addressed will be which cell types in the adult rat brain are immunopositive for PEDF.

Objective 3:

To test if age affects the PEDF protein production or binding of PEDF within the brain, the overall PEDF content of the brain, as well as PEDF serum content will be tested at various pre- and postnatal ages, and the PEDF immunolocation will be determined at various times during postnatal development.

III.3. Posttranslational modification of PEDF

PEDF is a multifunctional protein. It has been shown that depending on the type of posttranslational modification of the protein the function of PEDF can change.

Objective 1:

To test if age affects the glycosylation status of PEDF, this work addresses the question whether the posttranslational glycosylation of PEDF changes during postnatal development of the eye.

Objective 2:

To test if the glycosylation status varies between secreted and intracellular PEDF and if it is tissue specific, it will be investigated whether the glycosylation degree changes between cell lysates and conditioned media of cells of various origins.

Objective 3:

To investigate whether the differentiation status affects the glycosylation status of PEDF, it will be tested if the degree of glycosylation changes between differentiated and undifferentiated cells.

III. Materials and Methods

III.1. Materials

III.1.1. Cell culture

Chemical	Source
Accutase	Innovate Cell Technologies, USA
B27 supplement	Gibco BRL, Germany
Collagen type I	Sigma, Germany
DABCO (1,4-diazabicyclo(2,2,2)octane)	Sigma, Germany
Dispase II	Boehringer Mannheim
Dialyzed fetal calv serum (dFCS)	PAA, Germany
DNase I	Worthington Biochemicals
DMEM high glucose	Bio Whittaker
DMEM/F12	Boehringer, Germany
Dulbecco's phosphate buffered saline (PBS)	Sigma, Germany
Epidermal Growth Factor (EGF)	R&D Systems
Fibroblast growth factor (FGF)	R&D Systems
FCS	PAN, Germany
Fungizione	PAA, Germany
Glutamax	Gibco BRL, Germany
Glutamin	Sigma, Germany
Glycerol	Merck, Germany
Heparin	Sigma, Germany
Hanks balanced salt solution without Mg/Ca	PAN, Germany
Hiberante A	Gibco BRL, Germany
Laminin	Sigma, Germany
N2 supplement	Gibco BRL, Germany
Neurobasal medium	Gibco BRL, Germany
Polyvinylalcohol	Sigma, Germany
Tris	UBS

III.1.2. Enzymatic digestion

Chemical	Source
Recombinant N-glycosidase F	Roche, Germany
Sialidase/Neuraminidase from Clostridium perfringens	Roche, Germany

III.1.3. Western blotting

Chemical	Source
AEBSF(Aminoethylbenzenesulfonyl Fluorid)	Sigma, Germany
Acrylamid	BioRad
Bio-Rad DC protein assay	Hercules, CA
Chemoluminescent ECL detection reagent	Amersham Biosciences, England
Complete protease inhibitor cocktail tablets	Roche, Germany
Human recombinant PEDF	BioProducs, USA
Hyperfilm	Amersham Biosciences, England
Mercaptoethanol	Sigma, Germany
Nitrocellulose membrane Protran	Schleier&Schuell, Germany
Ponceau S	Sigma, Germany
Precision Plus Protein Standard	BioRad
Restore Western Blot stripping	Pierce
Tris-Glycine SDS Buffer	BioRad
Tween 20	Sigma, Germany

III.1.4. Immunodetection

Chemical	Source
Avidin-biotin complex, Vector stain ABC-HRP	Vector Laboratories, USA
Bovine serum albumin (BSA)	PAA
DAB (3,3'diaminobenzidinetetrahydrocholrid)	Vector Laboratories, USA
Goat preimmune IgG	Sigma, Germany
Human recombinant PEDF	BioProducs, USA
Hydrogen peroxide	Merck, Germany
2-Methylbutane	Aldrich, Germany
Mouse IgG	Sigma, Germany
Neo-Mount mounting medium	Merck, Germany
Paraformaldehyde	Aldrich, Germany
Peroxidase substrate kit	Vector Laboratories, USA
Rabbit IgG from serum powder	Paraformaldehyde
Rat VEGF165	R&D Systems
Sucrose	Sigma, Germany
Super Frost Plus glass slides	Menzel, Germany
Tissue-Tek Diatec	Sakura Finetek, Europe B.V.
Triton-X100	Sigma, Germany

III.1.5. Polymerase chain reaction

Gene Ruler 100bp DNA Ladder Fermentas	MBI
100bp Leiter Plus	PeqLab
NuSieve Agarose	Biozym
Random Primer	Roche
Superscript II	Invitrogen

III.1.6. Antibodies for Western blotting

Primary antibodies:	**dilution**	**Source**
rbt pcl α human PEDF	0.07 µg/ml	BioProducs Maryland, USA
gt pcl α ms PEDF	0.025 µg/ml	R&D Systems
rb pcl α VEGF A20	0.6 µg/ml	Santa Cruz, USA
mcl ms α - b-actin	1.5 µg/ml	Sigma, Germany
Secondary antibodies:		
preads. gt α-rb IgG peroxidase	1:300	BioScource-International
preads. dk α-gt IgG peroxidase	1:300	Jackson ImmunoResearch
preads. dk α-ms IgG peroxidase	1:300	Jackson ImmunoResearch

III.1.7. Antibodies for Immunohistochemistry

Primary antibodies:	**dilution**	**Source**
rbt pcl α PEDF	20 µg/ml	BioProducs Maryland, USA
rbt pcl α VEGF A20	20 µg/ml	Santa Cruz, USA
gt pcl α Glut-1 C20 (Glucose transporter 1)	1:200	Santa Cruz, USA
ms mcl α GFAP (Glial acidic fibrillary protein)	1:600	DAKO, Denmark
ms mcl α MBP (Myelin basic protein)	1:400	Chemicon, USA
ms mcl α NeuN (Neuronal Nuclei)	1:400	Chemicon, USA
ms mcl α ED1 (Ectodysplasin A)	1:300	Serotec
ms mcl α Ox 42 (macrophage complement receptor-3)	1:50	Serotec
gt pcl α vWF (von Willebrand Factor)	1:200	Santa Cruz, USA
sh pcl α vWF (von Willebrand Factor)	1:100	Serotec

Secondary antibodies:

dk-anti-gt IgG- Fluorescein (FITC)	1:400	Dianova
dk α rbt Rhodamine Red (RHOX)	1:400	Dianova
dk α gt Rhodamine Red (RHOX)	1:300	Dianova
dk-anti-ms IgG- Fluorescein (FITC)	1:200	Dianova
dk-anti-ms IgG-Rhox	1:100	Dianova
dk anti sh IgG Fluorescein (FITC)	1:350	Dianova
dk-anti-rbt-IgG(HL)Biotin-SP	1:300	Jackson ImmunoResearch

III.1.8. Solutions

Differentiation medium for RPE culture	Neurobasal medium, 1% FCS, B27 (50x), Pen/Strep, glutamin;
Digestion medium for cerebllear granule cells	Hiberante A, 2% (w/v) papain, 1% (w/v) DNase
Growth medium for cerebellar granule culture	DMEM/F12 medium, 0.2 M KCl, 0.2 M glutamin, Pen/Strep, 1% dFCS, N2 supplement;
Growth medium for RPE culture	Neurobasal medium, 20 ng/ml FGF, 20 ng/ml EGF, 20 ng/ml Heparin, B27 (50x), Pen/Strep, glutamin
Paraformaldehyde (4%)	dissolve 40 g paraformaldehyd in 800 ml H_2O_{dist} at 60°C while stirring and add 10 N NaOH until solution is clear; add 100 ml 10x PBS and fill up to 1 l with H_2O_{dist}; filter solution
PBS	dissolve 2 g KCl, 2 g KH_2PO_4, 80 g NaCl, 14.4 g $Na_2HP0_4x2H_2O$ in 800 ml H_2O_{dist} and adjust to 1 l; filter
Phosphatbuffer	154.8 ml 0.5 M Na_2HPO_4 and 45.2 ml 0.5 M NaH_2PO_4 fill up to 1 l with H_2O_{dist}

PPD digestion solution for RPE culture	2 g/100 ml papain**,** 100 mg/100 ml dispase II, 10 mg/ml Dnase I, 12.4 mM $MgSO_4$ in Hanks balanced salt solution without Mg/Ca;
PVA-DABCO	mix 12 g glycerol with 4.8 g polyvinylalcohol over night at room temperature; add 24 ml Tris pH8, heat for 30 minutes at 50^{o}C; DABCO; add 2.5 w/v DABCO, mix; centrifuge at 3000 g for 15 minutes; store at -20^{o}C;
RIPA buffer	0.1 M PBS, pH 7.4, 1% nonidet p40, 0.1% SDS, 0.5% sodium deoxycholate;
SDS Sample buffer	2 ml 0.625 M Tris/HCl pH 6.8, 0.2 g SDS, 5 ml glycerol, 0.5 ml β-mercaptoethanol, 0.1 ml bromophenol blue (1% in ethanol), 2.4 ml H_2O_{dist};
Sucrose solution	dissolve 150 g sucrose in 400 ml 0.1 M PO_4 and adjust to 500 ml with H_2O_{dist}; filter;
Western blocking solution	0.1 M PBS, pH 7.4, 0.1% Tween 20. 5% dry skim milk;
Western washing solution	0.1% Tween in PBS

III.1.9. Equipment and Instruments

Adobe Photoshop software, version 7.0	Adobe Systems, USA
AnalySIS software	Soft Imaging System GmbH, Muenster, Germany
Cell incubator	Heraeus, Germany
Culture hood	Cytair, Labortechnik Dunn, Germany
HP Precision Scan software 3.03	Adobe Systems, USA
ULT Freezor	Thermo Forma, Germany
Maxigel chamber	Biometra, Germany
Olympus BX51 microscope	Olympus, Germany
Photodocumentation for Ethidium bromide gels	LTF, Labortechnik, Germany
Thermocycler mastercycler gradient	Biozym, Germany

III.2. Methods

III.2.1. Animals

Experiments in this study were performed in accordance with the German guidelines for the use of experimental animals. Wistar and Long Evans rats were originally purchased from Charles River (Sulzfeld, Germany) and then bred in the animal facility of the University Clinic of Regensburg. Animals were housed under normal ambient light conditions on a 12-hour light-dark cycle and had free access to food and water. Rats were mated over night and the following day was taken as embryonic day (E) 0. Postnatal day (P) 0 was taken as the first 24 hours after birth. Animals were sacrificed with intraperitoneal injections of an overdose of sodium pentobarbital (200 mg/kg).

III.2.2. Western Blot analysis

To measure the PEDF and VEGF content of complete eyes, male Long Evans and Wistar rats were sacrificed at the following ages and n homogenates produced: P0 (7n Long Evans, 6n Wistar), 2 weeks (8n Long Evans, 10n Wistar) and 1 month (9n Long Evans, 10n Wistar), 2 months (9n Long Evans, 9n Wistar), 3 months (8n Long Evans, 9n Wistar), 8 months (9n Long Evans, 9n Wistar), 15-16 months (6n Long Evans, 4n Wistar) and 20-22 months (3n Long Evans, 3n Wistar). Complete eyes were rapidly enucleated, cleaned of surrounding tissue on an ice block, frozen in a mixture of dry ice and 2-methylbutane and stored at -80^{o}C until further processing**.** At the same time, complete brains were removed, shortly rinsed in PBS and frozen in the same way as eyes. The following number of brain homogenates were produced: embryonic day (E)15 (12n Long Evans, 11n W), E18.5 (8n Long Evans, 10n Wistar), P0 (7n Long Evans, 7n Wistar), P14 (5n Long Evans, 8n Wistar), 1 month (5n Long Evans, 5n Wistar), 2 months (5n Long Evans, 5n Wistar), 3 months (6n Long Evans, 5n Wistar), 7-8 months (8n Long Evans, 5n Wistar), 4n 15-22 months Long Evans and 2n 16-24 months Wistar**.** To receive serum, blood was collected from 34 Long Evans animals and sedimented for 20 minutes at 13000 rcf after coagulation.

Frozen eyes and brains were weight, minced with a razorblade in the presence of the appropriate amounts of ice-cold RIPA buffer (3 ml/g tissue) that was supplemented with protease inhibitors, homogenized with a manual pestle and triturated several times with a 20 or 23 gauge needle attached to a 2 ml syringe. Tissue homogenates were centrifuged at 10000 g for 20 minutes at 4^{o}C

and supernatants were removed and resedimented for an additional 20 minutes to yield solubilized extracts. Protein concentrations were determined with a Bio-Rad DC protein assay and homogenates stored in small aliquots at -80^{o}C to avoid repeated freeze-thaw cycles.

To test the protein content of different compartments in the eye, male or female Long Evans and Wistar rats were sacrificed at 6 weeks (4 animals per homogenate) and 18-19 months (2 animals per homogenate). Their eyes were quickly dissected on an ice block and the tissue pooled for homogenization. Eyes were cleaned of surrounding tissue and rinsed in RIPA buffer with protease inhibitors. After removal of the lens and any remaining aqueous liquor, the eyecup was placed into a new dish and the viscous vitreous was pulled off the retina with curved fine forceps and the retina was collected. The remaining RPE cell layer was collected together with the choroid. Protein homogenates of tissues were performed in triplicate as described above**.**

Changes in protein expression patterns were analyzed by Western blotting. Fifty µg of each sample were reduced in an SDS-Sample buffer containing 14.4 mM mercaptoethanol, resolved on a 1mm thick 10% SDS polyacrylamide gel in a cooled Maxigel chamber and then transferred onto a nitrocellulose membrane. To test for equal loading and transfer of samples onto the membrane, the membrane was shortly stained with 0.1% Ponceau S and scanned into the computer (HP Precision Scan software 3.03). To block

nonspecific binding, membranes were placed for one hour at room temperature in blocking solution. Blots were incubated in blocking solution over night at 4^{o}C with antibodies against purified human or mouse PEDF or a peptide mapping to the amino terminal of human VEGF (Santa Cruz, USA). Membranes were washed in 0.1% Tween-PBS three times for 15 minutes and incubated with the appropriate preadsorbed peroxidase linked secondary antibody for one hour at room temperature and washed three times for 15 minutes. Blots were activated with a chemo luminescent ECL detection reagent and exposed several times to Hyperfilm. As a positive control, and to calculate the PEDF and VEGF content, at least three different concentrations of human recombinant PEDF or rat VEGF165 between 2.5 and 60 ng were loaded on each gel. The same membranes were stripped and reblotted with a mcl ms α-β-actin antibody. As a negative control, primary antibodies were omitted and films were exposed for equivalent times as with specific antibodies, which showed no signal (not shown). The signal strength of PEDF and VEGF protein was calculated by digitizing the signals and analyzing their density using a NIH image 1.63 software system. Signals were then normalized to the ones of β-actin. Since homogenates of whole eyes were used, the strength of the actin signal decreased with increasing age of the animal due to an increasing ratio of liquid filled compartments in the retina (see Fig. IV.1.1 and Fig. IV.2.1). Therefore, the actin signal of the two strains was averaged at each developmental age and the PEDF signal normalized to the actin content of the respective age. Brain signals were normalized to the average β-actin signal

of all ages. To determine the amount of PEDF and VEGF in homogenates, values were compared to their correspondent standard curve.

III.2.3. Enzymatic digestion of proteins with N-Glycosidase F

For deglycosilation of proteins, a mixture of 50 µg of protein homogenate and 0.1% SDS was heated to 100^{o}C for 5 minutes to denature proteins. Trition X was added to a final concentration of 0.75%, as well as 4 units of N-Glycosidase F and the reaction micture was incubated over night at 37^{o}C in a water bath. After addition of the appropriate amount of loading buffer, the proteins were denatured again at 100^{o}C for 3 minutes before loading onto the gel. Western blots were processed as described above.

III.2.4. Enzymatic digestion of proteins with Sialidase

For desialylation of proteins, a mixture of 50 µg of homogenate and 500 mM Sodium phosphate solution of pH 6.0 was prepared and 0.25 units of Sialidase added. The reaction mixture was incubated over night at 37^{o}C in a water bath and stopped by heating the samples for 5 minutes at 100^{o}C. Western blots were processed as described above.

III.2.5. Retinal pigment epithelial cell cultures

Retinal pigment epithelial cultures were performed as previously described by Engelhardt and colleges (2005). Six 5-week old Long Evans rats were sacrificed. Eyes were enucleated, cleaned and placed in sterile PBS. The RPE layer was collected in PBS and centrifuged at 188 rcf at 4^{o}C for 10 minutes. The

tissue was then minced with a scalpel in the presence of a few drops of PPD digestion solution and further incubated in 5 ml of digestion solution for about 30-45 minutes at room temperature and triturated every 10 minutes. The digested tissue was centrifuged for 10 minutes at 188 rcf at 4 ^{o}C, triturated and washed two to three times in cold Neurobasal medium containing B27 supplement, Penicillin/Streptomycin and 2 mM glutamine until the tissue was broken down. Cells were taken up in 2-4 ml of growth medium without serum and placed in a plastic dish and incubated at 37^{o}C for 2 days. After 2 days spheres formed which were collected, centrifuged and taken up in twice the amount of growth medium and plated onto collagen type I (50 µg/ml) coated plastic dishes. Collagen caused the spheres to attach and grow out. Cultures were fed twice a week by performing a 50% medium change. For passaging of cells, 200 µl of accutase was added to the plate and incubated for several minutes at 37^{o}C until cells detached from the substrate. The collected cells were then centrifuged, washed with Neurobasal/B27 medium and plated in twice the amount of growth medium. Differentiation of the cells was achieved by removal of growth factors and addition of 1% FCS.

Differentiated and undifferentiated cell cultures were used for homogenization at passage 5. For that purpose, cultures were rinsed once with cold PBS, a small amount of RIPA buffer with protease inhibitors added for perforation of the membranes, and titurated with a yellow pipette tip. Cell lysates were centrifuged once at 10000 g for 20 minutes at 4^{o}C and supernatants removed. Protein concentrations were determined with a Bio-Rad DC protein

assay and cell lysates and conditioned media stored -80^{o}C in order to test their PEDF content by Western blot analysis. For immunohistochemical stainings, cells of passage 5 were plated onto glass cover slips that were coated with polyornithin (100 µg/ml) and laminin (5 µg/ml) and fixed for 10 minutes with 4% PFA.

III.2.6. Cerebellar granule cell cultures

Four male 7-day old Wistar rats were decapitated. The cerebellum was removed under sterile conditions and meninges and large blood vessels removed in a medium of Hibernate A and B27. The obtained tissue was minced in small pieces, transferred into a digestion medium and agitated at 30^{o}C for about 30 minutes. After settling of the tissue, the supernatant was discarded and the tissue triturated in Hibernate A and B27. Cells were centrifuged at 282 rcf for 10 min at 4^{o}C, taken up in growth medium and plated on polyornithine covered plastic dishes. For immunohistochemical staining, cells were plated on glass cover slips that were coated with polyornithine and laminine. Cells were fed every three days by performing a partial medium exchange. After 5 days in culture, cells were rinsed with PBS and used for homogenization and their conditioned media saved in order to test for PEDF. Cell Lysates were prepared as describes for RPE cell cultures.

III.2.7. Fibroblast cell culture

Fibroblast cultures were a kind gift of Lars Dreesman, Neurology, Naturwissenschaftliches und Medizinisches Institut Reutlingen. In short, ten small sections of skin from the back of neonatal green fluorescing CD rats were placed on the surface of plastic culture dishes for 10 minutes at 37^{o}C to allow skin fibroblasts to adhere to the dish. Cells were cultured in high glucose-DMEM medium containing 10% fetal bovine serum (FBS), penicillin/streptomycine, L-glutamine for 7 to 10 days at 37^{o}C and passaged by digestion with 0.1% trypsin and 0.02% ethylendiamine tetraacetic acid in PBS. For immunohistochemical staining, cells of passage 6 were plated on laminine coated glass cover slips for 2 days and fixed for 10 minutes with 4% PFA. For homogenization, cells of passage 6 were cultured for 2 days without FBS, and dissolved in Ripa buffer containing protease inhibitors, centrifuged for 20 minutes at 10000 g at 4^{o}C and stored at -80^{o}C.

III.2.8. Hepatocyte cell culture

Human hepatocytes were a kind gift of Dr. Thomas Weiss, Department of Surgery**,** University Clinic of Regensburg. Primary hepatocytes were isolated from adult human liver and cultured as described by Weiss and colleges (2003). In short, hepatocytes were cultured on collagen in DMEM medium containing 100 mU penicillin, 100 µg/ml streptomycin, 10 ng/ml glucagon, 125 mU/ml insulin and 60 ng/ml hydrocortisone without serum. For experimental use, cells were cultured

for 2 days, their conditioned media saved for PEDF analysis and cells were prepared for homogenization as described above.

III.2.9. Immunohistochemistry

For immunohistochemical stainings of eyes, tissues were prepared as following: deeply anesthetized animals were first perfused intracardially with 0.9% NaCl, and then with fresh 4% PFA. Eyes were enucleated, placed in 4% PFA over night at 4^{o}C and then equilibrated in a 30% sucrose solution until the next day. Eyes were cleaned of surrounding tissue and part of the cornea and the lens were removed. The remaining eye cup was embedded in Tissue-Tek and frozen in a mixture of 2-methylbutane and dry ice. Several eyes of the same age from both strains or one eye of each developmental age of both strains were frozen in the same Tissue-Tek block. Cryosections of 8 µm were collected on Super Frost Plus glass slides and neighboring slices were stained to detect PEDF and VEGF protein. In total 5x P0, 5-6x P14, 3x 3 month and 3x 15-17 month old Wistar and Long Evans rat eyes were stained and analyzed.

For immunohistochemical studies of brains, the brains of deeply anesthetized male Long Evans were removed and frozen in a mixture of dry ice and 2-methylbutane and placed in Tissue-Tek. In total, brains from 7x P0, 5x P7, 6x P14, 6x P28, 6x 3 months and 7x 15-17 months old male Long Evans animals were processed. Brain cryosections of 7 µm were collected on Super Frost Plus glass slides, dried for 2 hours and fixed with fresh 4% PFA up to 2 hours. After

washing with 0.1% Triton X 100 in PBS cryosections were stained on the same day to detect PEDF protein.

All eye and brain sections were first incubated in 3% milk and 0.1% Triton X-100 in PBS for 60 minutes at room temperature to block nonspecific binding. Each specific antibody was diluted in blocking solution and incubated overnight at 4^{o}C in a humidified chamber. Slides were washed three times for 15 minutes with PBS plus 0.1% Triton X-100. For DAB stainings, endogenous peroxidase activity was blocked for 30 minutes in 0.3% hydrogen peroxide in washing solution. Sections were then incubated with peroxidase labeled secondary antibody for at least two hours at room temperature in a humidified chamber, washed three times, incubated for 1-2 hours with an avidin-biotin complex and developed with a commercially available DAB peroxidase substrate kit. Slides were mounted using Neo-Mount mounting medium.

For double labeling, fluorescent staining was performed. Blocking and washing solutions were the same as described above. While eyes were incubated over night at 4^{o}C in the dark with a combination of employed primary antibodies, brains were first stained for rbt pcl α-PEDF on the first day and on the second day with the other primary antibody of choice in 1% BSA. After incubation of each primary antibody, slices were washed 3 times and incubated with the appropriate fluorescing secondary antibody. Cryosections were washed and mounted with PVA-DABCO.

Control slides were performed in parallel with actual stainings and were either treated as following: omitting the primary antibody, substituting the primary

antibody with preimmune IgGs at the same concentration of the corresponding primary antibody and preadsorption of the anti-PEDF antibody with an excess of PEDF of 100 times its molarity at 4^{o}C over night and centrifugation for 30 minutes at 20000 g. Tissues were analyzed with transmitted light (bright field) or fluorescent light using an Olympus BX51 microscope. Images were taken directly from the microscope to a computer image system (analySIS). Similar areas of the eye of both rat strains were used for stainings as well as pictures. Processing of images of stainings and their controls was performed in parallel and was performed using Adobe Photoshop software which included adjustment of brightness and contrast to achieve similar background levels and scaling to final size.

III.2.10. Measurement of retinal ganglion cell staining intensity

For measurement of retinal ganglion cell (RGC) staining intensity, eyes of 3 different Long Evans and Wistar animals at P0, P14, 3 months and 15-17 months were stained in parallel for PEDF using DAB. Images of RGC's were taken with the same camera and light settings and the staining intensity of 20 RGC's per animal was measured using the software ImageJ 1.33U. Obtained values were adjusted for background absorption outside the stained retina. For statistical purposes data from Long Evans and Wistar eyes were combined.

III.2.11. Statistical Analysis

All results are expressed as the mean ± standard error of the mean (SEM). To determine if age had a significant effect on the PEDF or VEGF content of eyes, brains, serum or the staining intensity or RGC's, first a Kruskal-Wallis test was performed. If the Kruskal-Wallis test showed a significant effect of age, then a Mann-Whitney-U test was used to test if there were differences in the expression of PEDF and VEGF between Wistar and Long Evans strains at each postnatal age. A Spearman rank correlation test was employed to determine the changes in PEDF and VEGF protein expression over time. Differences were considered statistically significant when $P < 0.05$.

III.2.12. Polymerase chain reaction (PCR)

cDNA from various human blood cells was a kind gift of Dr. Michael Rehli and Dr. Sven Heinz**,** Department of Hematology, University Clinic of Regensburg. For PCR of the PEDF gene a Taq PCR Master Mix was employed. The following primers were used for the detection of human PEDF mRNA: 5'-tat gac ttg atc agc agc cc-3' (forward) and 5'-agc ttc atc tcc tgc agg ga -3' (reward) which yield a 663 bp long product, and of GAPDH mRNA: 5'- ggt cgg tgt gaa cgg att tg -3' (forward) and 5'-ggt cgg tgt gaa cgg att tg-3' (reward) which yield a 318 bp long product. PCR amplifications were performed for 30 cycles. Each cycle included denaturation at 94^{o}C for 45 seconds, annealing of primers at 61^{o}C and extension of primers at 72^{o}C for each 1 minute. PCR products were separated on a 1.5%

agarose gel which contained 0.5% ethidium bromide and specific DNA bands were examined under an ultraviolet transilluminator.

IV. PEDF protein content and localization in the eye and its comparison to VEGF.

IV.1. Is there a difference in the PEDF content or protein location between pigmented and albino rat eyes indicating that PEDF might be related to the albino phenotype?

Introduction:

In the eye, PEDF has been detected in several cell types of various species (Becerra et al., 2004; Behling et al., 2002, Karakousis et al., 2001; Ogata et al., 2002, Ortego et al., 1996; Wu et al., 1995) and exists in basically all compartments of the eye, including the vitreous, RPE, retina, choroid, ciliary body, lens and cornea. In the vitreous of several speices, PEDF has been estimated to be between 1-2 µg/ml or 20-40 nM and is about 10-fold lower than in the interphotoreceptor matrix (Barnstable and Tombran-Tink, 2004). Within the retina, RPE cells secrete PEDF into the interphotoreceptor matrix and are the main source of PEDF production.

In addition to its neuroprotective effects on photoreceptor cells (Cao et al., 2001; Cayouette et al., 1999; Jablonski et al., 2000) and retinal neurons (Cao et al., 1999; Ogata et al., 2001a), it has been shown that PEDF supports normal Mueller glia development (Jablonski et al., 2001), induces the morphogenesis of photoreceptor neurons of Xenopus laevis (Jablonski et al., 2000), as well as the

differentiation and maturation of pigment granules of albino RPE cells from Wistar rats in retinal cultures (Malchiodi-Albedi et al., 1998).

Albinism is generally heterogeneous but manifests the same abnormalities, which include an underdevelopment of the central retina, abnormal chiasmatic routing of temporal retinal axons, reduction of about 30% of rod photoreceptor numbers and the lack of mature melanosomes. It has been shown that there is a relation between the melanin content and rod numbers, ocular rhodopsin, as well as the degree of misrouting at the chiasm (Donatien and Jeffery, 2002) indicating the importance of melanin in the eye.

Since there are indications that PEDF plays a role in the differentiation and maturation of pigment granules, it could be therefore hypothesized that lower levels of PEDF might be related to the albino phenotype in comparison to normally pigmented eyes. In order to test this hypothesis, the PEDF content and localization were compared in pigmented Long Evans and albino Wistar rats. For this purpose, homogenates of complete rat eyes and retinas were prepared from birth until adulthood and their PEDF content measured by Western blot analysis. Retinas from both rat strains were immunohistochemically stained for PEDF to analyze and compare their protein location.

Results:

Whole eye homogenates from male Long Evans and Wistar rats of various postnatal ages were separated by Western blot analysis. As a positive control and to calculate the PEDF content in the eye various concentrations of human

recombinant PEDF were used. The purified PEDF protein migrated at the same molecular weight as the protein found in the eye samples and was detected slightly below 50 kDa (Fig. IV.1.1b). Omitting the primary antibody showed no staining (data not shown) and both anti-PEDF antibodies gave the same results (data not shown), indicating the specificity of the primary antibodies.

At birth, PEDF levels were high and Long Evans eyes had virtually the same amount of PEDF as Wistar eyes and contained 1.27±0.06 and 1.28±0.02 ng/µg protein, respectively (Fig. IV.1.1a). Although at P28 and 3 months there was a maximum decrease of PEDF of 22% compared to levels at P0, the PEDF content remained high during the first three months of age. There was no significant difference between the two rat stains at any of the ages tested and in total, Wistar rat eyes contained 1.8 % less PEDF compared to Long Evans eyes. A Spearman rank correlation showed no significant effect of age on the overall PEDF content in the eye for both Long Evans (n=41, r=0.057, P=0.72) and Wistar rats (n=43, r=-0.2, P=0.2). Overall, Western data indicate that there was no significant difference in the PEDF content of complete eyes between the two strains (P=0,847, n=84).

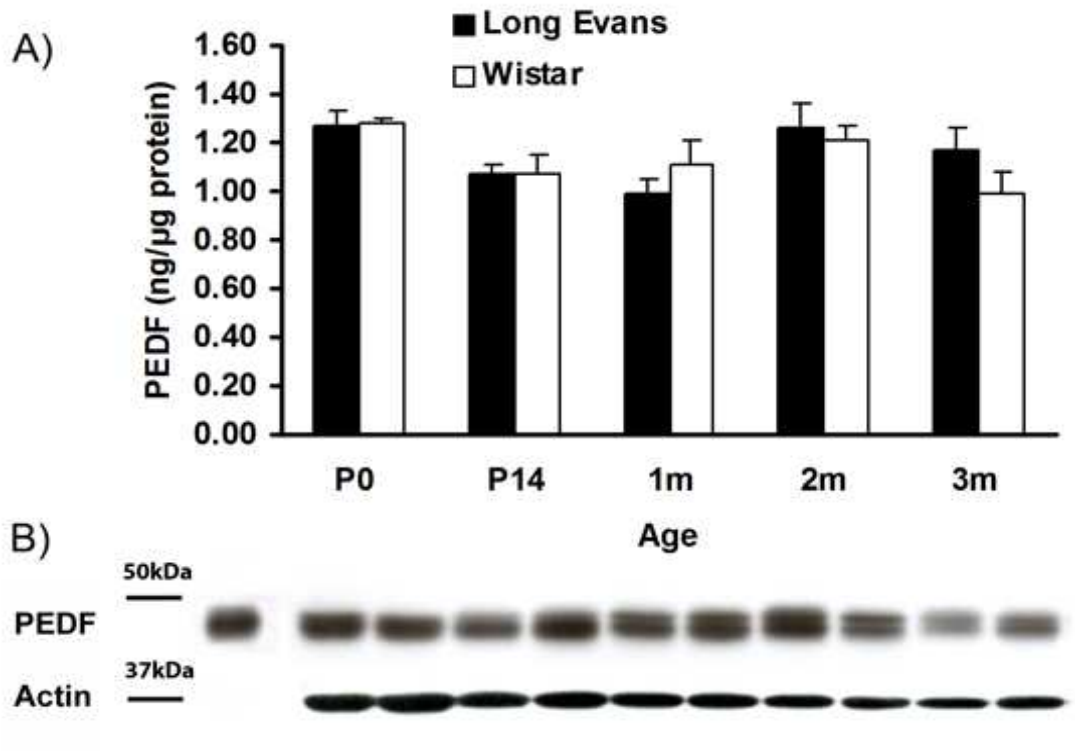


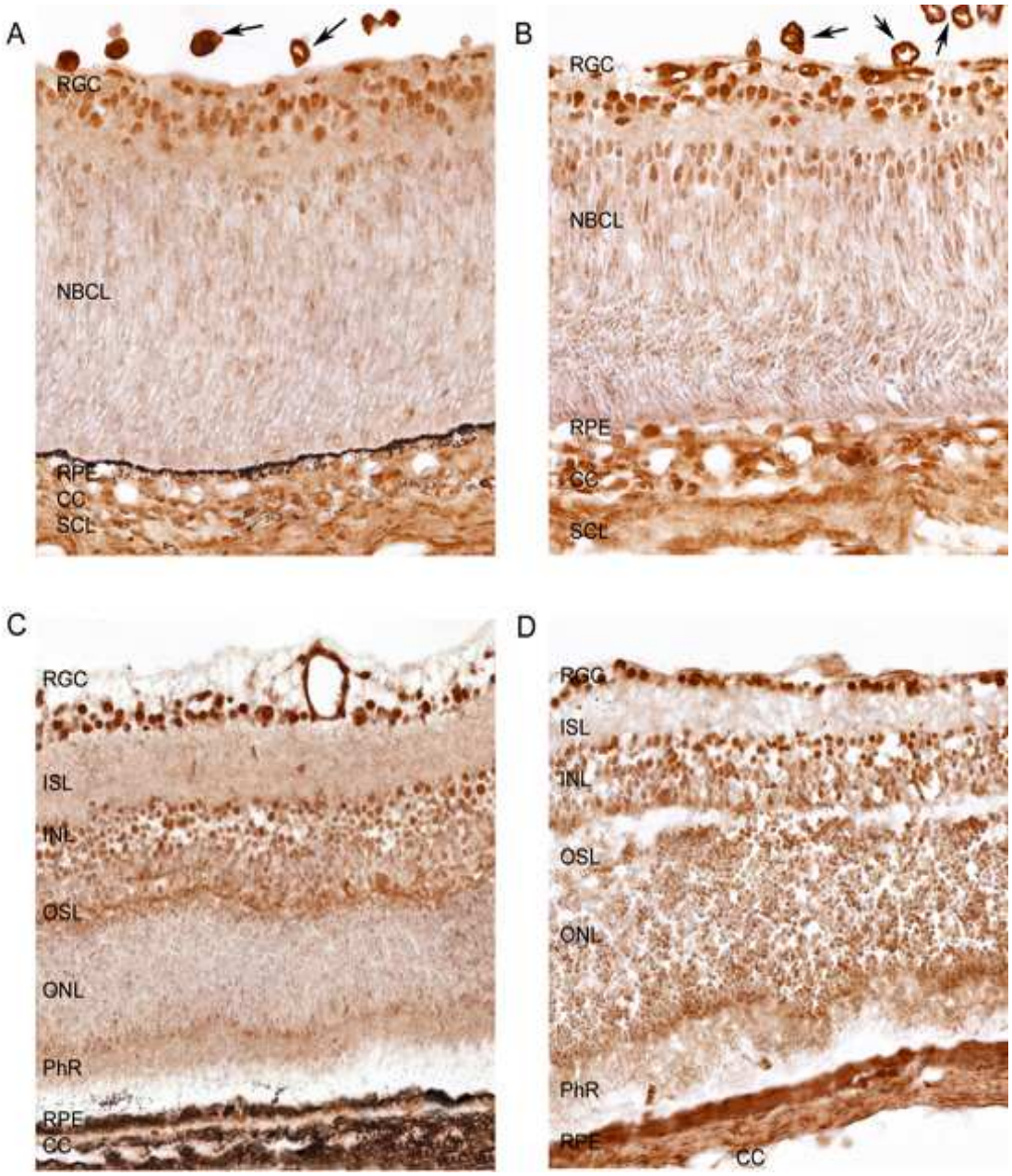
Fig IV.1.1. PEDF content of complete Long Evans and Wistar rat eyes from birth until 3 months of age.

A) 50 µg of complete eye homogenates of animals between PO and 3 months were analyzed by Western blot analysis. PEDF was normalized to the average actin signal at each age and then compared to a standard curve of PEDF (20-50 ng, not shown). Data are shown as mean ± SEM. Black bars represent eyes of Long Evans rats and white bars Wistar eyes. A Mann-Whitney-U test showed that overall there was no significant difference between the two rat strains (P=0.847, n=84)**.** Although there was a significant difference in the PEDF content in eyes between P0 and P14 (P=0.006, n=40) and between 1 and 2 months (P=0.031, n=37), a Spearman Ranks test showed that overall age had no significant effect on the PEDF content in either strain (Long Evans: n=26, r=-0.845, P<0.000; Wistar: n=24, r=-0.737, P<0.000).

B) Underneath the graph a Western Blot of one representative sample per age is shown for PEDF and actin. PEDF samples comigrated with the positive control (lane 1, 50 ng) and were detected slightly below 50 kDa.

To examine if there are differences between the two rat strains in the PEDF protein localization within the retina that could be correlated with the albino

phenotype of Wistar rats, retinas were immunohistochemically stained for PEDF at P0, P14 and 3 months of age (Fig. IV.1.2). Stained slices were compared to the appropriate IgG controls, which were negative in all cases (Fig. IV.1.2H). Preadsorption of the PEDF antibody also showed no staining (Fig. IV.1.2G), furthermore indicating the specificity of the PEDF staining.



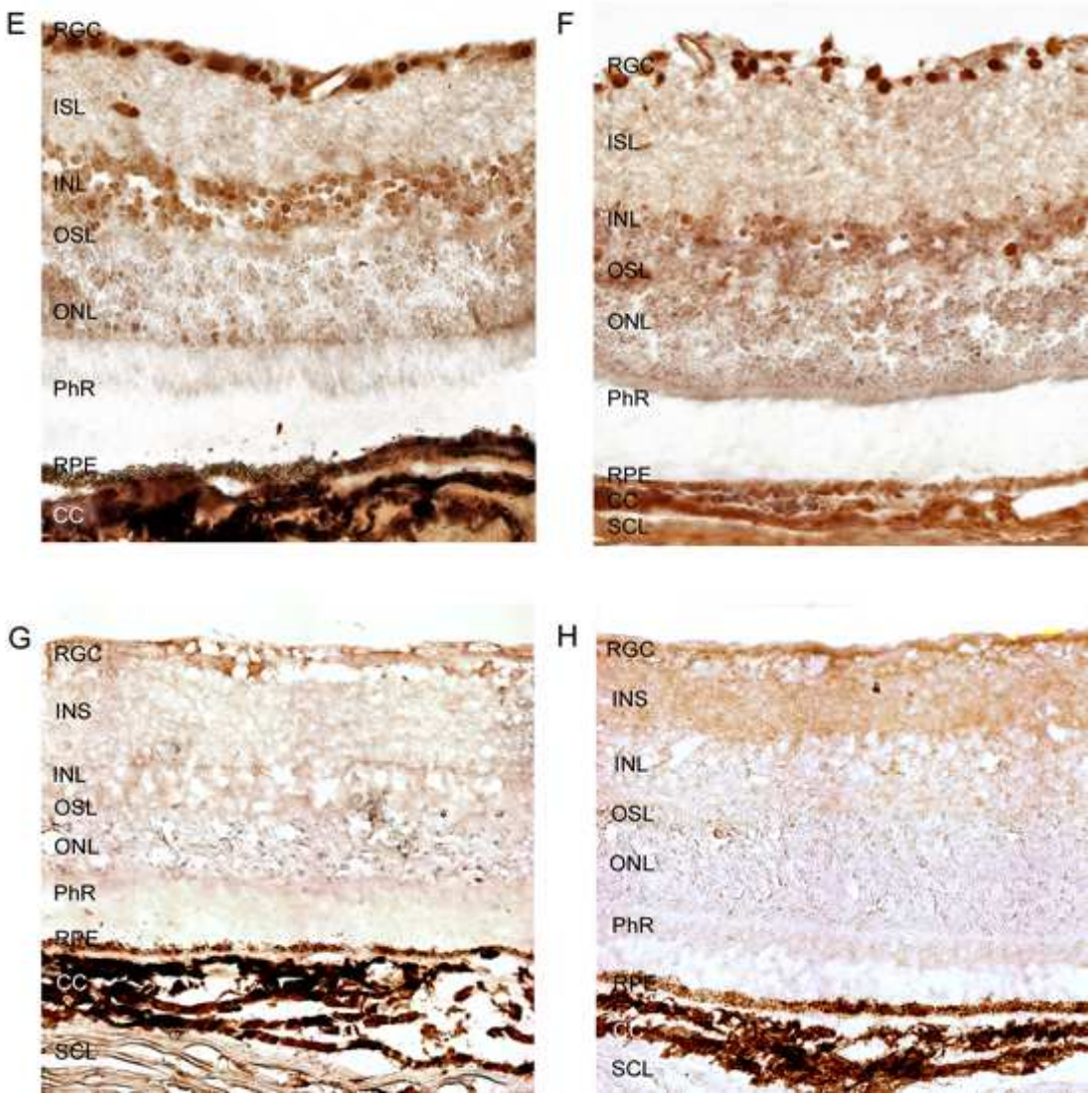


Fig. IV.1.2) DAB immunocytochemical localization of PEDF in Long Evans (left panel) and Wistar (right panel) retinas at PO (A, B), P14, (B, C) and 3 months (E, F). Picture (G) shows a preadsorption control and (H) an IgG control of the 3 month old Long Evans retina. Note the strong staining of vessels on the surface of the retina (arrows), as well as the clear reaction of RGC somata for PEDF in both strains. Abbreviations: CC, choroid; INL inner nuclear layer; INS, inner synaptic layer; ONL, outer nuclear layer; OSL, outer synaptic layer; NBCL, neuroblastic cell layer; PhR, photoreceptors; RGC, retinal ganglion cells; RPE retinal pigment epithelial cells; SCL, sclera;

Prominent staining for PEDF was seen in retinal ganglion cells (RGC) at all ages in both rat strains. To test if a difference in staining intensity might indicate a difference in PEDF content, the RGC staining intensity was measured. Measurements indicated that the staining intensity increased with increasing age and was the highest at 3 months of age (Fig. IV.1.3). There was a significant increase in the staining intensity of RGC's between P0 and P14 (P=0.002, n=12), but not between P14 and 3 months of age (P=0.132, n=12). Although the RGC staining intensity between Long Evans and Wistar eyes could not be statistically compared at each age, it appeared as if RGC's of both strains stained to similar degrees (Fig. IV.1.3 and IV.1.2).

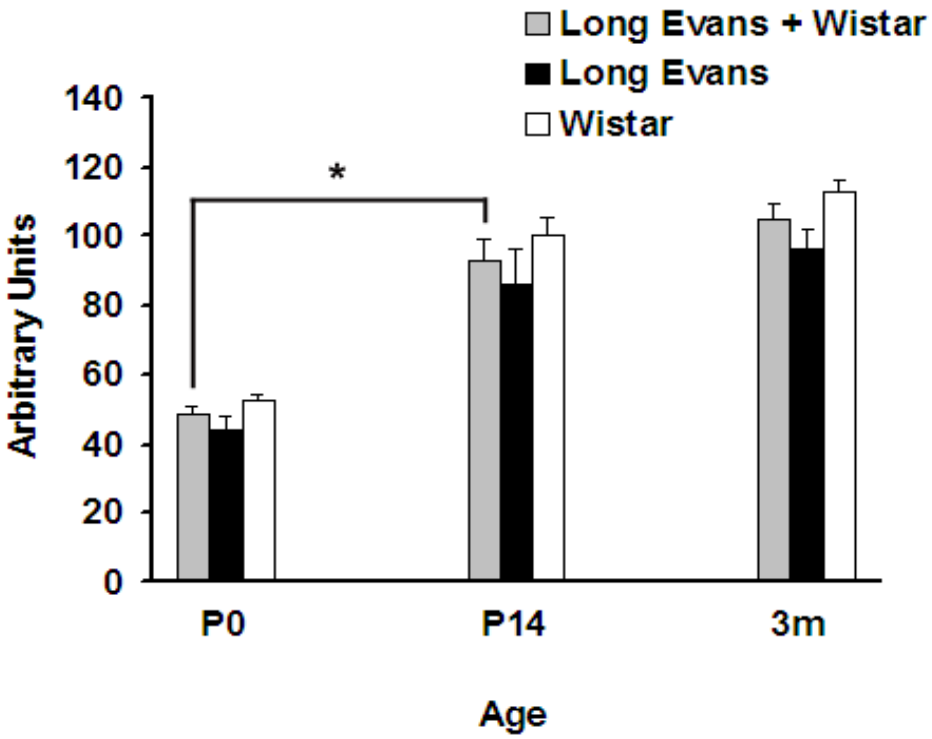


Fig IV.1.3) RGC staining intensity of PEDF of retinas between birth and 3 months.
PEDF DAB staining of retinas from three different Long Evans (black bars) and Wistar (white bars) animals was performed in parallel and the staining intensity of 20 RGC's from each animal was measured and adjusted to the background. A Kruskall Wallis test for Long Evans and Wistar RGC's combined (gray bars) showed a significant overall effect of age on RGC staining intensity (P=0.002, n=18). When ages were compared among each other, a Mann-Whitney-U test showed that there was a significant difference in the RGC staining intensity between P0 and P14 (P=0.002, n=12), but not between P14 and 3 months of age (P=0.132, n=12). Significant changes between ages are indicated by asterisks.

Overall, there was no obvious difference in the location of the PEDF signal between rat strains. At each age tested, the different retinal layers seemed to stain to similar degrees in Long Evans and Wistar rats as supported by the measurement of the RGC staining intensity. There was an increase in the overall staining intensity after birth, but retinas at two weeks stained to similar degrees as at three months. Since overall there were no obvious differences in the staining location between the two rat strains and the staining intensities appeared similar as well, the following description of the PEDF staining accounts for both rat strains.

The PEDF staining of the inner nuclear layer (INL) was not as strong as for RGC's, and not all cells in the INL stained for PEDF. Cells in the INL facing the RGC layer tended to stain more often than the ones facing the outer nuclear layer (ONL). This was already apparent at P0 when the different retinal layers had not fully differentiated yet and only cells of the neuroblastic cell layer facing the RGC layer stained for PEDF. In general, the ONL stained relatively weak for PEDF, but was somewhat stronger stained at 2 weeks of age compared to adulthood and only some cells of the ONL showed a rather weak signal for

PEDF, while others were negative. Photoreceptors weakly stained for PEDF at 2 weeks, but not at 3 months of age.

PEDF immunoreactivity of RPE cells was very intense at all ages tested and the nuclei of RPE cells heavily stained for PEDF as determined by nuclear staining (see also Fig. IV.2.4). In the Wistar rat, the staining intensity of the choroid was fairly strong between birth and adulthood. Due to the pigmentation it was difficult to determine if the immunoreactivity of the choroid of Long Evans rats was comparable to that of Wistar rats. The sclera also showed fairly prominent staining for PEDF at all ages. A strong PEDF staining of blood vessels was observed in both strains at all ages, which was however especially strong at birth, when the antibody labeled the nuclei and cytoplasm of endothelial cells on the surface of the retina.

To verify that Long Evans and Wistar retinas in fact contained similar amounts of PEDF as indicated by immunohistochemical stainings, and to examine the PEDF content of the retina in more detail, homogenates were prepared from retinas of 6 weeks old rats (Fig. IV.1.4). Preliminary Western blot results showed that young Long Evans and Wistar retinas appeared to contain similar amounts of PEDF, although there was a tendency for Wistar retinas to contain slightly less PEDF.

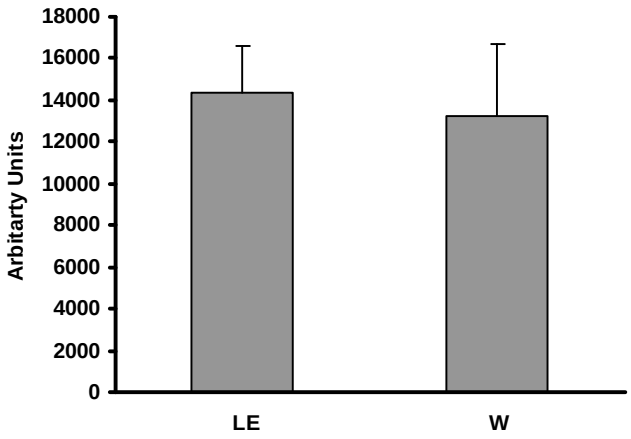


Fig. IV.1.4) PEDF content of retinas of 6 weeks old Long Evans and Wistar rats. 50 µg of three homogenates of retinas of 6 weeks old Long Evans (LE) and of Wistar (W) rats were analyzed by Western blot analysis. PEDF signals were normalized to the average actin. Data are shown as mean ± SEM.

Discussion:

Western results showed that the PEDF content in the entire eye was not significantly different in both rat strains from birth until young adulthood and did not significantly change with age, indicating that the PEDF content of entire eyes does not differ between albino and pigmented eyes and thus might not be related to the albino phenotype.

However, PEDF can accumulate to different degrees in different compartments of the eye, and the retina is not the primary source of PEDF within the eye. To determine if more subtle differences in PEDF distribution or production might be related to the albino phenotype, special attention was put on the retina that also contains melanosomes. The immunohistochemical staining of retinas for PEDF of this study are in good agreement with another study that

investigated the location of PEDF in the mouse retina between embryonic day 14.5 and 3 month old animals (Behling et al., 2002). Rat strains were similar in respect to the specific PEDF protein location and expression, as indicated by measurement of the RGC staining intensity, furthermore indicating that the PEDF protein might not be related to the albino phenotype. The finding that the PEDF content of 6 week old retinas appeared to be similar furthermore indicates that the two rat strains did not differ in the PEDF content and localization of the retina.

Interestingly, the overall PEDF staining signal was the weakest at birth, although the PEDF content of the entire eye was the highest at this age. Since previous reports show that the PEDF content in the retina is relatively low compared to other compartments in the eye, the immunohistochemical staining intensity of the retina may therefore not be a good indicator for the PEDF content of the entire eye. One explanation that might explain the apparent discrepancy between the PEDF content of complete eyes and the immunohistochemical staining intensity at birth is that the antibody appeared to label only the already differentiated RGC and RPE layer, while the undifferentiated neuroblastic cell layer was for the most part negative for PEDF. This is supported by previous reports that show that *in vitro* undifferentiated RPE cells do not produce PEDF contrary to differentiated cells (Ohno-Matsui et al., 2001). This is insofar interesting, since PEDF is upregulated in young, quiescent cells of the G0 state, and low in replicating cells, so that it is thought that PEDF might have an inhibitory effect on the cell cycle (Pignolo et al., 1993 and 2003). A lack of PEDF in undifferentiated replicating cells would also be in line with reports that have

shown that PEDF induces the differentiation of various cell types including RPE cells, Muller glia and photoreceptors. Thus, a low immunohistochemical PEDF signal of the retina at birth may not be contradictory to a high PEDF content of the entire eye.

Presently, it is not known whether PEDF might have some kind of function in the cell before its secretion into the extracellular space. The fact, that PEDF is produced by RPE cells (Tombran-Tink et al., 1995), binds to them (Aymerich et al., 2001) and has been shown to induce their pigmentation in culture (Malchiodi-Albedi et al., 1998), indicates that an autocrine mechanism and therefore also its secretion might be important for its function. This is also supported by the finding that nuclei of endothelial, RPE cells and potentially also RGC's stained for PEDF, which suggests that the protein may be taken up by the cell and function as a signaling molecule in the cell body, as is the case for nerve growth factor (Delcroix et al., 2004). In fact, it has been shown that PEDF is taken up by injured motor neurons, transported retrogradely and reduces their cell death (Houenou et al., 1999), also suggesting that PEDF may function as a signaling molecule in the nucleus.

Taken together, the data of this study indicate that there were no differences in between rat strains from birth until young adulthood in the PEDF protein content of entire eyes, and probably also not of the retina, as well as in the PEDF location within the retina, which supports the idea that PEDF might not be related to the lack of pigmentation in albino Wistar eyes.

It is not clear why in complete retinal cultures PEDF was able to induce pigmentation of RPE cells of neonatal Wistar eyes (Malchiodi-Albedi et al., 1998), although the present study indicates that pigmented Long Evans and albino Wistar eyes do not differ in the PEDF content and localization. Further studies would be necessary to clarify if there might be differences in between strains regarding the PEDF content of the retina and of the RPE layer especially during embryogenesis and at younger ages than 6 weeks. It would be also important to know if differences in the amount and location of the PEDF receptor might exist in the RPE layer and if cell culture conditions might possibly induce the expression of the PEDF receptor or of yet another factor in the reaction cascade of PEDF, thereby inducing the pigmentation of RPE cells.

It would be furthermore interesting to find out if there are differences in the posttranslational processing of PEDF in the retina between the two rat strains, since it has been shown that differences in posttranslational processing can change the function of PEDF (Maik-Rachline et al., 2004). It also has been reported that there are differences in the posttranslational processing of PEDF depending on where the protein is produced in the eye. For instance, the N-terminal of PEDF from the bovine interphotoreceptor matrix has been found to be unblocked (Wu et al., 1995), but is blocked in bovine vitreous and in human blood (Wu and Becerra, 1996; Peterson et al., 2003), indicating that PEDF is processed differently in different compartments of the eye. Thus, changes in posttranslational processing could possibly explain why certain functional

aspects of PEDF might differ in between the two rat strains although they do not differ in the PEDF content and location in the eye.

IV.2. Is a loss of PEDF protein content or a change in PEDF localization related to the degeneration of the Wistar retina at old age?

Introduction:

Several studies provide evidence for the neuroprotective effect of PEDF on various cell types in the eye (Tombran-Tink and Barnstable, 2003b; Barnstable and Tombran-Tink, 2004). Pretreatment with PEDF protects rat retinal neurons from hydrogen peroxide-induced cell death in culture (Cao et al., 1999), transiently delays the death of photoreceptors cells in the rd/rd and rds/rds mouse models of retinitis pigmentosa (Cayouette et al., 1999), protects photoreceptors from light induced damage (Cao et al., 2001), and has a neuroprotective effect on the inner layers and ganglion cells of the retina under ischemic conditions (Ogata et al., 2001a).

It has been shown previously that at old age, the Wistar retina shows signs of degeneration including the loss of photoreceptors (Sullivan et al., 2003; Weisse, 1995), while the retina of old pigmented Long Evans rats appears morphologically intact. In fact, this age-dependent, ambient light-induced degeneration of the Wistar rat retina has been shown to be related with AMD in humans (Sullivan et al., 2002). Because of its neuroprotective effect in the retina against phototoxic and oxidative stress, it could be therefore hypothesized that a loss of PEDF might be related to the observed degeneration in old Wistar retinas compared to morphologically intact Long Evans retinas.

To test this hypothesis, complete eyes of Wistar and Long Evans rats between 3 and 24 months of age and retinas from 18-19 month old animals were homogenized and their PEDF protein content measured by Western Blot analysis. In order to compare the localization of PEDF protein within the retina, eyes from 15-16 month old animals were stained immunohistochemically for PEDF.

Results:

As previously shown the PEDF content of complete rat eyes was the highest at birth (1.27±0.06-1.28±0.02 ng/µg protein) and remained fairly high until 3 months of age in both rat strains (Fig. IV.1.1). After 3 months however, PEDF decreased and reached its lowest level at the oldest age tested, at which time the PEDF content was only about 22.8 - 26.6% from values at birth, or 0.29±0.03 and 0.34±0.03 ng/µg protein for Long Evans and Wistar rats, respectively (Fig. IV.2.1). At 8 (P=0.031, n=18) and 15-16 months of age (P=0.048, n=9), there was a significant difference in the amount of PEDF between Long Evans (8 months: 0.51±0.04 ng/µg and 15-16 months: 0.42±0.05 ng/µg) and Wistar rats (8 months: 0.72±0.07 ng/µg and 15-16 months: 0.63±0.05 ng/µg). Between 3 and 22 months, Wistar eyes contained a total of 23% more PEDF than Long Evans eyes.

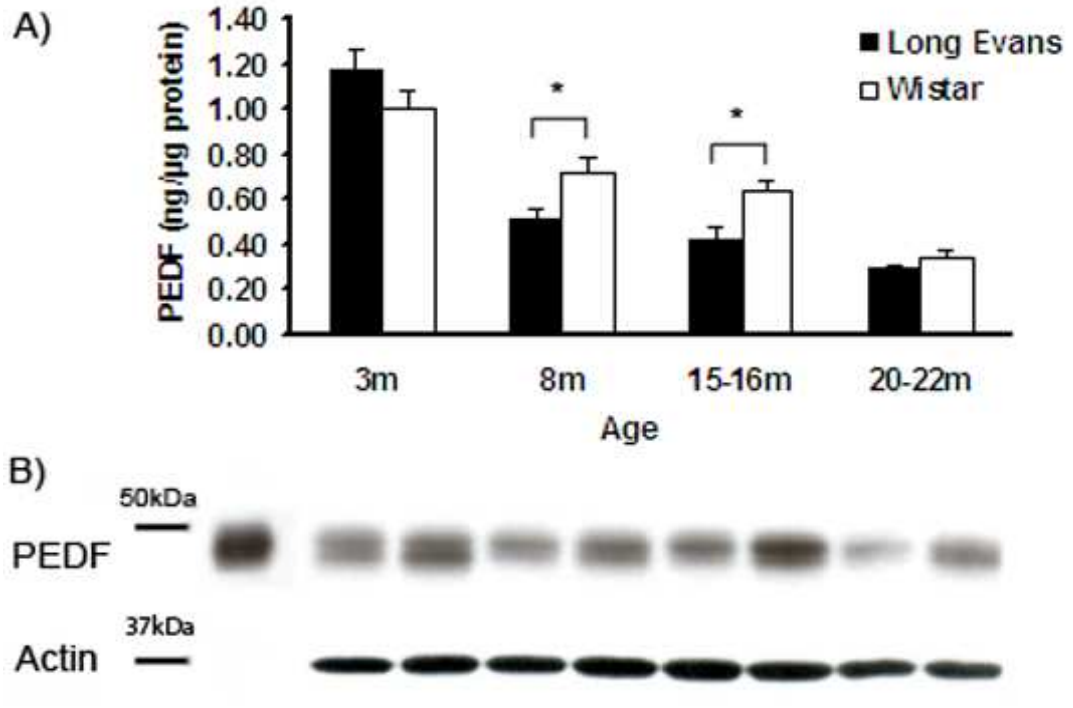


Fig IV.2.1) PEDF content of complete Long Evans and Wistar eyes from adulthood until old age.

A) 50 µg of complete eye homogenates of animals between 3 and 22 months were analyzed by Western blot analysis. PEDF was normalized to the average actin signal at each age and then compared to a standard curve of PEDF (20-50 ng, not shown). Data are shown as mean ± SEM. Black bars represent eyes of Long Evans rats and white bars Wistar eyes. A Mann-Whitney-U test showed that overall there was no significant difference between the two rat strains ($P=0.145$, $n=50$), although at 8 ($P=0.031$, $n=18$) and 15-16 months ($P=0.048$, $n=9$) Wistar eyes contained significantly more PEDF than Long Evans eyes. A Spearman Ranks test showed that overall the PEDF content in both strains significantly decreased with increasing age (Long Evans: $n=26$, $r=-0.845$, $P<0.000$; Wistar: $n=24$, $r=-0.737$, $P<0.000$). Significant changes between rat strains are indicated by asterisks.

B) Underneath the graph a Western Blot of one representative sample per age is shown for PEDF and actin. PEDF samples migrated at the same height as the positive control (lane 1, 50 ng) and were detected slightly below 50 kDa.

To determine if the overall protein content of the entire eye was reflected by the staining pattern in the retina, eyes from 15-16 months old Long Evans and Wistar rats were stained immunohistochemically for PEDF. Appropriate IgG and preadsorption controls were negative in all cases (Fig. IV.2.2.C and D) indicating the specificity of the PEDF staining.

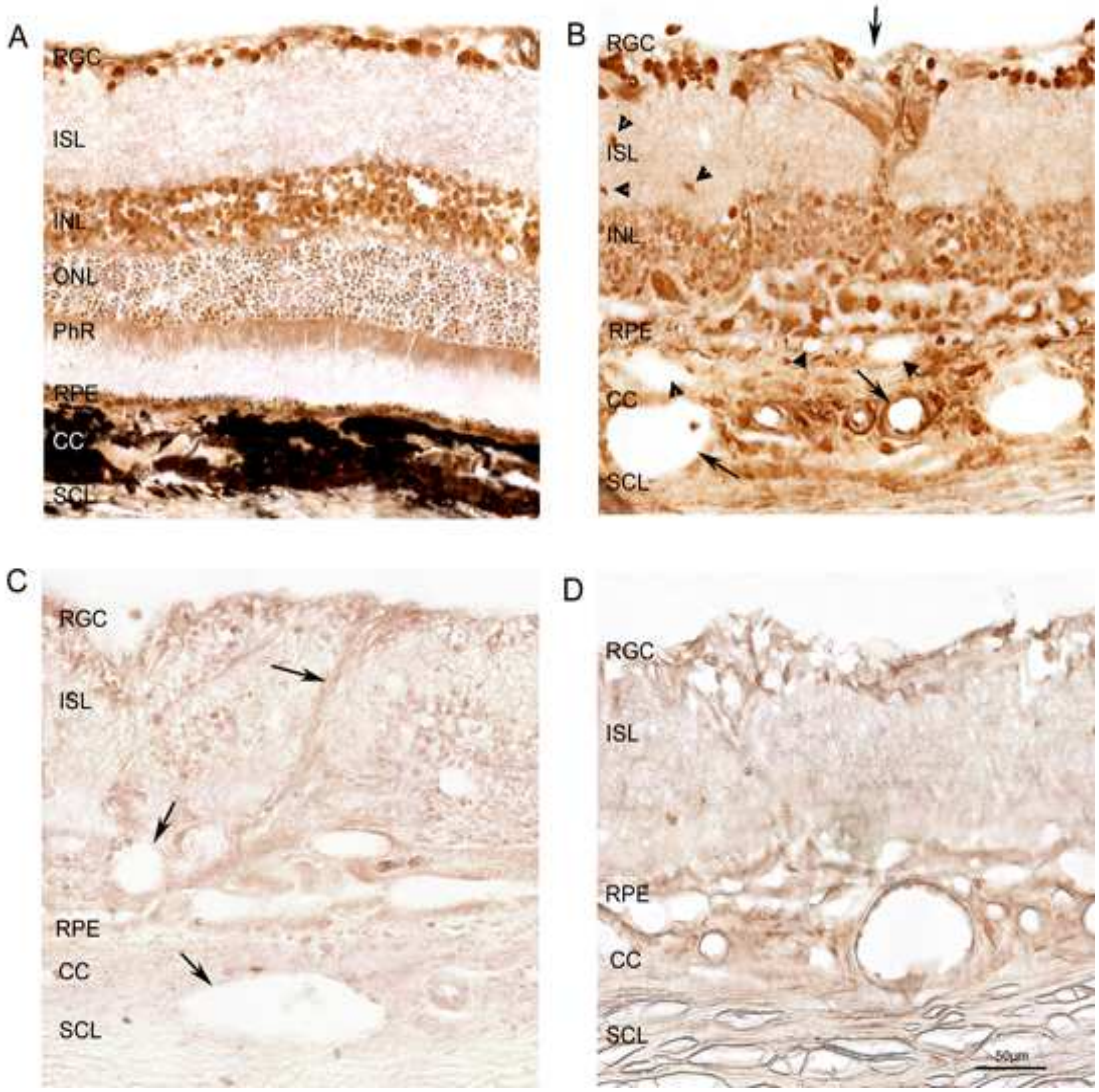


Fig. IV.2.2) DAB immunocytochemical localization of PEDF in a 15-16 month old Long Evans (A) and Wistar (B) retina. Picture (C) shows an IgG control and (D) a preadsorption control of the same Wistar retina as shown in (B). The pictures show the large lumen of vessels in the choroid and vessels that transverse the entire retina (arrows) as well as holes in close vicinity to the RPE layer in the Wistar retina (arrow heads). Also note the appearance of some cells in the ISL of the Wistar retina as indicated by arrow heads. Abbreviations: CC, choroid; INL inner nuclear layer; ISL, inner synaptic layer; ONL, outer nuclear layer; PhR, photoreceptors; RGC, retinal ganglion cell layer; RPE retinal pigment epithelial cell layer; SCL, sclera;

Figure IV.2.2B shows a typical example of a degenerated Wistar retina at old age. In all Wistar eyes tested, the photoreceptor layer, as well as the outer nuclear layer was degenerated nearly completely almost all over the entire eye, and remnants could only be found in some areas of the retina. Age-matched Long Evans retinas (Fig. IV.2.2A), however, appeared morphologically intact. Holes were found in the close vicinity of the RPE layer of old Wistar retinas contrary to Long Evans eyes. Furthermore, especially large lumina of blood vessels could be observed frequently in the choroid of old Wistar eyes, while the choroid of old Long Evans retinas did not appear different from 3 months old animals. In some cases blood vessels also transversed the entire Wistar retina, which was not observed in old Long Evans retinas.

In general, the same retinal layers that were labeled for PEDF at younger ages also stained in both rat strains at old age, although with lower intensity. Measurement of the staining intensity of RGC somata indicated that although the staining of RGC's was still prominent at high age, it was significantly decreased compared to 3 month old animals ($P=0.015$, $n=12$) (Fig. IV.2.3). Although it was not possible to perform a statistical analysis in between rat strains, it appeared

that RGC somata of both rat stains stained with similar intensities for PEDF at old age (see Fig. IV.2.3 and IV.2.2). In old Long Evans retinas, photoreceptors were labeled for PEDF, while the few leftover photoreceptors in Wistar retinas did not stain for PEDF (not shown). Fluorescent stainings showed, that in Wistar retinas the nuclei of RPE cells stained very faint for PEDF compared to strongly labeled nuclei of RPE cells of Long Evans retinas (Fig. IV.2.4). Furthermore, the RPE layer of old Wistar rats contained a large amount of auto fluorescent lipofuscin which could not be observed in age-matched Long Evans eyes (Fig. IV.2.4).

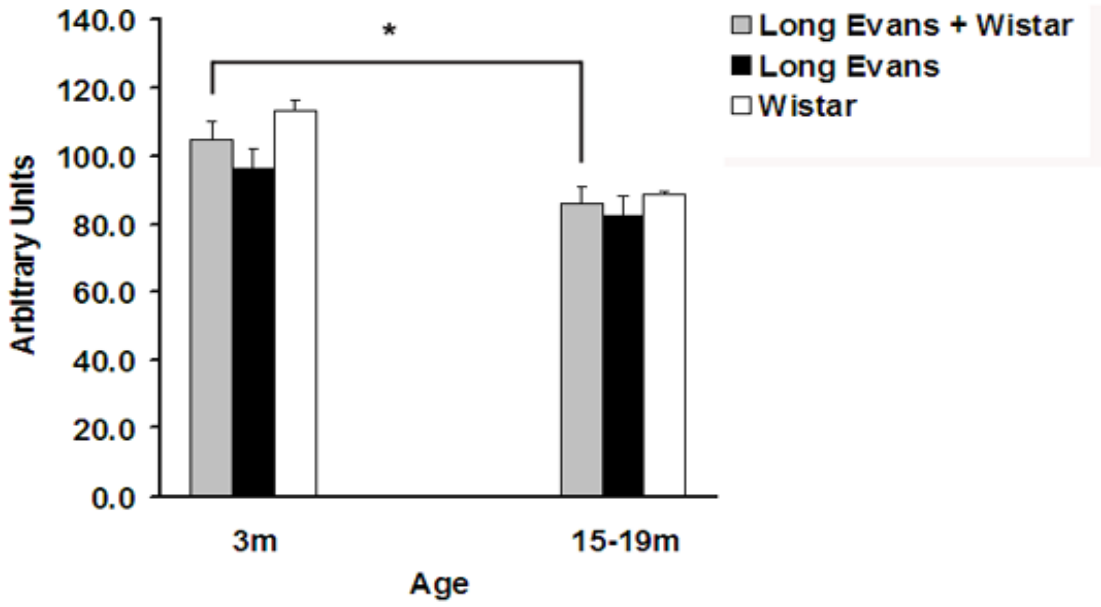


Fig IV.2.3) RGC staining intensity of retinas at 3 and 15-19 months.
PEDF DAB staining of retinas from three different Long Evans (black bars) and three Wistar (white bars) animals was performed in parallel and the staining intensity of 20 RGC's from each animal was measured and adjusted to the background. A Kruskall Wallis test for Long Evans and Wistar RGC's combined (gray bars) showed a significant effect of age on the RGC staining intensity ($P=0.015$, $n=12$). Significant changes between ages are indicated by asterisks.

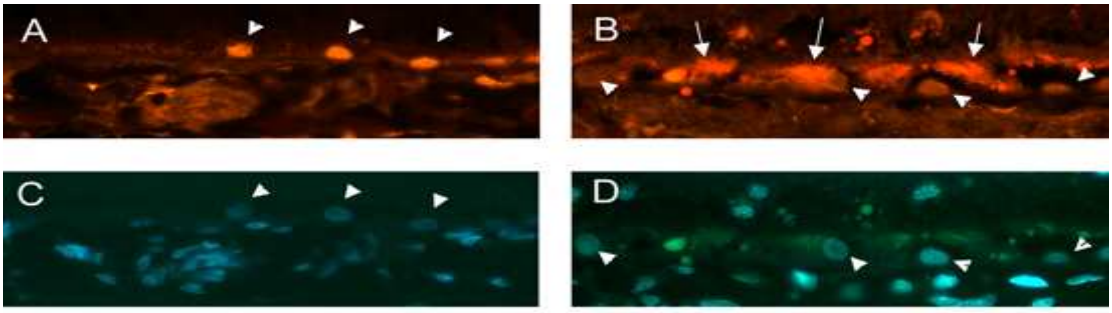


Fig. IV.2.4) Fluorescent PEDF staining of RPE cells in Long Evans (A) and Wistar (B) retinas at old age. Pictures C and D show the corresponding Hoechst stainings to indicate the location of RPE nuclei as shown by arrow heads. PEDF images of both rat strains were taken with the same camera settings and their contrast adjusted in parallel. The figure shows that RPE cells of Long Evans eyes stained much stronger than those of Wistar eyes, which contained however a large amount of auto fluorescent lipofuscin as indicated by arrows.

Interestingly, the choroid of old Wistar retinas was widened and contained a large amount of strongly stained cells that had not been observed at younger ages (Fig. IV.2.2). At old age, only the nuclei of cells in the sclera were positive for PEDF as determined by nuclear staining (data not shown). Furthermore, in the Wistar retina somata could also be observed in the ISL, a layer that normally does not contain any cell bodies (Fig. IV.2.2).

Since signs of retinal degeneration usually are accompanied by proliferation and migration of microglia, a double labeling of PEDF and the microglia/macrophage markers Ox42 and ED1 was performed to test if these cells might stain for PEDF. Only a small amount of cells stained for Ox42 and ED1 that were mainly located on the surface of the retina (Fig. IV.2.5; data for ED1 are not shown) which indeed also stained for PEDF.

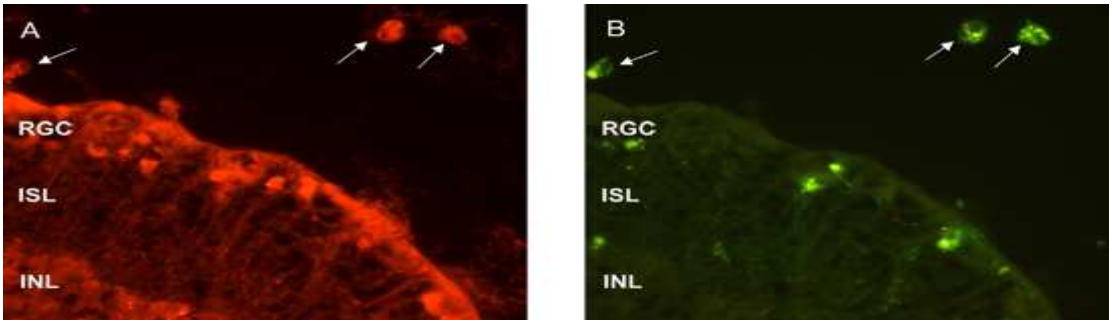


Fig. IV.2.5) Fluorescent double staining of a degenerated Wistar retina at old age with PEDF (A) and Ox42 antibody (B). The figure shows that microglia on the surface of the retina immunoreacted with both PEDF and Ox42 antibodies which is indicated by arrows.

In order to determine the PEDF content of the retina at old age, retinal homogenates of 18-19 month old Wistar and Long Evans rats were prepared. Preliminary data indicate that old Wistar retinas contained about 45% less PEDF than age-matched Long Evans retinas (Fig. IV.2.8). In comparison with 6 week old animals, old Long Evans retinas even contained slightly more PEDF (about 10%), while old Wistar retinas showed a decrease of PEDF of 35%.

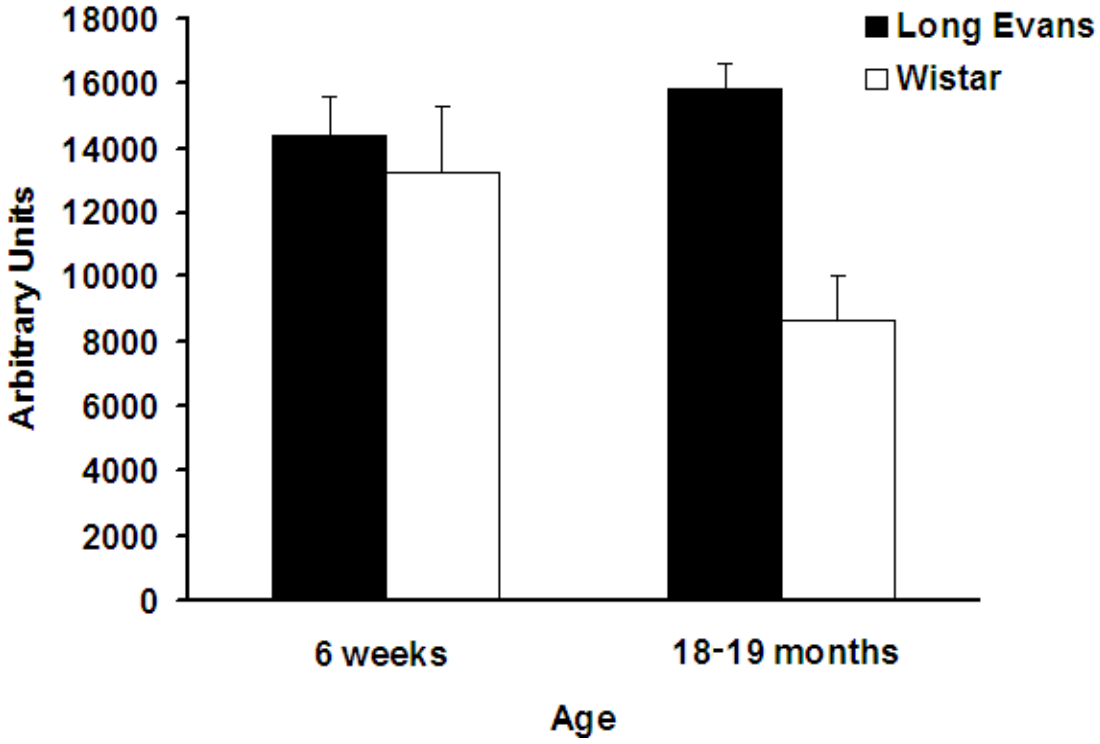


Fig. IV.2.8) PEDF content of retinas of 6 weeks and 18-19 month old Long Evans and Wistar rats. 50 µg of three homogenates of different retinas of each Long Evans (black bars) and Wistar rats (white bars) were analyzed by Western blot analysis. PEDF signals were normalized to the average actin. Data are shown as mean ± SEM.

In summary, although in the entire eye PEDF significantly decreased with increasing age in both strains, in old animals the same retinal layers stained for PEDF as in young adult animals and no obvious difference in staining location could be found in between strains. Overall, the staining was reduced compared to 3 months old animals as indicated by the measurement of RGC staining intensity. Nuclei of RPE cells of old Wistar retinas stained however much less compared to Long Evans eyes and retinas of old Wistar rats contained less PEDF than of Long Evans rats.

Discussion:

As hypothesized, Western results showed that in entire eyes there was a significant downregulation of PEDF with increasing age in both rat strains. This is in agreement with a previous study that has shown that PEDF is downregulated with increasing age in the aqueous humor of humans (Ogata et al., 2004). PEDF has also been shown to be downregulated in senescent cells, such as fibroblasts and RPE cells (Pignolo et al., 1993 and 2003; Tombran-Tink et al., 1995). Thus, the downregulation of PEDF in the entire old eye appears to be a natural phenomenon that might be associated with the decline of the cell cycle rate and might not be related to the observed degeneration of old Wistar retinas.

Contrary as hypothesized, between 8 and 16 months of age Wistar eyes contained significantly more PEDF than Long Evans eyes, suggesting that a low PEDF content in the entire eye is not associated with the observed degeneration of the Wistar retina. Ogata and colleges (2003) have shown that there is a significant difference in the PEDF content in the vitreous between men and women at old age. Although only male rats were tested, it is possible that the difference in PEDF content of Wistar and Long Evans rat eyes between 8 and 16 months might be genetic and therefore strain specific. Considering its neuroprotective effect in the eye, it could be hypothesized that the increased content of PEDF in entire Wistar eyes, as well as its still prominent staining in degenerated Wistar retinas might be a compensatory mechanism to prevent retinal degeneration, although unsuccessfully. In fact, PEDF has been shown to be upregulated in the vitreous of rhegmatogenous retinal detachment, where it

has been suggested to act as a neuroprotective agent for the detached retina (Ogata et al., 2001b). This idea would however not be in agreement with preliminary data that suggest that Wistar retinas contain less PEDF than Long Evans retinas.

Interestingly, in contrast to younger ages and age-matched Long Evans retinas, PEDF-stained cell bodies appeared in the choroid and ISL of old Wistar eyes. In fact, migration of neurons, as well as extension of Muller cell processes through holes in the Bruch's membrane into the choroidal region has been shown to occur in degenerated retinas of old Wistar rats (Sullivan et al., 2003). Sullivan and colleges also showed that the causal agent in this degenerative process was light, although albino rats were maintained under normal animal house lightening conditions of 750 lux. The appearance of somata within the choroid and the ISL therefore suggests a migration of cells within old Wistar retinas. The reason for the observed remodeling and migration of cells within the retina is presently unknown, but may potentially serve to retard retinal degeneration (Sullivan et al., 2003).

Similar to Western blot results of entire eyes, some aspects of immunohistochemical data of the retina also suggest that PEDF may not be related to the degeneration of the retina at old age. First, In general the same retinal layers stained for PEDF at old age in both strains compared to younger ages. Second, the overall retinal staining remained similarly strong in both

strains, although RGC staining intensities were significantly lower at old age compared to 3 month old animals, thereby reflecting the overall decrease of PEDF with increasing age.

However, other, more subtle changes indicate that a decrease of PEDF within the retina could be related with the degeneration of old Wistar retinas. First, RPE cells -the main PEDF source in the retina- stained obviously less in old Wistar retinas compared to Long Evans retinas as indicated by fluorescent stainings, and the remaining photoreceptor cells in old Wistar retinas did not stain for PEDF contrary to Long Evans photoreceptors. This would be in line with a decreased production of PEDF by RPE cells, since they secrete PEDF into the interphotoreceptor matrix (Becerra et al., 2004). In fact, *in vitro* experiments have shown that senescent RPE cells, RPE cells under oxidative stress or irradiated with near-ultraviolet light all have a reduced PEDF production (Tombran-Tink et al., 1995; Ohno-Matsui et al., 2001; Matsunaga et al., 1999; Li et al., 1999). Recently it has been made possible to visualize senescent cells by staining for beta galactosidase and it was shown that replicative senescence of RPE cells also takes place in old monkey eyes (Hjelmeland et al., 1999), suggesting that *in vivo* PEDF production may be in fact also downregulated in RPE cells at old age.

Second, another characteristic of senescent RPE cells *in vivo* is the progressive accumulation of auto fluorescent lysosomal material (lipofuscin) (Hjelmeland et al., 1999) which was also observed in old Wistar retinas of this study. Drusen are the earliest morphological features of AMD in humans and are

basal deposits of granular material with widened collagen or vesicles and membranous profiles external to the RPE (Green, 1999). The observation that holes, or possibly Drusen, were in close contact with the RPE layer, furthermore suggest a malfunctioning of the RPE layer which could cause a decrease in PEDF production. In support of this idea is the finding of Sullivan and colleges (2003) who have shown a degeneration of the RPE layer and Bruch's membrane in old Wistar retinas.

Third, preliminary data suggest that the degenerated Wistar retina contains less PEDF than the morphological intact Long Evans retina whose PEDF levels were even slightly increased compared to retinas of young animals. Since Wistar eyes do not contain any pigments, that among other things reduce the photochemical stress in the retina, the lack of melanin and the increased reactive oxygen species could potentially lead to a lower PEDF production within the Wistar retina at old age.

Because PEDF supports the normal development of photoreceptor neurons after removal of the RPE (Jablonski et al., 2000), it is therefore likely that a decrease in the PEDF production by RPE cells due to pathological changes in the ageing eye could contribute to the degeneration of photoreceptors. Thus, a decreased concentration of PEDF within the old Wistar retina due to a malfunctioning RPE layer might contribute to the observed degeneration including the loss of photoreceptor cells.

It is unlikely, however, that the observed decrease in RPE staining intensity for PEDF and the decreased PEDF content of Wistar retinas are the sole reason for the observed degeneration and one can only speculate why at this age, only the Wistar retina showed signs of degeneration. One explanation could be the lack of pigmentation in Wistar eyes. Melanin, a dopa derived pigment, acts as an optical aid to avoid light scattering, is known to function as an antioxidant, contributes to the protection of photoreceptors from light damage and has been implicated to play a role during development of the retina (Sanyal et al., 1988; Jeffery, 1997). Consequently, retinas from albino Wistar rats were shown to be more susceptible to ischemic damage than pigmented retinas (Safa and Osborne, 2000). It is therefore likely that the lack of pigmentation is also an important factor that contributes to the observed degeneration of the retina.

Taken together, a naturally occurring decrease of PEDF in complete eyes at high age in combination with a lower PEDF content in the albino retina might not be able to protect photoreceptors from extensive light-induced cell damage, and could therefore contribute to the degeneration of the albino eye at high age.

In summary one can say that the downregulation of PEDF in the entire aging eye appears to be a normal process. Western blot results of whole eyes furthermore indicate that there seem to be strain specific differences in the PEDF content at old age. The downregulation of PEDF in the old albino retina might be a contributing factor to the observed retinal degeneration as indicated by Western blot results of retinas and the reduced staining of RPE cells at high age.

IV.3. VEGF/PEDF ratio and VEGF protein expression during early postnatal angiogenesis of the rodent eye.

The retinal vasculature in the rodent is unique in respect to its postnatal development. During vasculogenesis a primitive vascular plexus forms de novo and a superficial layer of vessels forms first and originates from the optic nerve. The vessels spread outwards radially as a network of capillaries and reach the edge of the retina in the second postnatal week. In a second phase, a deep vascular system is formed by sprouting perpendicularly as far as the inner nuclear layer and extends branches parallel to the retinal surface. Thus, between P1 and P18, a gradient of vascular development and subsequent remodeling takes place between the optic disc and the retinal margin, as well as the superficial and deep layers.

VEGF is a major mediator of vascularisation in the eye and an important growth factor during the development of the retina. It is capable of initiating angiogenesis by promoting endothelial cell proliferation as well as chemoattraction, and maintains the viability of immature blood vessels (Alon et al., 1995). VEGF also facilitates pericyte recruitment of immature vessels which appears to be an important step in the maturation of vessels (Benjamin et al., 1998).

Besides its neuroprotective effect, PEDF also functions as a strong anti-angiogenic factor in the eye (Tombran-Tink and Barnstable, 2003b). The protein inhibits the migration of endothelial cells *in vitro* (Duh et al., 2002) and was more

effective than other angiogenesis inhibitors such as angiostatin, thrombospondin-1, and endostatin, placing it among the most potent natural inhibitors of angiogenesis (Dawson et al., 1999). PEDF is probably responsible for the normal avascularity of several ocular compartments and addition of anti-PEDF antibodies to rat corneas was shown to induce the invasion of new vessels into these corneas (Dawson et al., 1999). Loss of its expression leads to increased ischemia-induced retinal neovascularisation (Dawson et al., 1999) and treatment of mice with systemic or intra-ocular injections of recombinant PEDF results in a significant decrease of choroidal and retinal neovascularisation (Mori et al., 2001; Stellmach et al., 2001; Duh et al., 2002).

It has been shown that under pathological conditions such as diabetic retinopathy, retinopathy of prematurity, macular degeneration and glaucoma, regions of the retina become ischemic. Ischemia increases the production of angiogenic stimulators and decreases the production of angiogenic inhibitors, thereby breaking the balance between positive and negative regulators of angiogenesis. It is therefore thought that alterations in the ratio of VEGF to PEDF may be an underlying cause of pathological neovascularisation (Gao et al., 2001; Tombran-Tink and Barnstable, 2003a).

Thus far, little is known about the relative protein ratio of VEGF to PEDF in the normal eye during physiological angiogenesis early in postnatal development. It is hypothesized that during early developing life angiogenic stimulators outweigh angiogenic inhibitors (see also Fig. I.5.2.1; Gao et al., 2002a). To test the hypothesis that the VEGF/PEDF ratio is similarly elevated during early

postnatal angiogenesis, as has been shown in pathological conditions, the relative protein content of VEGF to PEDF was measured in complete rat eyes from birth until 3 months and the protein location of VEGF was compared to the one of PEDF by immunohistochemical means.

Results:

The same samples that were used to measure the PEDF content in complete rat eyes were used to calculate the overall amount of VEGF (Fig. IV.3.1). As a positive control, several concentrations of recombinant rat VEGF were loaded onto the gel. VEGF showed a migration pattern similar to the molecular weight of the protein in the eye samples and was slightly above 20 kDa. Omitting the primary antibody showed no staining (data not shown).

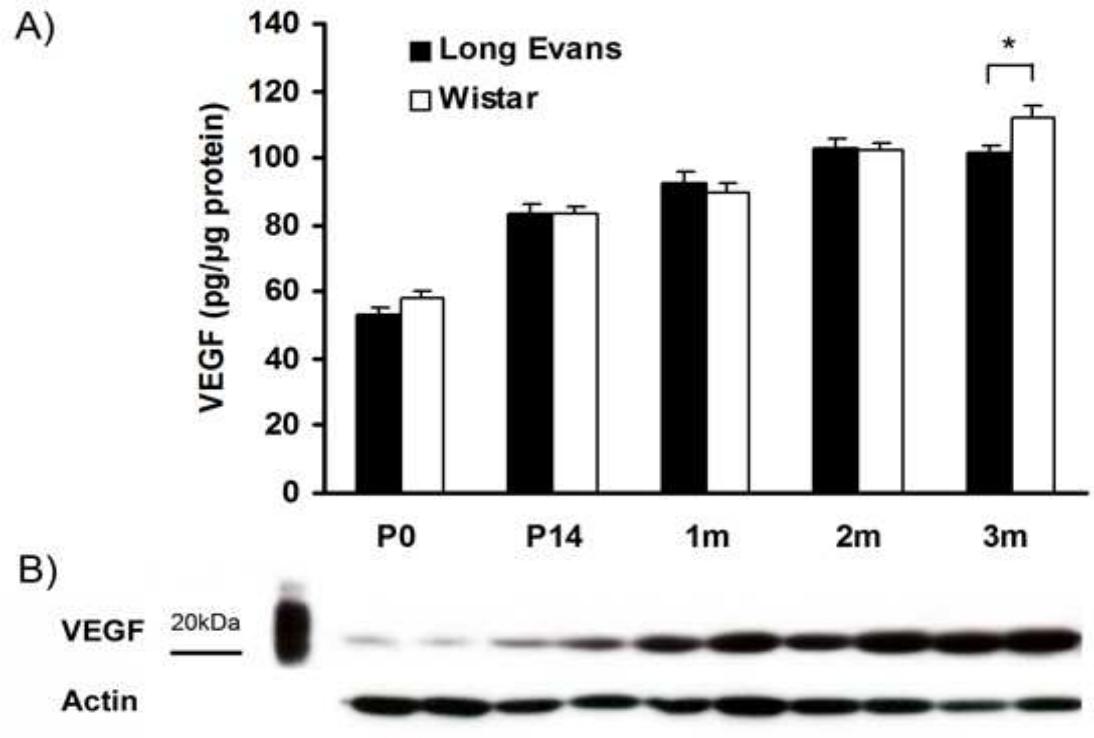


Fig. IV.3.1) VEGF content of complete Long Evans and Wistar rat eyes from birth until 3 months of age.

A) 50 µg of complete eye homogenates of animals between PO and 3 months were analyzed by Western blot analysis. VEGF was normalized to the average actin signal at each age and then compared to a standard curve of VEGF (2.5-10 ng, not shown). Data are shown as mean ± SEM. Black bars represent eyes of Long Evans rats and white bars Wistar eyes. A Mann-Whitney-U test showed that overall there was no significant difference in the VEGF content of eyes between the two rat strains (P=0.576, n=85), although at 3 months Wistar eyes contained significantly more VEGF than Long Evans eyes (P=0.027, n=17)**.** A Spearman Rank test showed that overall there was a significant increase in VEGF with age in both strains (Long Evans: n=41, r=0.794, P<0.000; Wistar: n=44, r=0.915, P<0.000). Significant changes between rat strains are indicated by asterisks.

B) Underneath the graph a Western Blot of one representative sample per age is shown for VEGF and actin. VEGF in samples migrated at similar height as the positive control (lane 1, 2.5 ng) and was detected slightly above 20 kDa.

The overall VEGF content in the eye was the lowest at birth (Long Evans = 53±2.6 pg/µg and Wistar = 58±2.0 pg/µg protein) and there was no significant difference between the rat strains at any of the ages tested, except at 3 months when Wistar eyes contained slightly, but significantly more VEGF than Long Evans eyes (Long Evans 101.2±2.6 pg/µg protein, Wistar 112±3.6 pg/µg protein, P=0.025, n=17). VEGF levels steadily increased from birth on and at 3 months Long Evans and Wistar eyes contained 1.91 and 1.93 times more VEGF than at birth, respectively. A Mann-Whitney-U test showed that overall there was no significant difference in the VEGF content between strains (P=0.576, n=84), but a significant change of VEGF was observed with increasing age for both strains (Spearman rank correlation for Long Evans n=41, r=0.794, P<0.000 and Wistar n=44, r=0.915, P<0.000). Wistar rat eyes contained a total of 2.9% more VEGF compared to Long Evans eyes.

PEDF and VEGF values from individual eye samples were used to calculate the ratio between VEGF and PEDF (Fig. IV.3.2). The VEGF/PEDF ratio was the lowest at birth and there was 22-24 times more PEDF than VEGF present in Wistar and Long Evans rat eyes, respectively. Between birth and two weeks of age, this ratio doubled and increased significantly ($P<0.000$, $n=30$), but remained fairly stable until 3 months and during this time there was 10.7-12.8 times more PEDF than VEGF in eyes of both rat strains. Between birth and 2 months, there was no significant difference in the VEGF/PEDF ratio when comparing the two rat strains at each individual age. At 3 months, however, there was a significant difference in the VEGF/PEDF ratio between Long Evans and Wistar rats ($P=0.036$, $n=17$), since Wistar eyes contained slightly less PEDF, but somewhat more VEGF than Long Evans eyes. A Spearman rank correlation showed a significant overall effect of age in respect to the VEGF/PEDF ratio for Long Evans ($n=41$, $r=0.445$; $P=0.004$) and Wistar eyes ($n=43$, $r=0.6$; $P<0.000$) due to the shift in the VEGF/PEDF ratio after birth. Between P14 and 2 to 3 months there was, however, no significant effect of age on the VEGF/PEDF ratio for Wistar ($n=29$, $r=0.169$; $P=0.384$) and Long Evans eyes ($n=34$, $r=0.02$; $P=0.912$), respectively.

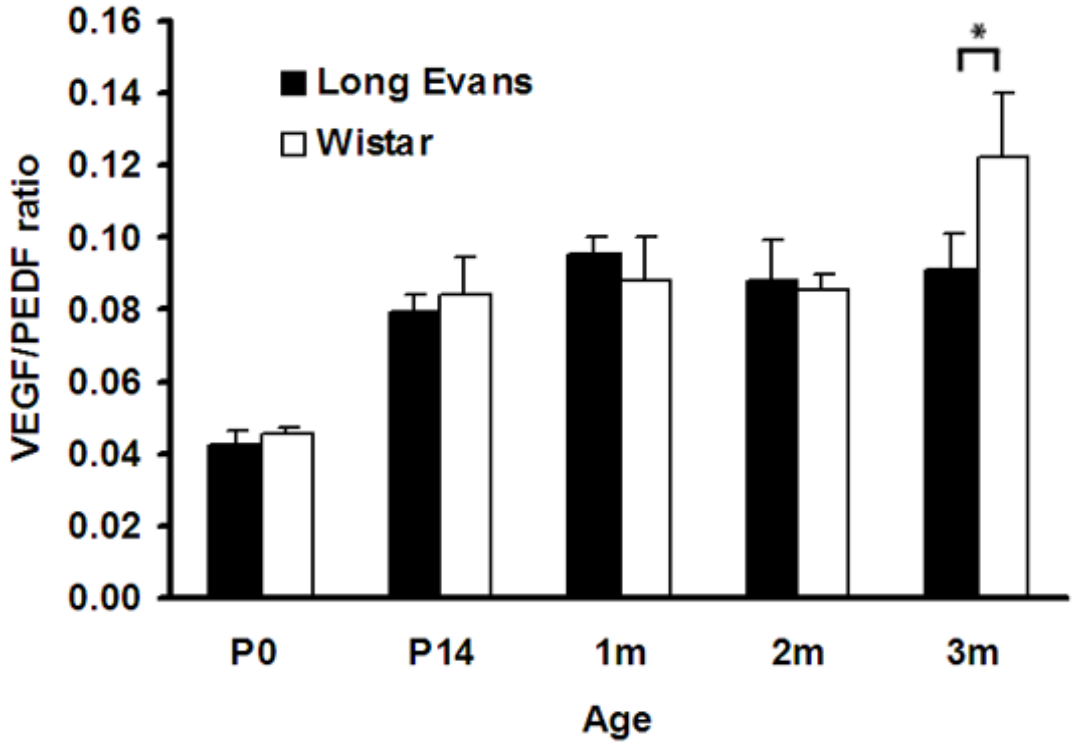
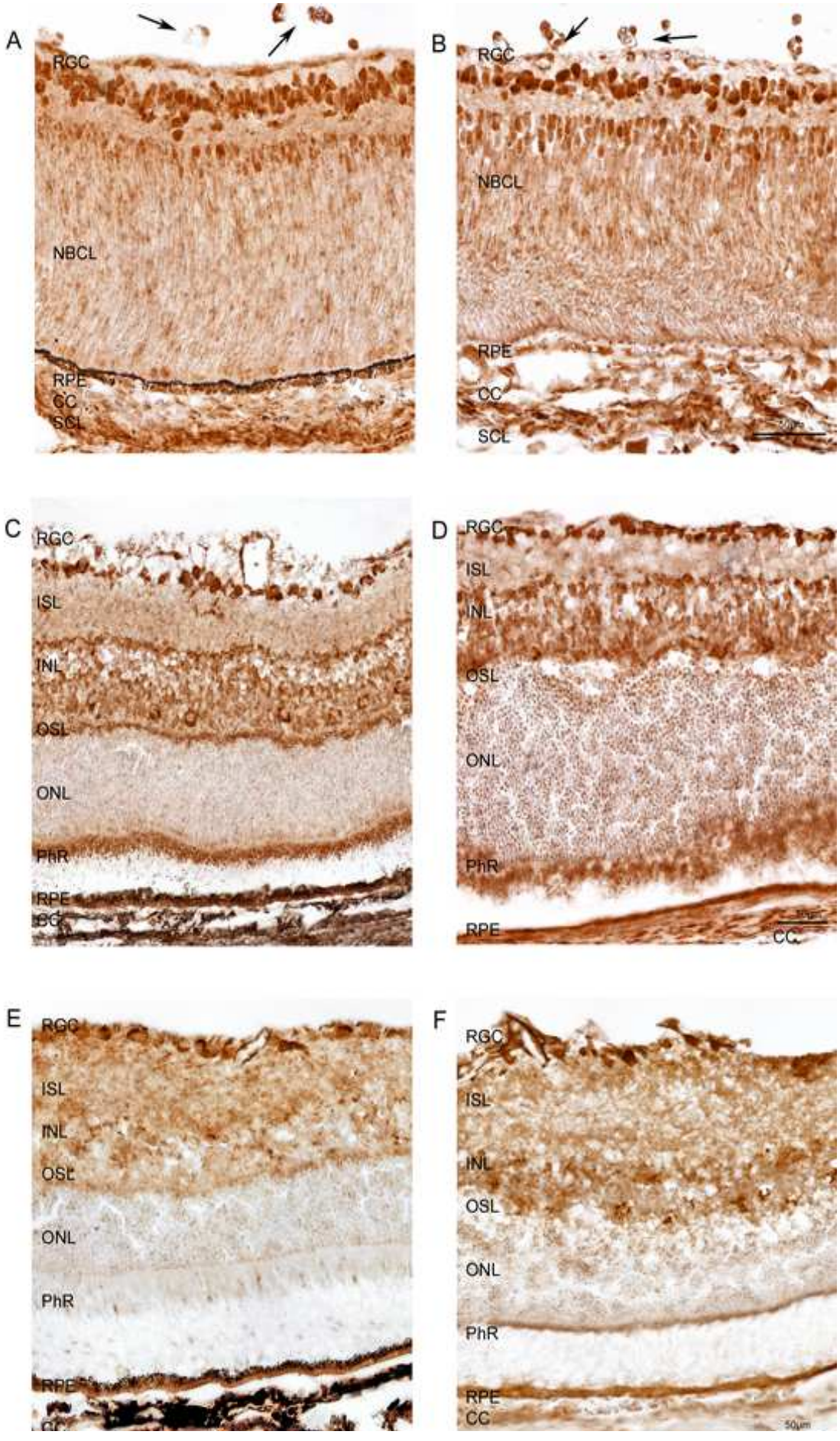


Fig. IV.3.2) VEGF/PEDF ratio of complete Long Evans and Wistar rat eyes from birth until 3 months of age.
The estimated amount of VEGF and PEDF of each sample was used to calculate the VEGF/PEDF ratio. Data are shown as mean ± SEM. Black bars represent eyes of Long Evans rats and white bars Wistar eyes. A Mann-Whitney-U test showed that overall there was no significant difference in the VEGF/PEDF ratio between Long Evans and Wistar eyes ($P=0.403$, $n=84$), although at 3 months the VEGF/PEDF ratio was significantly higher in Wistar eyes compared to Long Evans eyes ($P=0.036$, $n=17$). Overall, there was a correlation between the VEGF/PEDF ratio and age for both strains (Spearman rank test for Wistar: $n=43$, $r=0.6$, $P<0.000$ and Long Evans: $n=41$, $r=0.445$, $P=0.004$). However, between P14 and 2 to 3 months, respectively, there was no significant effect of age on the VEGF/PEDF ratio in Wistar ($n=29$, $r=0.168$, $P=0.384$) and Long Evans eyes ($n=34$, $r=0.02$, $P=0.912$). Significant changes between rat strains are indicated by asterisks.

To investigate the localization of VEGF protein in the eye, retinas at birth, 2 weeks and 3 months old Long Evans and Wistar rats were stained for VEGF (Fig. IV.3.3) and compared to the appropriate IgG controls, which were negative in all cases (see Fig. IV.1.2 G).


A
RGC
NBCL
RPE
CC
SCL
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RGC
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C
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Fig. IV.3.3) DAB immunohistochemical staining of Long Evans (left panel) and Wistar (right panel) retinas for VEGF at P0 (A and B), P14, (C and D) and 3 months (E and F) of age. The pictures show the weak staining of vessels on the surface of the retina (arrows) for VEGF, as well as the clear reaction of RGC somata for VEGF in both strains. Abbreviations: CC, choroid; INL inner nuclear layer; INS, inner synaptic layer; ONL, outer nuclear layer; OSL, outer synaptic layer; NBCL, neuroblastic cell layer; PhR, photoreceptors; RGC, retinal ganglion cells; RPE retinal pigment epithelial cells; SCL, sclera;

Since no obvious differences in staining location or staining intensities for VEGF protein were observed between the two rat strains, the following paragraph describes a general staining of both strains. Although VEGF staining was already fairly strong at birth, it appeared to be the most intense at two weeks of age and decreased again somewhat in adulthood.

As for PEDF, the RGC layer intensely stained for VEGF. Most cells of the INL stained for VEGF, although not quite as prominent as in the RGC layer. Comparable to the PEDF signal, cells in the ONL showed a slight VEGF signal at 2 weeks of age which decreased however to nearly nondetectable levels at 3 months of age. Like PEDF, VEGF stained photoreceptors relatively intense at 2 weeks of age, which also decreased to nearly nondetectable levels at 3 months of age. Comparably to PEDF, the RPE layer appeared to increase in VEGF staining after birth in both strains. Similarly to PEDF, a VEGF signal was detected in the choroid at all ages.

PEDF stained blood vessels in retinas of both strains as indicated by double labeling with the vessel marker Glut-1 (Fig. IV.3.4). However, no colocalization of VEGF and the vessel marker Glut-1 could be observed, although both proteins were in very close vicinity, especially at the surface of the

retina (Fig. IV.3.5). Vessels within the retina however clearly were only stained for Glut-1 but not for VEGF (data not shown).

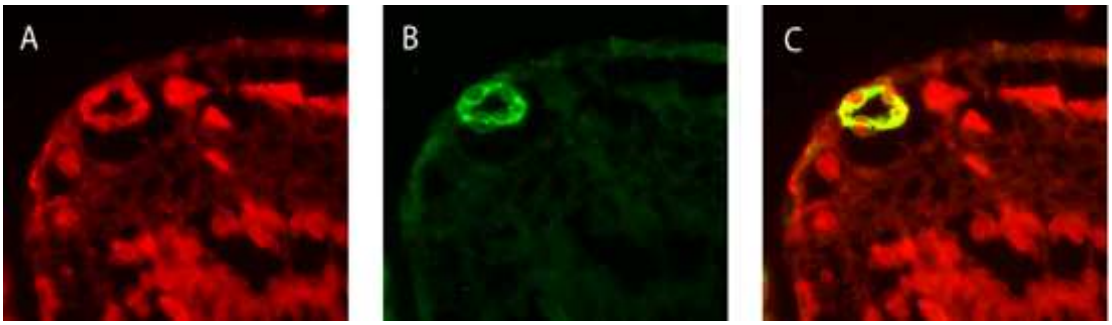


Fig. IV.3.4 Fluorescent double labeling of a vessel with PEDF (A) and Glut-1 antibody (B) of a 3 month old Wistar eye. Image (A) and (B) were super imposed which shows the double labeling of a vessel with PEDF and Glut-1 antibody as indicated by the yellow color (C).

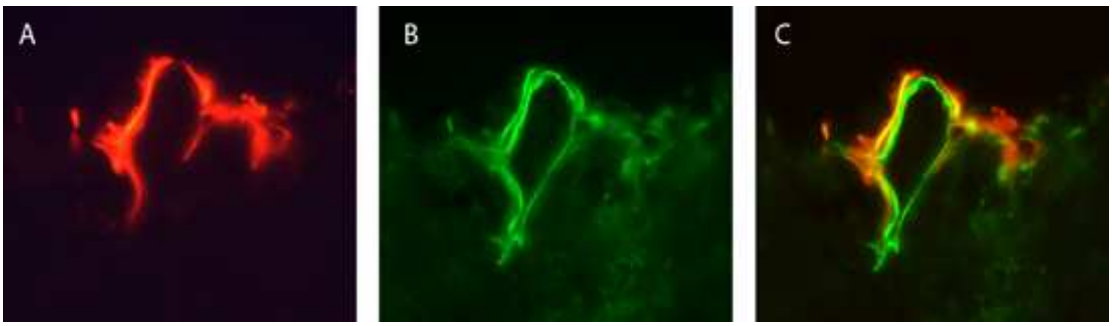


Fig. IV.3.5 Fluorescent double labeling of a vessel with VEGF (A) and Glut-1 antibody (B) of a 3 month old Long Evans eye. Image (A) and (B) were super imposed as shown in (C) which shows the close localization of VEGF and Glu-1 positive structures on the surface of the retina that was however not completely colocalized.

Discussion:

The results of this study show that Wistar and Long Evans retinas similarly stained for VEGF protein from birth until young adult age in regards to staining location and intensity, suggesting that angiogenic stimulators do not significantly outweigh angiogenic inhibitors during physiological angiogenesis that occurs in early postnatal development. Previous studies have shown that VEGF mRNA parallels retinal vascularisation in a temporal and spatial manner in rat neonates (Murata et al., 1996; Yi et al., 1998), although low VEGF protein levels can still be detected after completion of vascularisation (Chan et al., 2000). In these works, cells expressing VEGF in the retina included the RPE layer, INL and RGC layer, which were also labeled in this study. In the present study, VEGF protein was readily detectable also in adulthood. However, VEGF labeling appeared to be the most intense at 2 weeks of age, at a time when the vascular network is not quite complete yet, which is in line with previous reports (Murata et al., 1996; Yi at al, 1998).

At birth, complete eyes contained far more PEDF than VEGF and the VEGF/PEDF ratio even increased with age which would implicate a more stimulatory environment for vessel growth. Thus, the data of complete rodent eyes do not support the hypothesis that during normal vessel growth a high VEGF to PEDF ratio is needed for vessels to grow as has been suggested before and has been shown to be the case in pathological conditions. However, one needs to be careful interpreting these results since the whole eye contains vascularised areas and compartments that are devoid of vessel (see also Fig.

I.5.1.1). Furthermore, PEDF accumulates to different degrees in different compartments of the eye and the VEGF content in the retina was not tested. Also, the employed antibodies could have different sensitivities for the stained rat tissue and the employed positive control. This would however only affect the absolute concentrations of the growth factors, but not the change of their respective ratio over time.

If immunohistochemical stainings in fact mirrored the PEDF and VEGF content in the retina, then at birth rodent retinas contained about equal amounts of both growth factors, or possibly slightly more VEGF. This would indicate more of a stimulatory environment for vessel growth compared to data from complete eyes and be in support of the above hypothesis.

However, outgrowing vessels on the surface of the retina just after birth clearly stained strong for PEDF, which would not be in line with the idea of PEDF as an antiangiogenic agent. Although on the surface of the retina, VEGF stained structures in the close vicinity of vessels that were most likely glia cells, vessels themselves were not labeled for VEGF. Furthermore, photoreceptor cells stained stronger for VEGF than for PEDF, although it has been shown that PEDF has a neuroprotective effect on photoreceptor cells (Cao et al., 2001) and normally there are no vessels in this layer.

One hypothesis that might explain these observations would be that at early postnatal age, PEDF might function more as a neuroprotective factor in the retina rather than an antiangiogenic agent and/or that the function of PEDF changes over time. This idea is supported by several findings. First, it has been

shown that depending on the phosphorylation status of PEDF, the protein can function as an antiangiogenic factor or a neurotrophic agent (Maik-Rachline et al., 2004). Thus, depending on the existence of specific intra- and extracellular protein kinases PEDF can have different effects. Second, PEDF was proven to even have a synergistic action on VEGF-induced bovine retinal endothelial cell proliferation when cells were previously cultured with VEGF. PEDF however had an inhibitory effect on cell growth when cells were not previously stimulated by VEGF (Hutchings et al., 2002). Thus, depending on the phenotype PEDF can exert opposite effects on endothelial cells. This idea is supported by data that show that during pathological neovascularisation PEDF only induced apoptosis of endothelial cells that are activated and participate in neovascularisation, and that PEDF did not harm existing vessels (Stellmach et al., 2001). Third, PEDF is even able to protect cultured retinal pericytes surrounding the microvasculature from advanced glycosylation end product-induced apoptosis through its antioxidative properties (Yamagishi et al., 2002). All these data therefore indicate that PEDF may not necessarily always act as an antiangiogenic agent on vessel growth and could have even a stimulatory effect during physiologically occurring angiogenesis. Clearly, more research needs to be done in order to find out under which conditions PEDF has an inhibitory or even stimulatory effect on endothelial cell growth.

In total, there was a high degree of overlap in the location of the two growth factors in the retina. Both growth factors seemed to stain many of the

same cell types and were located in close vicinity to each other, suggesting that their regulation may be closely correlated with each other in many cell types. In fact, intravitreal injections of the angiogenic inhibitor plasminogen kringle 5 both downregulates VEGF and upregulates PEDF in the entire retina, as well as in cultured retinal vascular cells (Gao et al., 2002b). Furthermore, VEGF secreted by RPE cells upregulates the expression of PEDF via VEGFR1 in an autocrine manner (Ohno-Matsui et al., 2003), indicating that PEDF and VEGF protein expression are closely linked.

In conclusion, the VEGF and PEDF protein content of complete eyes and the immunohistochemical stainings of retinas do not support the idea that during angiogenesis that occurs early in postnatal development angiogenic stimulators significantly outweigh angiogenic inhibitors. However, further experiments that focus more on the VEGF and PEDF content in the retina will be needed to answer this question in more detail. The data of this study suggest that the expression of VEGF and PEDF may be closely correlated during times of physiologically occurring angiogenesis in the rodent eye, and that under normal physiological conditions PEDF may function more as a neurotrophic and neuroprotective factor rather than an antiangiogenic factor in the retina.

IV.4. Is a change of the VEGF/PEDF ratio and VEGF protein expression related to the observed neovascularisation in old Wistar eyes?

Introduction:

In the eye ischemic events underlie the progression of several blinding diseases. Low oxygen levels have been shown to increase the production of VEGF which is known to be a key angiogenic factor involving neovascularisation. On the other hand, there is evidence that PEDF induces apoptosis of endothelial cells that are participating in neovascularisation (Stellmach et al., 2001). It has been previously shown that the balance of VEGF and PEDF is disturbed during pathological neovascularisation (Ohno-Matsui et al., 2001; Gao et al., 2001), and there are indications that species specific alterations in the ratio of VEGF to PEDF may be the reason for the high sensitivity of some rat strains to neovascularisation after hyperoxia (Gao et al., 2002a).

The growth of neovascular membranes from the choroid into the retina is a major cause of blindness in AMD. Besides retinal degeneration that has been linked to AMD, old Wistar eyes also showed signs of neovascularisation while age-matched Long Evans retinas appeared morphologically intact. To test the hypothesis that a change in the VEGF/PEDF ratio might be related to the observed neovascularisation and degeneration at old age, the VEGF/PEDF ratio was measured in Wistar and Long Evans eyes between 3 and 22 months of age and the VEGF protein location was compared to the one of PEDF in 15-17 months old rat retinas by immunohistochemical means. Since VEGF A has been

found to be the major form of VEGF responsible for neovascularisation (Carmeliet and Collen, 1999), an antibody specific to VEGF A was employed.

Results:

As previously shown in figure IV.3.1, the VEGF content of complete rat eyes significantly increased and roughly doubled between birth and 3 months of age. After 3 months however, a Kruskal Wallis test showed that age had no significant effect on the VEGF content of combined Long Evans and Wistar eyes any more ($P=0.094$, $n=51$), and at 22 months, Long Evans and Wistar eyes contained 2.45 and 1.96 times more VEGF than at birth, respectively (Fig. IV.4.1). When rat strains were tested individually, a Spearman Rank test indicated that age still had an effect on the VEGF content in Long Evans eyes ($n=26$, $r=0.654$, $P<0.000$), but not in Wistar eyes ($n=25$, $r=0.794$, $P=0.055$). However, no significant difference could be found in between rat strains at any age tested. In total, Wistar rat eyes contained 2.8% more VEGF compared to Long Evans eyes.

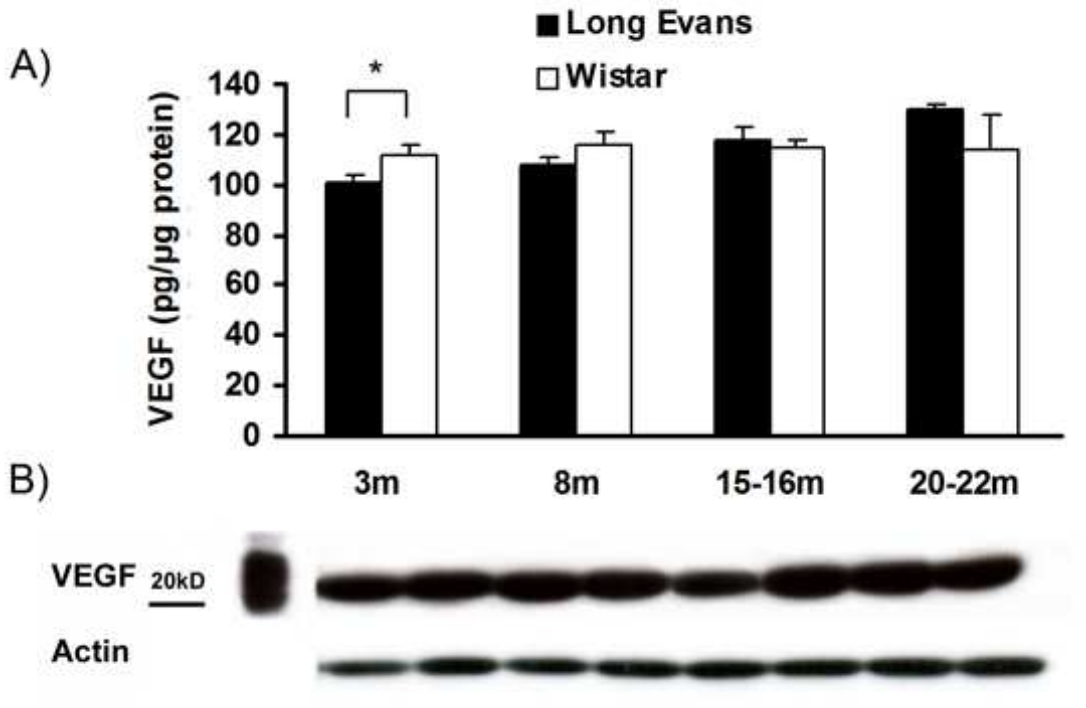


Fig IV.4.1) VEGF content of complete Long Evans and Wistar rat eyes from 3 months until high age.

A) 50 µg of complete eye homogenates of animals between 3 and 22 months were analyzed by Western blot analysis. VEGF was normalized to the average actin signal at each age and then compared to a standard curve of VEGF (2.5-10 ng, not shown). Data are shown as mean ± SEM. Black bars represent eyes of Long Evans rats and white bars Wistar eyes. A Kruskal Wallis test showed that for Long Evans and Wistar eyes combined, overall there was no significant difference in the VEGF content over age ($P=0.094$, $n=51$). However, when testing the strains separately, a Spearman Rank test showed that there was a correlation between the VEGF content in Long Evans eyes and age ($n=26$, $r=0.654$, $P<0.000$), but not for Wistar eyes ($n=25$, $r=0.055$, $P=0.794$). Significant changes between rat strains are indicated by asterisks.

B) Underneath the graph a Western Blot of one sample per age is shown for VEGF and actin. PEDF samples migrated at the same height as the positive control (lane 1, 2.5 ng) and were detected slightly above 20 kDa.

As has been shown before, the VEGF/PEDF ratio was the lowest at birth, but remained fairly stable until 3 months (Fig. IV.3.2 and Fig. IV.4.2). At older ages however, the VEGF/PEDF ratio increased dramatically due to decreasing PEDF and slightly increasing VEGF levels, so that at 20-22 months there was only 2.2 and 3 times more PEDF than VEGF in Long Evans and Wistar eyes, respectively, compared to 24-22 times more PEDF than VEGF at birth (Fig. IV.4.2). At 8 and 15 months, the VEGF/PEDF ratio of Long Evans eyes was significantly higher than that of Wistar eyes (8 months: $P=0.024$, $n=18$; 15 months: $P=0.024$, $n=9$), since Wistar eyes contained significantly more PEDF than Long Evans eyes. Between 3 and 22 months, a Spearman rank correlation showed a significant overall effect of age on the VEGF/PEDF ratio for Long Evans ($n=24$, $r=0.74$, $P<0.000$) and Wistar eyes ($n=26$, $r=0.906$, $P<0.000$) which increased over time.

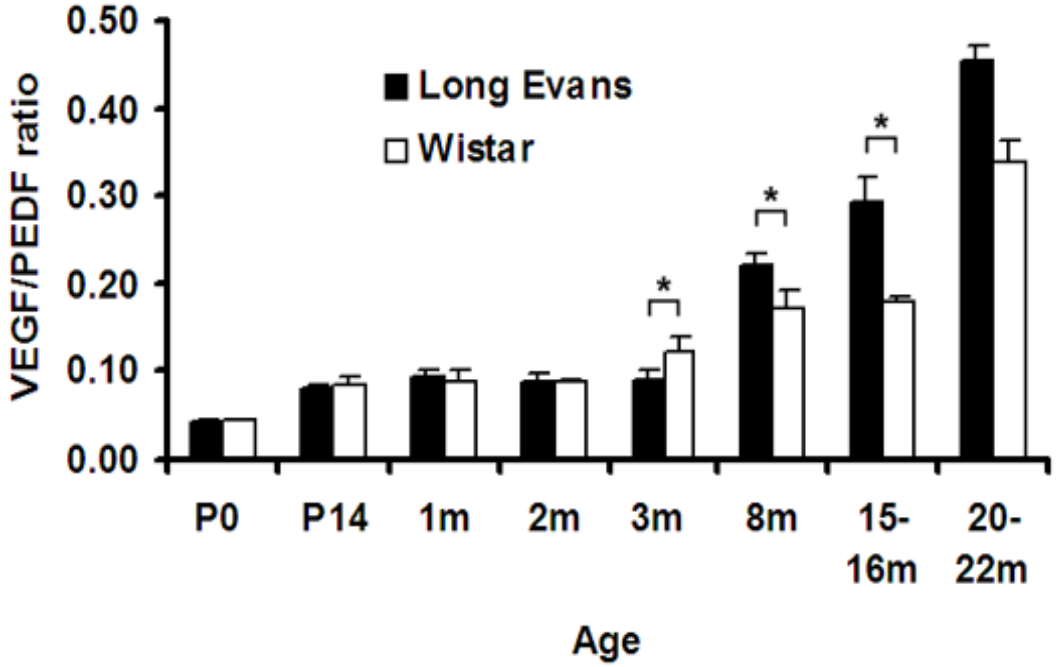


Fig. IV.4.2) VEGF/PEDF ratio of complete Long Evans and Wistar rat eyes from birth until old age.
The estimated amount of VEGF and PEDF of each sample was used to calculate the VEGF/PEDF ratio. Data are shown as mean ± SEM. Black bars represent eyes of Long Evans rats and white bars Wistar eyes. A Mann-Whitney-U test showed that overall there was no significant difference in the VEGF/PEDF ratio between Long Evans and Wistar eyes (P=0.756, n=117), although at 3, 8 and 15 months there was a significant difference in the VEGF/PEDF ratio between strains (3 months: P=0.036, n=17; 8 months: P=0.024, n=18; 15 months: P=0.024, n=9). Overall, the VEGF/PEDF ratio significantly increased with age for both strains (Spearman rank test for Wistar: n=58, r=0.807; P<0.000 and Long Evans: n=59, r=0.809; P<0.000). Significant changes between rat strains are indicated by asterisks.

According to the VEGF/PEDF ratio of complete eyes, Long Evans rats should be more susceptible to neovascularisation than Wistar rats. Therefore, retinas from 15-17 months old Long Evans and Wistar eyes were stained for VEGF to test for any strain specific differences within the retina in more detail.

Stainings were compared to the appropriate IgG controls, which were negative in all cases (see Fig. IV. 2.2). As has been shown already in figure IV.2.2, vessels transversed the entire Wistar retina in some areas indicating the growth of new blood vessels, which could not be observed in Long Evans retinas. Furthermore, only the choroid of Wistar retinas contained a high amount of blood vessels with an especially large diameter which had not been detected to this extent at younger ages.

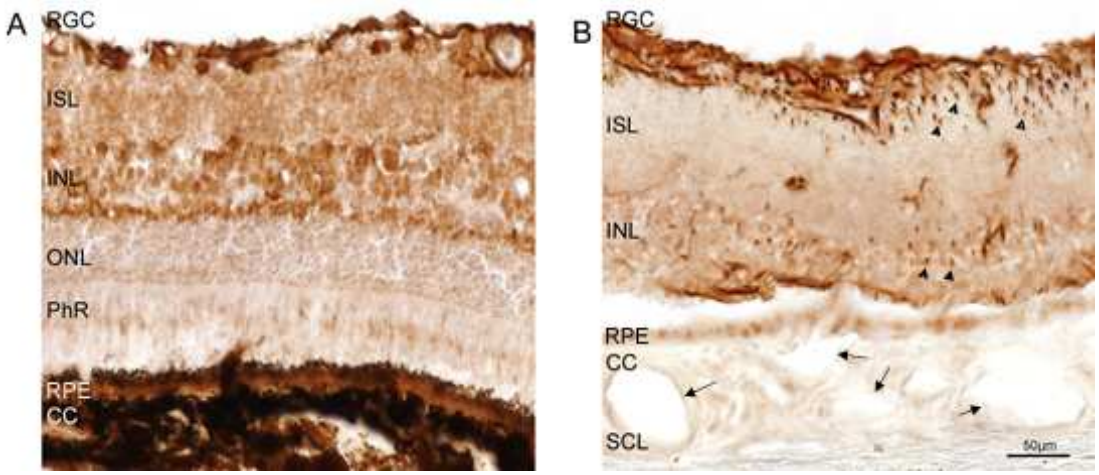


Fig. IV.4.3) DAB immunohistochemical staining of 15-17 months old Long Evans (A) and Wistar (B) retinas with VEGF antibody.
The pictures show the decreased staining of the RPE layer as well as somata of RGC and INL cells in the old Wistar retina compared to the Long Evans retina. The appearance of fiber-like structures in the ISL is indicated by arrow heads. Large vessels in the choroid of Wistar retinas are marked by arrows. Abbreviations: CC, choroid; INL inner nuclear layer; ISL, inner synaptic layer; ONL, outer nuclear layer; PhR, photoreceptors; RGC, retinal ganglion cells; RPE retinal pigment epithelial cells; SCL, sclera;

As for PEDF, the RGC layer intensely stained for VEGF in both rat strains. In old Wistar retinas one could however observe a shift of VEGF staining from RGC somata and from somata from the INL to GFAP stained fibers that appeared in the RGC layer, ISL and INL of the retina (Fig. IV.4.4 and IV.4.3). This shift in VEGF staining location was much less pronounced in old Long Evans retinas where VEGF/GFAP double labeled fibers could only be detected on the surface of the retina, especially in the vicinity of large vessels (Fig. IV.4.5). No GFAP/PEDF double labeling could be detected in any of the rat strains at old age (Fig. IV.4.6 and Fig. IV.4.7).

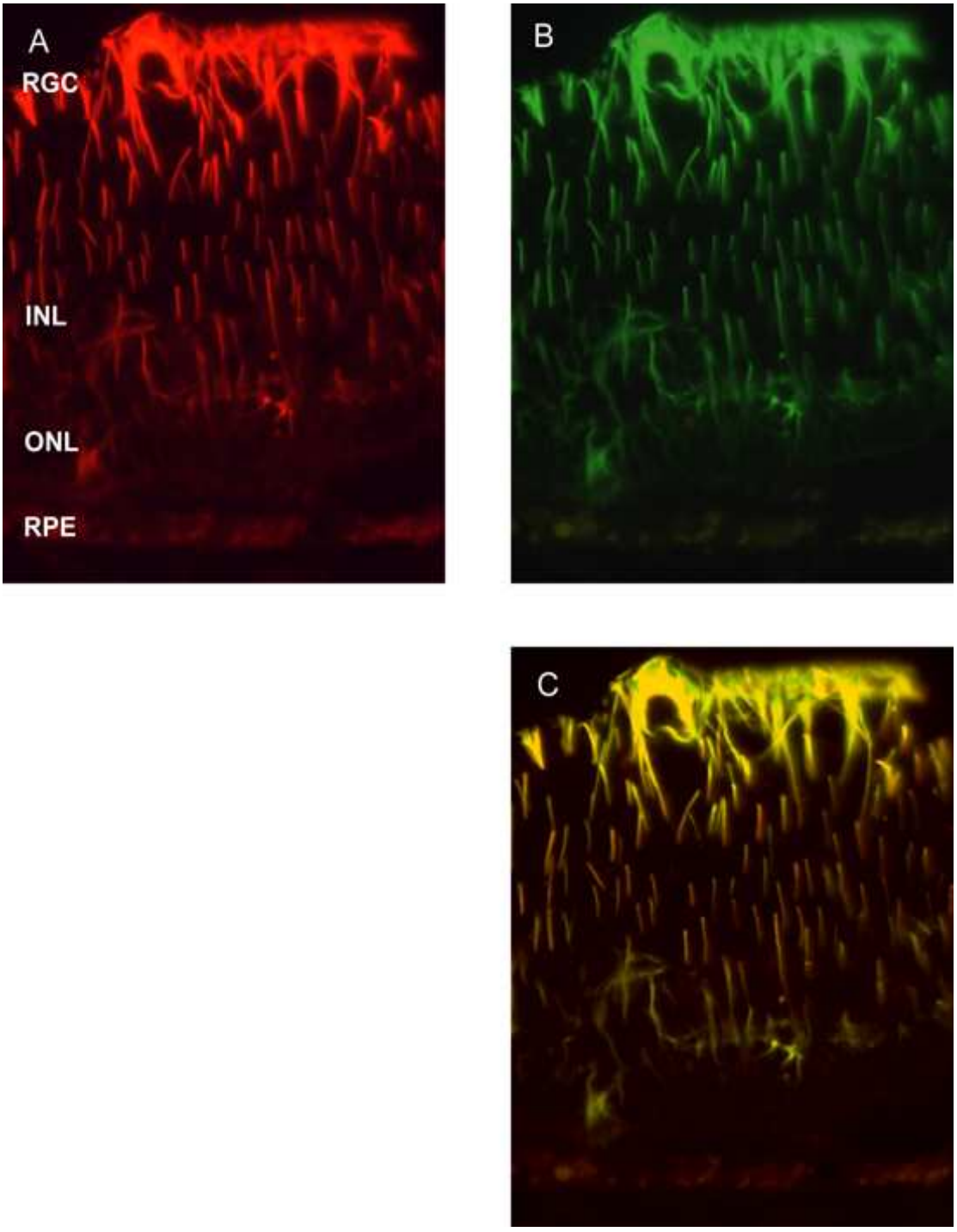


Fig. IV.4.4. Fluorescent double labeling of an old Wistar retina with VEGF (A) and GFAP antibody (B). The image shows the lack of VEGF stained somata in the INL. An overlay of the VEGF and GFAP staining is seen in (C) which shows a complete overlap of VEGF and GFAP stained structures in old Wistar retinas as shown by the yellow color. Abbreviations: INL inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cells; RPE retinal pigment epithelial cells;

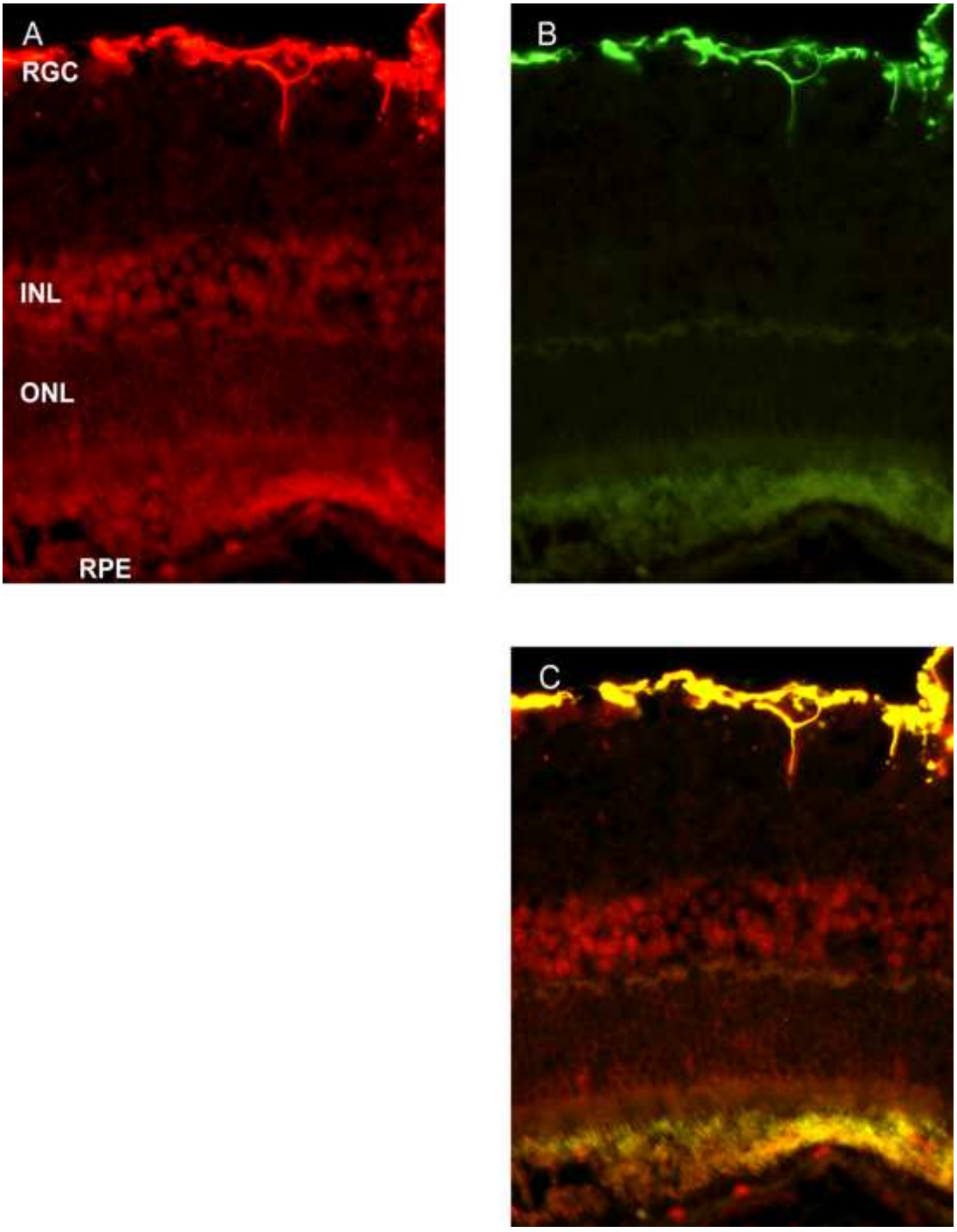


Fig. IV.4.5. Fluorescent double labeling of an old Long Evans retina with VEGF (A) and GFAP antibody (B). An overlay of the VEGF and GFAP staining is shown in (C) which shows that in old Long Evans retinas VEGF/GFAP double labeled structures were only located at the surface of the retina as indicated by the yellow color. Abbreviations: INL inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cells; RPE retinal pigment epithelial cells;

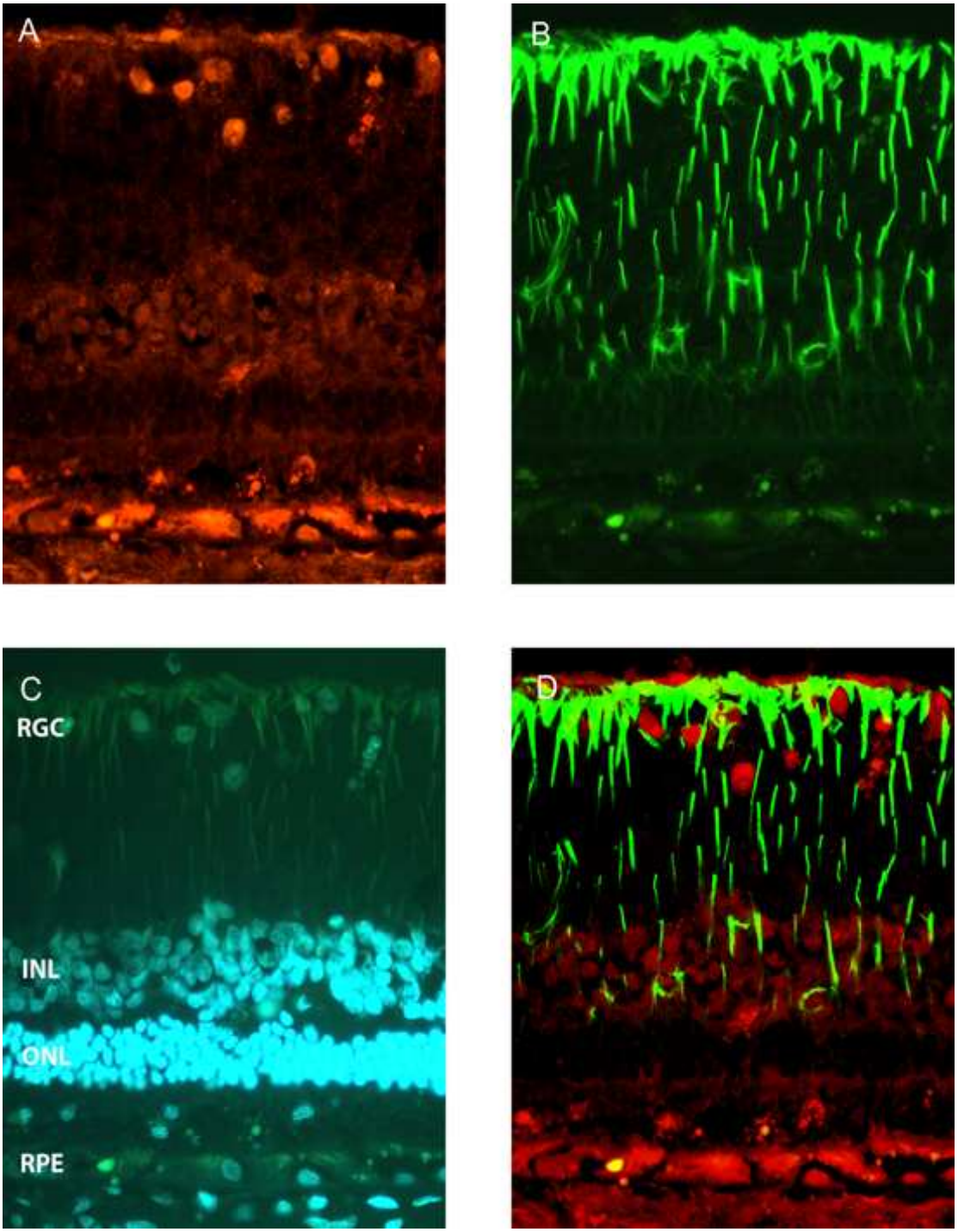


Fig. IV.4.6. Fluorescent double labeling of an old Wistar retina with PEDF (A) and GFAP antibody (B). A nuclear staining of the same area with Hoechst is shown in (C) and an overlay of the PEDF and GFAP staining in (D). The figure shows that there was no colocalization of PEDF and GFAP labeled structures anywhere in the retina. Abbreviations: INL inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cells; RPE retinal pigment epithelial cells;

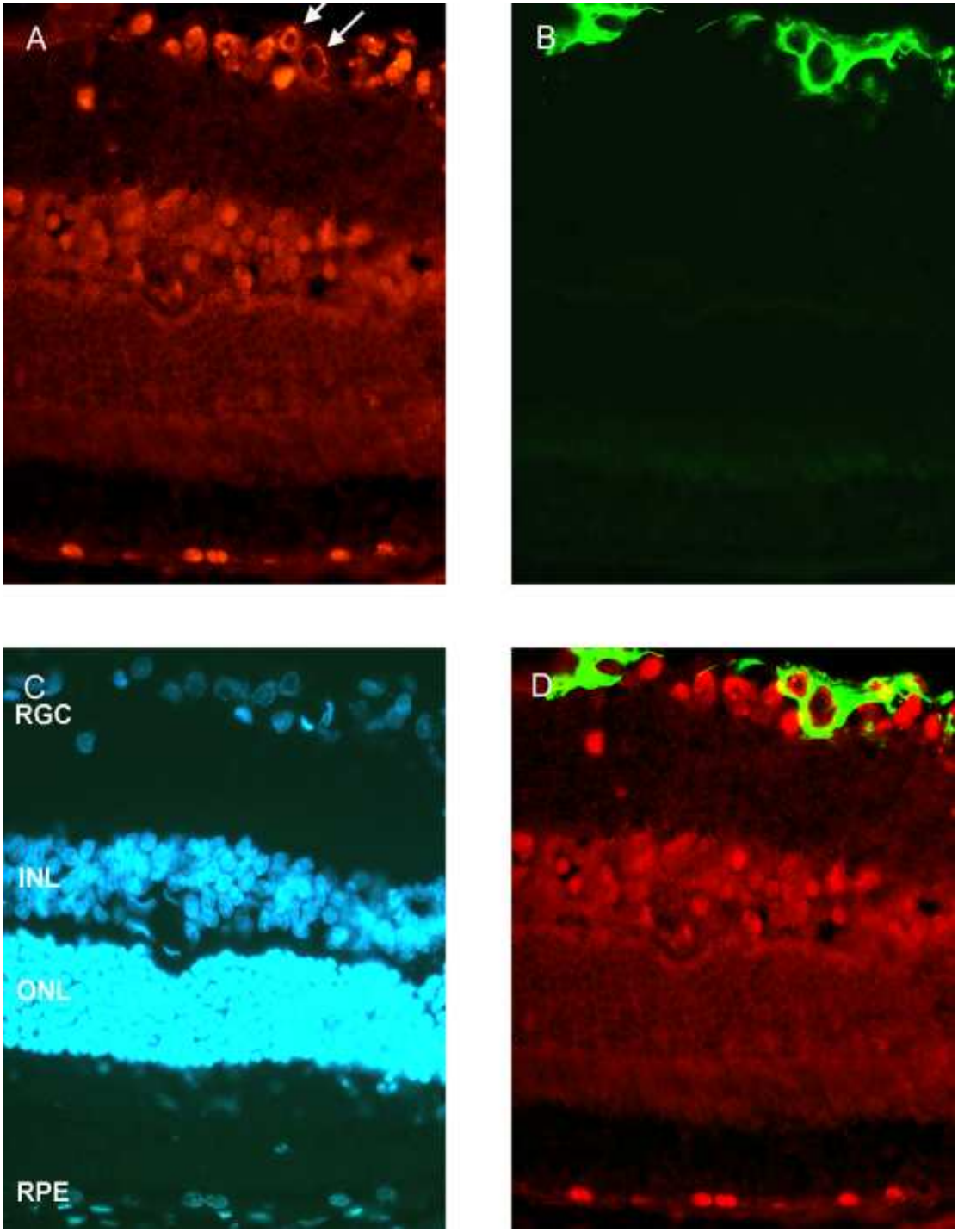


Fig. IV.4.7. Fluorescent double labeling of an old Long Evans retina with PEDF (A) and GFAP antibody (B). A nuclear staining of the same area with Hoechst is shown in (C) and an overlay of the PEDF and GFAP staining in (D). The figure shows that there was no PEDF/GFAP colocalization, although GFAP positive astrocytes were in close vicinity to PEDF positive vessels (arrows). Abbreviations: INL inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cells; RPE, retinal pigment epithelial cells;

In Long Evans retinas the ONL and photoreceptor layer weakly stained for both PEDF and VEGF. A strong VEGF staining was detected in the RPE layer of Long Evans retinas while the VEGF labeling of RPE cells of old Wistar retinas had decreased a lot (Fig. IV.4.3). The VEGF signal in the choroid of old Wistar eyes had decreased to nearly background levels compared to 3 month old animals, while the same layer showed a strong staining for PEDF (see Fig. IV.2.2). Due to the pigmentation in Long Evans rats the staining of both growth factors in the choroid was difficult to determine in this strain.

In summary, in general PEDF stained the same cell types and layers in both rat retinas at old age although with slightly changing intensities. The same was also true for VEGF regarding Long Evans retinas. In old Wistar retinas there was, however, an obvious shift in VEGF protein expression from RGC somata and cells of the INL to newly appearing GFAP labeled fibers in the RGC layer, ISL and INL.

Discussion:

Western blot results indicate that complete Wistar and Long Evans eyes contained similar amounts of VEGF. In Wistar eyes, the VEGF content did not significantly change after young adulthood was reached, while it slightly, but significantly increased with increasing age in Long Evans eyes. Since morphologically intact Long Evans eyes even had a significantly higher VEGF/PEDF ratio between 8 and 15 months, they should be more susceptible to

neovascularisation than Wistar eyes. The data therefore suggest, that a high VEGF/PEDF ratio of complete eyes cannot be responsible for the observed signs of neovascularisation in old Wistar rats and that the two rat strains may have a different susceptibility to neovascularisation in regards to the VEGF/PEDF ratio.

However, one needs to be careful to compare data of complete eyes with the retina. Since the retina contributes only a relatively small amount of PEDF to the overall PEDF content in the entire eye, the finding that complete eyes of old Long Evans rats had a high VEGF/PEDF ratio may not be contradictory to the hypothesis that a high VEGF/PEDF ratio in the retina (and not complete eyes) might be related to pathological neovascularisation. Indeed, within the retina there are several indications that a change in immunohistochemical staining patterns of VEGF and PEDF at old age might contribute to an unbalanced expression of the two growth factors and might therefore be related to retinal neovascularisation.

First, the findings of this study that RPE cells of old Wistar retinas stained less for PEDF, and that Wistar retinas contained less PEDF than those of Long Evans rats would contribute to an increased VEGF/PEDF ratio within the Wistar retina. This would be in line with the report that RPE cells produce less PEDF under oxidative stress, while their VEGF production is increased (Ohno-Matsui et al., 2001).

Second, old Wistar retinas also showed an obvious shift in VEGF protein expression from RGC somata and cells of the INL to GFAP labeled fibers. GFAP

has been previously shown to label Muller glia cells in the eye during retinal stress and in pathological states including AMD (Fan et al., 1996; DiLoreto et al., 1995; Wu et al., 2003). The observed shift in VEGF expressing cells is also in line with a previous study that has shown an upregulation of VEGF in the retina of patients with diabetes and disciform AMD in astrocytes and Muller cells (Sueishi et al., 1996; Amin et al., 1997). Also, activated or VEGF expressing Muller cells have been associated with photoreceptor cell loss and neovascularisation (Amin et al., 1997; Roque and Caldwell, 1990; Perez and Perentes, 1994). Activation of Muller cells was also reported to occur during photoreceptor degeneration, and precedes retinal vascularisation in Royal College of Surgeons rats with inherited retinal dystrophy (Roque and Caldwell, 1990). In Roque and Caldwell's study RGC's as well as RPE cells of AMD retinas showed an apparent decrease in VEGF expression which is in line with the data of this study. These reports together with the findings of the present study support the idea that the staining of Muller cells for VEGF is associated with the observed degeneration and revascularization of old Wistar retinas. It has been also shown that RPE removal from the retina causes degenerative changes in Muller cells, such as a failure to form adherents junctions with photoreceptor cells, suppression of glutamine synthetase expression, and, notably, the expression of GFAP. Interestingly, PEDF addition aborted these pathological changes in Muller cells including the expression of GFAP (Jablonski et al., 2001). Given these observations, it is likely that PEDF might also be able to reduce the VEGF expression of these cells. A decrease in PEDF within the retina might

therefore contribute to the VEGF expression of Muller glia which seems to be a prerequisite for retinal degeneration and revascularization.

A third indication for an unbalanced expression of the two growth factors is the finding that similar to cells of the INL and RGC's, RPE cells of old Wistar rats showed a strong decrease in VEGF staining and the choroid practically did not stain for VEGF any more. It has been shown that RPE cells preferentially secrete VEGF towards their basolateral side (Blaauwgeers et al., 1999) and that RPE cells have a decreased expression of VEGF during AMD (Roque and Caldwell, 1990), which might explain why practically no VEGF could be localized in the choroid any more at old age. There is further evidence that only continuously administered VEGF can induce the fenestration of endothelial cells (Ribatti et al., 2001; Eriksson et al., 2003), as is typically the case in the choroid. Thus, a lack of VEGF in the choroid due to a decreased VEGF expression by RPE cells could potentially explain the observed widened vessels in the choroid of old Wistar eyes.

Thus, in the degenerated albino retina the strength and cellular location of the expression of the two growth factors was not as closely correlated as observed in younger animals which could contribute to the observed degeneration and neovascularisation of the retina.

In summary, one can say that although the VEGF/PEDF ratio of the entire eye significantly increased in both rat strains at old age, a high VEGF/PEDF ratio in the entire eye cannot be associated with degenerative diseases of the eye, but

may be a naturally occurring process at old age that could however make certain older individuals more susceptible to degenerative processes including neovascularisation. The decreased labeling of RPE cells for PEDF in old Wistar retinas in combination with a decrease of PEDF in the entire retina compared to Long Evans retinas supports the idea that a shift in the VEGF/PEDF ratio within the retina may be associated with the observed retinal neovascularisation as well as degeneration. Furthermore, the uncoupling of the protein expression of the two growth factors due to the observed increase in VEGF staining of glia cells in the old Wistar retina may be also an important indicator for retinal damage and may even be a prerequisite for vessel growth.

IV.4. Final conclusions of chapter IV

- The data of this study show that the protein expression of PEDF in the entire eye was high at birth until young adulthood, but decreased from thereon with increasing age of the animal. This appeared to be a natural phenomenon independent of retinal degeneration or vascularisation.
- Overall there was no correlation between the lack of pigmentation in Wistar eyes and the PEDF content of complete eyes or its protein localization within the retina at younger ages.
- A decrease of PEDF production by RPE cells in old Wistar eyes could contribute to the observed reduction of PEDF within the entire retina and might therefore contribute to the observed retinal degeneration and neovascularisation.
- While the protein localization of PEDF and VEGF appeared to be closely regulated in the healthy eye, the expression of the two growth factors was uncoupled during pathophysiological conditions at advanced age. These changes were especially evident regarding the VEGF location which shifted from RGC and INL cells to Muller glia cells which might contribute to a VEGF induced neovascularisation.
- In the complete eye PEDF outweighed VEGF during early physiologically occurring angiogenesis, suggesting that early during development PEDF may act more as a neurotrophic and differentiating factor than as an angiogenic one.

- At old age the VEGF/PEDF ratio in entire eyes increased significantly. This increased ratio and the uncoupling of the location of the two growth factors at old age compared to birth suggest that vessel growth during normal physiological angiogenesis and pathological neovascularisation may be controlled in a different way.

V. PEDF protein content and localization in the rat brain during postnatal development.

V.1. Can PEDF protein be immunolocated in the adult rat brain?

Introduction:

Several studies provide evidence that PEDF does not only have a neuroprotective effect on various cell types in the eye, but also on other cells in the CNS (Tombran-Tink and Barnstable, 2003b; Barnstable and Tombran-Tink, 2004). The neurotrophic effect of PEDF on cells in the brain has been demonstrated mainly by *in vitro* experiments by its ability to support neuronal survival, to protect neurons against various neurotroxic effects and in some cases to induce neuronal differentiation. So far, it has been shown that PEDF has survival and/or differentiating effects on primary cultures of rat cerebellar granule neurons (Taniwaki et al., 1995 and 1997; Araki et al., 1998), immature primary rat hippocampal neurons (DeCoster et al., 1999), and avian and murine spinal motor neurons (Houenou et al., 1999; Bilak et al., 1999 and 2002). PEDF has also been shown to stimulate the metabolism and chemokine production of microglia cells and thereby indirectly inhibits the proliferation of astroglia(Yabe et al., 2005; Sugita et al., 1997; Takanohashi et al., 2005). Thus far, PEDF was shown to have protective effects on Muller glia function in the retina and enhances the growth and survival of Schwann cells in the peripheral nervous system. Although both Muller and Schwann cells have been shown to also

produce PEDF (Eichler et al., 2004a and b; Crawford et al., 2001), so far, however, there is no evidence that PEDF has neuroprotective effects on further glia cells or that PEDF is produced by other types of glia cells in the brain.

Previous research has shown that PEDF might be produced in the brain, since its mRNA was found in homogenates of various human brain areas (Tombran-Tink et al., 1996) and PEDF was proven to exist in CSF (Bilak et al., 1999; Kuncl et al., 2002). However, it is also known that PEDF exits in rather high concentration in the blood (Peterson et al., 2003; Maik-Rachline et al., 2004). The finding of our laboratory, that PEDF mRNA is expressed by monocytes, macrophages, granulocytes and dendritic cells of the blood suggests that PEDF may be produced by blood cells as well (see Fig. V.1.1). It is therefore not clear whether PEDF protein is in fact produced by cells in the CNS, or if the protein and its mRNA that have been found in brain homogenates is in fact derived from blood cells. With the exception of a PEDF immunohistochemical reaction of human, monkey and rat motor neurons and ependymal cells in the spinal cord (Bilak et al., 1999 and 2002), thus far, there are no studies about the specific localization of the PEDF protein in the healthy CNS during postnatal development. To find out more about the localization of the protein in the CNS, PEDF immunohistochemistry was performed to determine if there are any PEDF positive cells in the adult rat brain.

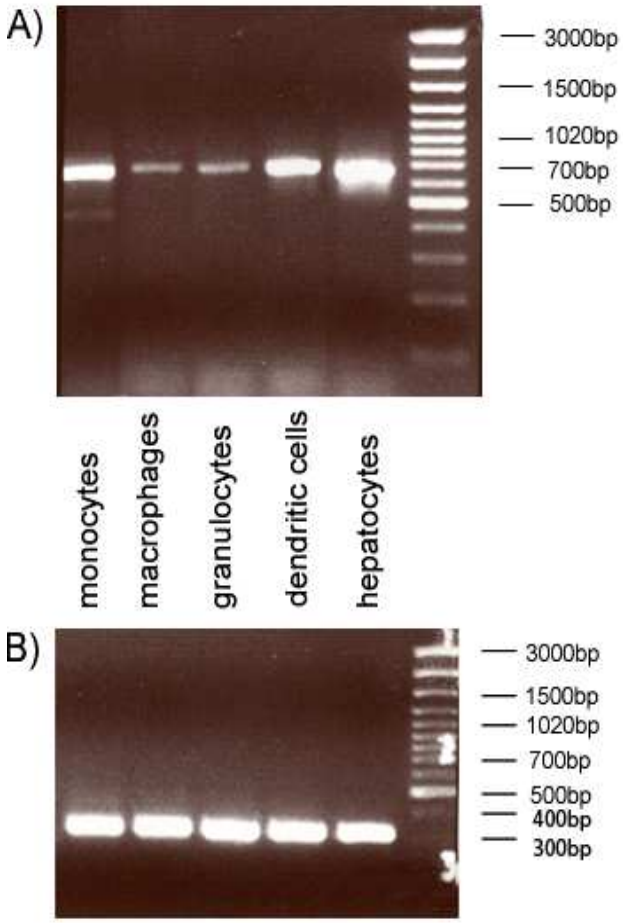


Fig. V.1.1) PEDF gene expression of human blood cells.
PCR products of the PEDF gene (A) and the house keeping gene GAPDH (B) indicate PEDF gene expression in monocytes, macrophages, granulocytes and dendritic cells. Human hepatocytes were used as an internal positive control since they are known to strongly express the PEDF gene.
During testing whether PEDF was upregulated in the brain after a trauma, it was discovered that PEDF increased not only in lesioned brain homogenates, but also in those sham-lesioned controls that occasionally contained some blood. Because of this finding, blood serum and mRNA of the following cells were tested for PEDF expression.

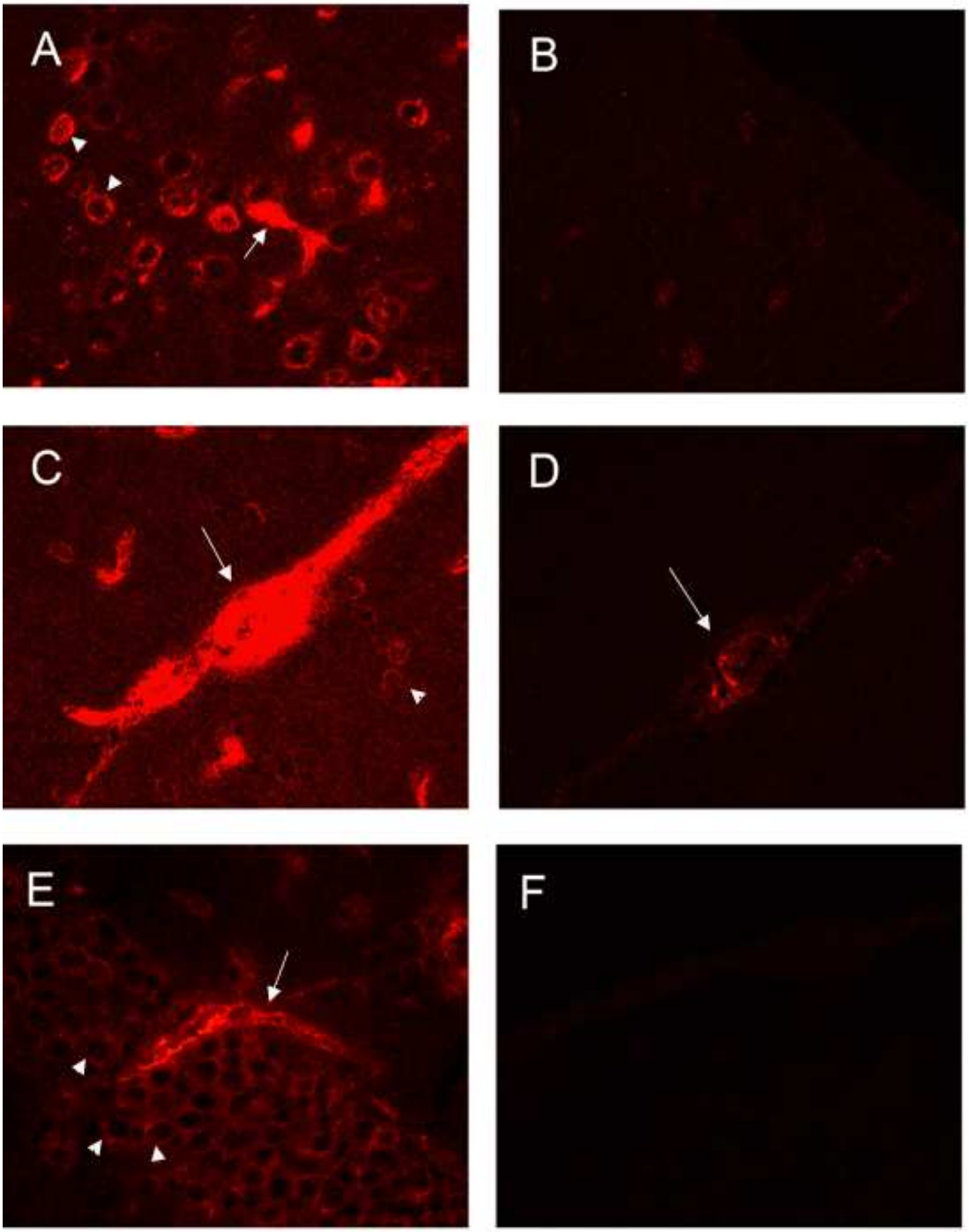
Results:

Immunohistochemical labeling for PEDF was performed in fluorescence and DAB. As can be seen in figure V.1.2, the PEDF-antibody clearly labeled cells and vessels in the cortex and dentate gyrus of the hippocampus of a 3 month old Long Evans rat brain. To verify the specificity of the staining, fluorescent stainings were performed in parallel with the appropriate IgG control, which was negative (Fig. V.1.2F). As a further control, a preadsorption of the PEDF-antibody with an excess of PEDF protein was performed (Fig. V.1.2B and D). Preadsorption of the antibody completely abolished the cellular staining and reduced the strong staining of vessels significantly, indicating the specificity of the antibody staining. Stainings were performed several times with similar results.

DAB stainings produced basically the same results as fluorescent stainings. Preadsorption of the antibody completely abolished cellular signals and strongly, but not completely reduced staining of vessels (Fig. V.1.3). As an internal control for the preadsorption, the nonspecific protein BSA was used in the same concentration as PEDF in order to verify that the excess of PEDF did not cause any nonspecific steric hindrance of the binding of the antibody (Fig. V.1.3A). This internal control showed the same results as the PEDF staining, further validating the specificity of the PEDF staining.

In total, seven different antibodies from different species and against different parts of the human PEDF protein were tested by immunohistological means as well as Western Blot analysis. Although all antibodies showed similar results only one antibody produced good results in both Western blot analysis and immunohistochemical stainings and could be abolished by preadsorption with an excess of PEDF protein.

Fig. V.1.2) Fluorescent PEDF immunohistochemistry in the adult rat brain (next page).
Seven µm thin sagittal cryosections of a 3 month old Long Evans rat brain were fixed with 4% PFA and stained with a rabbit polyclonal antibody against human PEDF (1 ng/µl) and a secondary dk α-rbt RHOX antibody (1:400) (A, C, E). Controls were performed by substituting the primary PEDF antibody with the appropriate concentration of rbt IgG (F) or preadsorbing the primary PEDF antibody with a 100 times excess of PEDF protein over night and subsequent centrifugation (B, D) and show an abolished and dramatically reduced staining for PEDF, respectively. The PEDF antibody was treated the same as the preadsorption control but without addition of the PEDF protein. The data show staining of the PEDF antibody in areas of the cortex (A, B), an area between hippocampus and thalamus (C, D) and the dentate gyrus (E, F) and were taken with the same camera settings and adjusted for contrast and brightness in parallel. The cellular staining for PEDF is indicated by arrow heads and vessels are marked by arrows.


A
B
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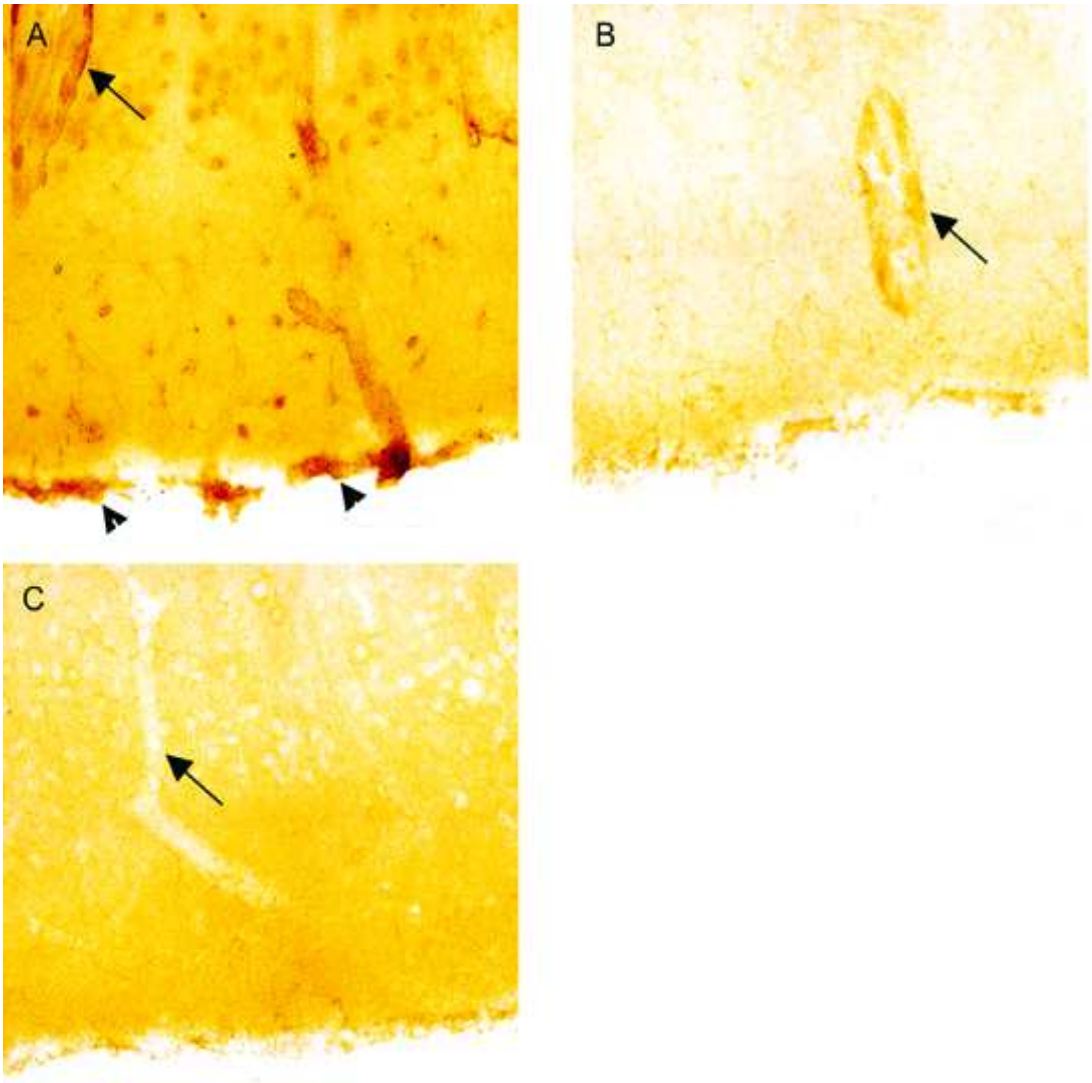

Fig. V.1.3) PEDF DAB immunohistochemistry in the adult rat brain.
Seven µm thin coronal cryosections of a 3 month old Long Evans rat brain were fixed with 4% PFA and immunoreacted with a polyclonal rabbit antibody against human PEDF (0.66 ng/µl) in the presence of 100 times excess of BSA (A). Further controls were performed by substituting the primary PEDF antibody with the appropriate concentration of rbt IgG (C) and using a preadsorbed primary PEDF antibody with a 100x excess of PEDF protein over night and subsequent centrifugation (B) which abolished and dramatically reduced staining for PEDF, respectively. The data show the strong PEDF staining of cells, vessels (arrows), as well as meninges (arrow heads).

Discussion:

Fluorescence and DAB stainings indicate that the employed PEDF antibody labels cells and vessels in the adult rat brain. The specificity of the cellular immunostaining in the brain was indicated by two different controls, both of which were negative. Although vessels still showed a low signal after preadsorption of the antibody, the signal was significantly reduced, suggesting that these structures also specifically stain for PEDF. Due to the intense labeling of vessels with PEDF, a higher dilution of the antibody in combination with an excess of PEDF should be performed to abolish the signal completely.

The clear staining of cells and vessels for PEDF in the dentate gyrus of the hippocampus and the cortex suggests that PEDF might be produced in the adult rat brain or at least bind to these cells. The finding furthermore indicates that PEDF in the brain might not only be derived from serum or cells in the blood, since PEDF in the blood should not be able to pass into the brain tissue through the blood brain barrier.

V.2. What is the distribution of PEDF immunopositive cells in the adult rat brain?

In order to determine which cell types in the adult rat brain were PEDF positive, double stainings were performed with the neuron specific marker NeuN, the astrocyte marker GFAP, the oligodendrocyte marker MBP and the vessel markers vWF and Glut-1. Glut-1 is also used as a marker of the blood brain barrier and stains all vessels in the brain.

There was a clear colocalization of the vessel markers Glut-1 (Fig. V.2.1) and vWF with PEDF (Fig. V.2.2). PEDF appeared to label all vessels in the entire brain, although with different intensities and larger vessels were stained stronger than smaller vessels.

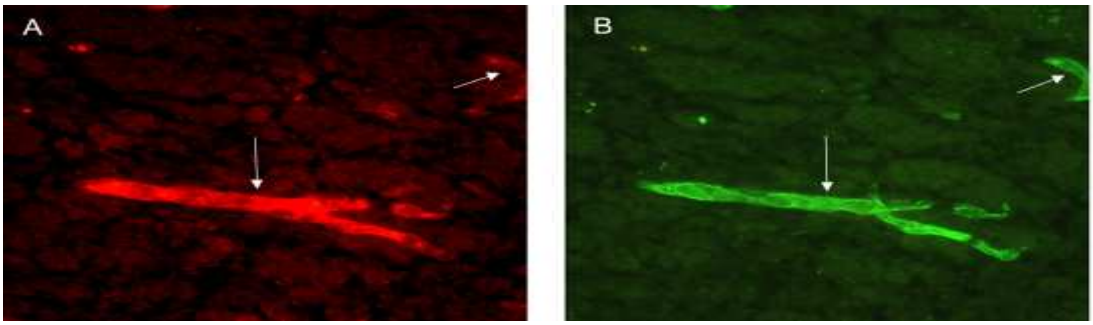

Fig. V.2.1) Fluorescent double labeling of a 3 month old Long Evans rat brain with PEDF (A) and Glut-1 antibody (B). The data show the weaker staining of smaller vessels for PEDF (right arrow) compared to larger vessels (left arrow) while all vessels immunoreacted equally strong with the Glut-1 antibody.

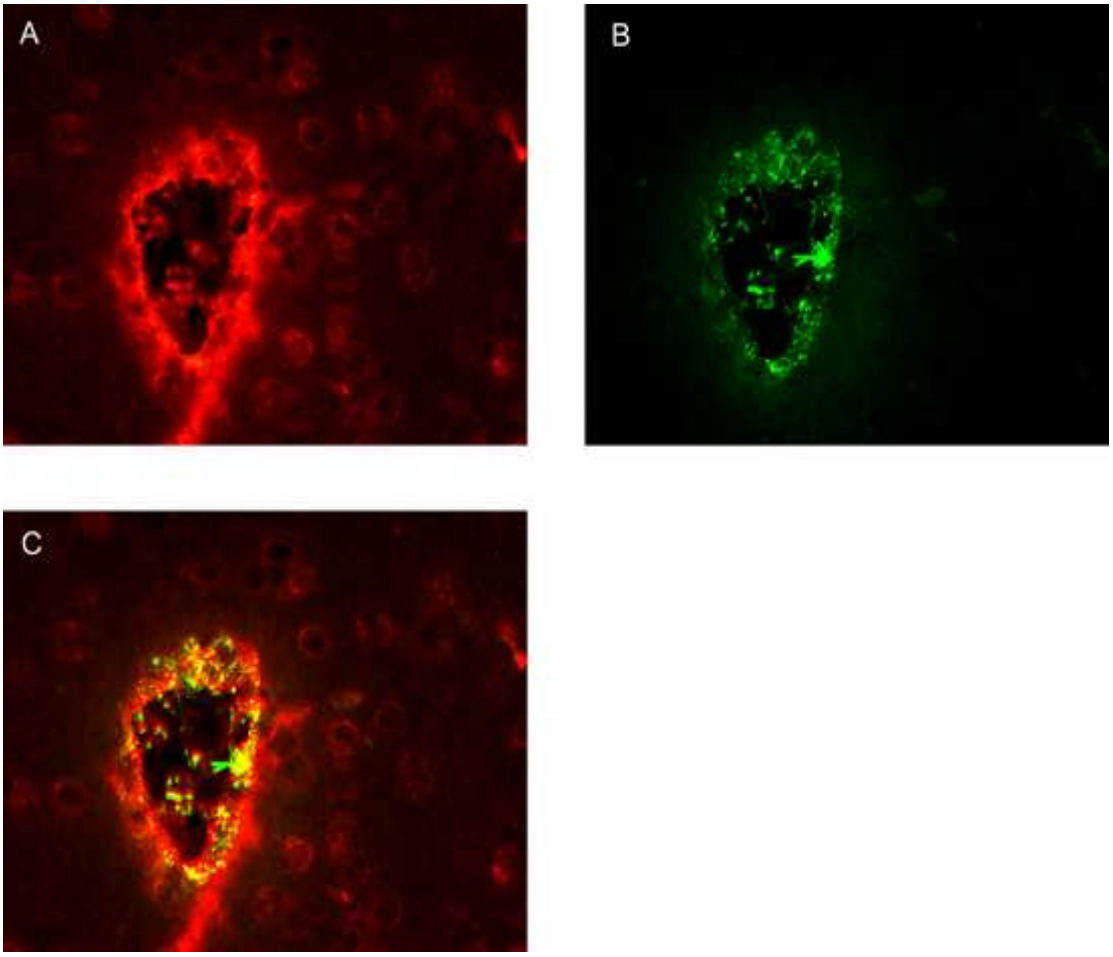

Fig. V.2.2) Fluorescent double labeling of a 3 month old Long Evans rat brain with PEDF (A) and vWF antibody (B). Images of PEDF and vWF stainings were super imposed to show that antibodies stained endothelial cells of the same vessels (C).

Since PEDF mainly labeled the soma of cells and GFAP is strongly expressed in the ramifications of glia cells, it was difficult to determine whether any structures in the brain were double labeled with GFAP and PEDF (Fig. V.2.3). The finding that in most areas of the brain the PEDF-labeled structures were mainly large and round, and astrocytes have a star-shaped size and are relatively small suggests that in these areas of the brain astrocytes were not stained for PEDF and might therefore not produce and/or bind PEDF (see Fig.

V.2.7- V.2.14). In the corpus collosum, however, PEDF-labeled structures appeared to be more typical for astrocytes and it appeared as if there was colocalization of some GFAP positive structures with PEDF (Fig. V.2.3). A double labeling of PEDF with Glut-1 in the same area indicated however, that the majority of larger PEDF labeled structures were vessels (Fig. V.2.4). Since the colocalization seemed to occur only in the close vicinity of vessels (Fig. V.2.3C), and it is known that astrocytes are often closely wrapped around vessels, it is therefore likely that the apparent double labeling in these areas was no true colocalization and that astrocytes that are surrounding vessels also do not stain for PEDF.

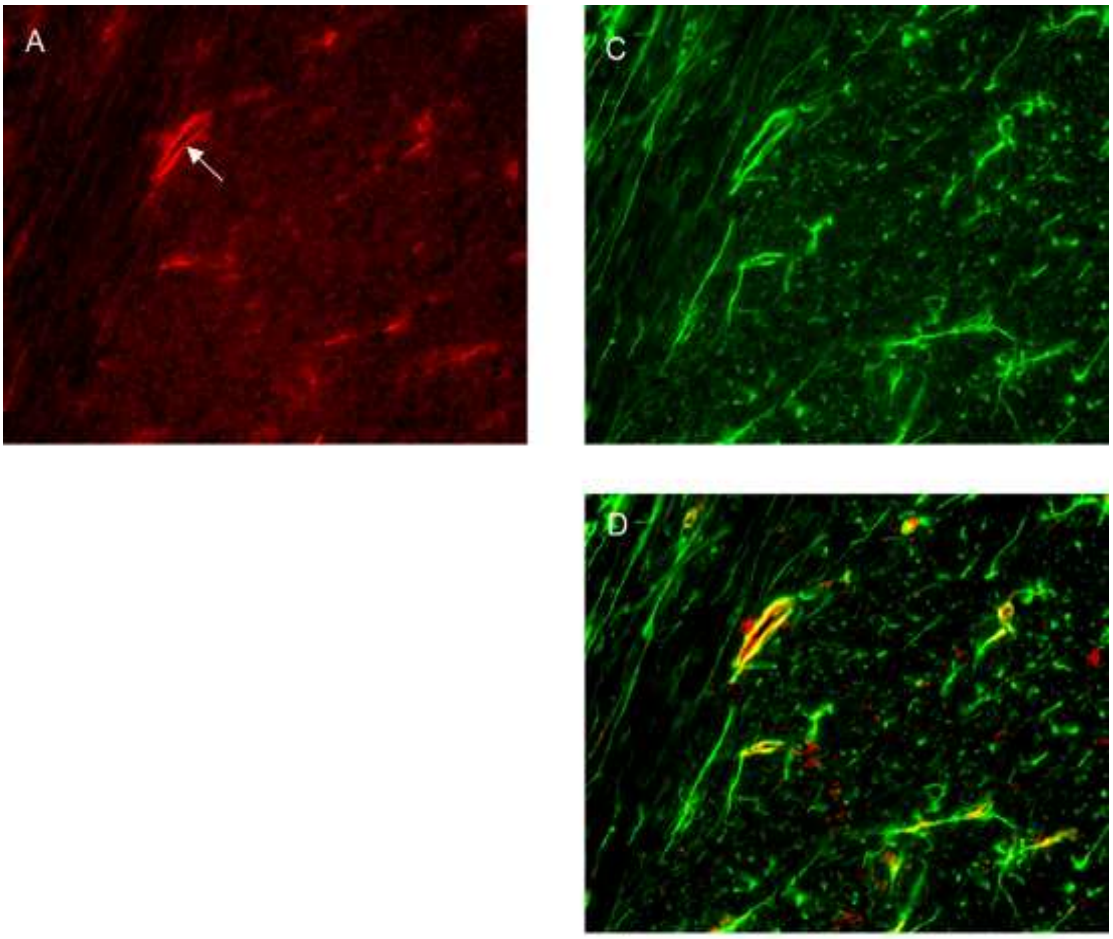

Fig. V.2.3) Fluorescent double labeling of the corpus collosum of a 3 month old Long Evans rat brain with PEDF (A) and GFAP antibody (B). Images for PEDF and GFAP were super imposed to show double labeling (C). Glia cell processes are shown in green. The presence of only a few yellow marked structures that were mainly in the vicinity of vessels (arrow) suggests that the majority of glia cells do not stain for PEDF.

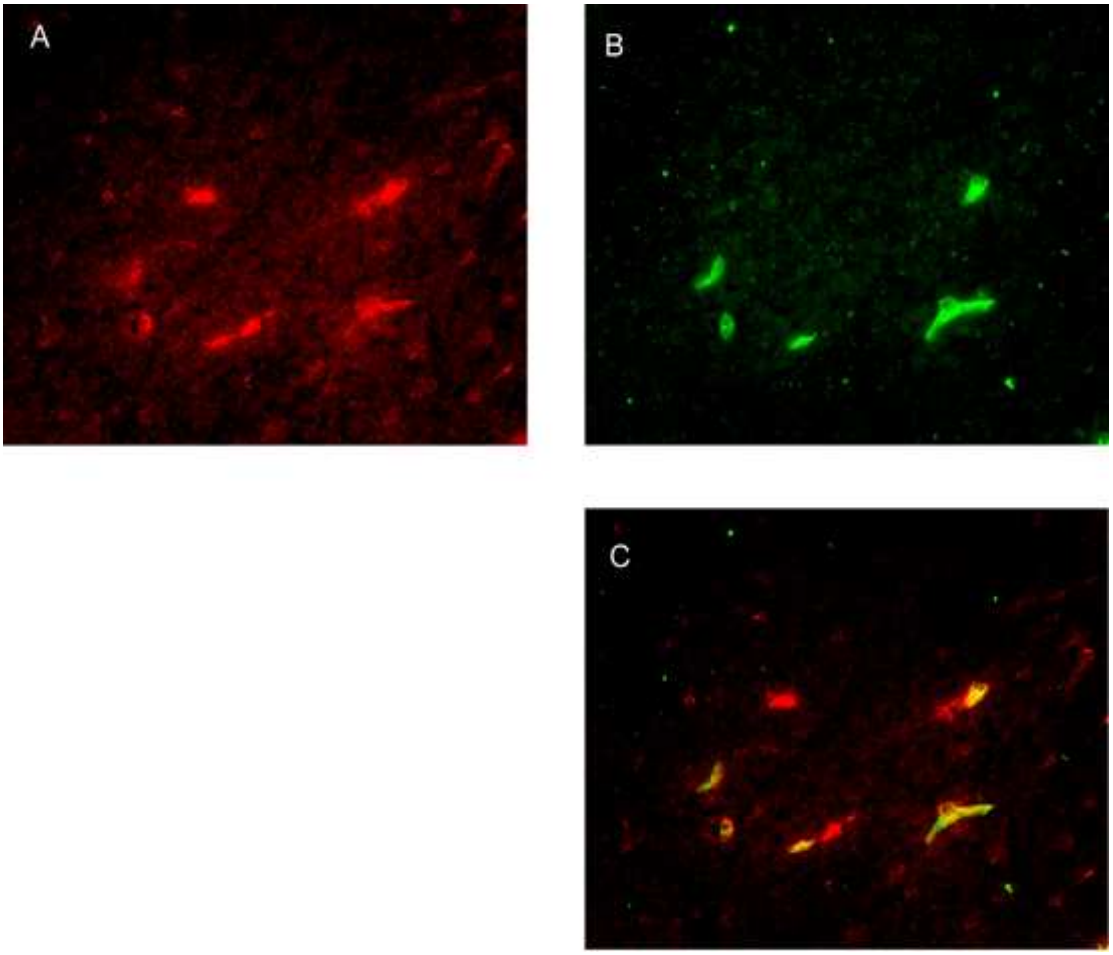

Fig. V.2.4) Fluorescent double labeling of the corpus collosum of a 3 month old Long Evans rat brain with PEDF (A) and Glut-1 antibody (B). Images for PEDF and Glut-1 were super imposed to show double labeling (C). The data show that in the corpus collosum the PEDF antibody mainly labeled vessels as indicated by the yellow color, but also some other undetermined structures.

Also, no double staining with PEDF could be detected anywhere in the brain with the oligodendrocyte marker MBP (Fig. V. 2.5), although both markers were located in close vicinity in the meningeal layers.

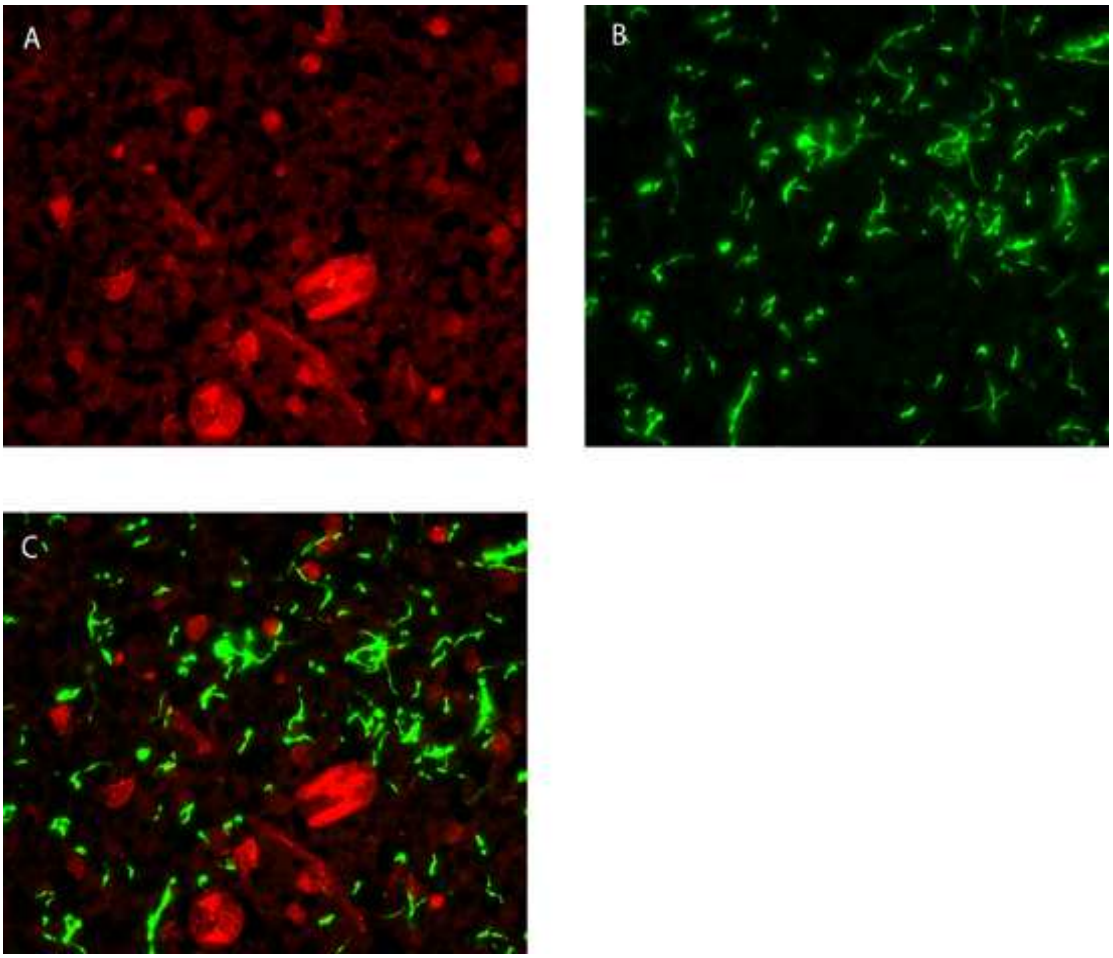

Fig. V.2.5) Fluorescent double labeling of the olfactory bulb of a 3 month old Long Evans rat brain with PEDF (A) and MBP antibody (B). Oligodendrocytic processes are shown in green. Images for PEDF and MBP were super imposed and show that there were no double labeled structures, suggesting that PEDF may not be expressed in oligodendrocytes (C).

Similar to vessels, the majority of neurons of most brain areas were clearly labeled for both Neu-N and PEDF (Fig. V.2.7-V.2.14). However, neuronal labeling was not as intense as the one of endothelial cells. The PEDF signal

appeared to be mainly localized in the cytoplasm of neurons and the perinuclear region. In some cases, such as in the olfactory bulb, it appeared as if PEDF especially labeled the nucleolus, since little dot-like structures could be observed in some neurons (Fig. V.2.6).

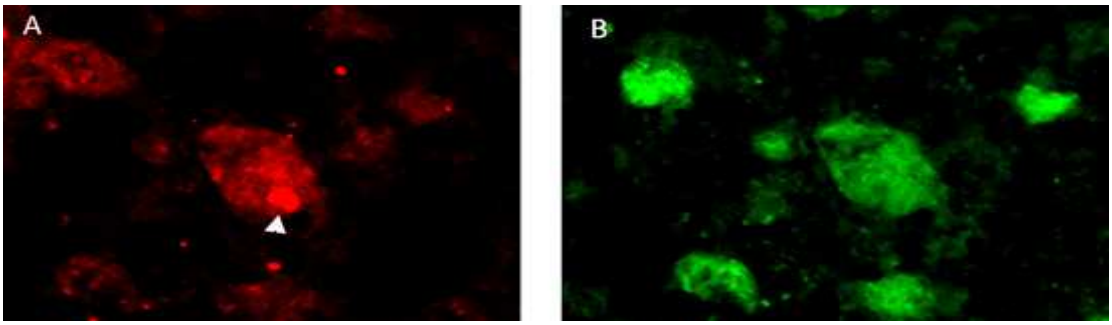

Fig. V.2.6) Fluorescent staining of the olfactory bulb of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (B). Neuronal nuclei are shown in green. The picture shows a cell with a dot-like structure in the nucleus that is stained against PEDF (arrow head) suggesting that PEDF protein is localized in the nucleolus.

It appeared that all NeuN positive cells in the entire dentate gyrus of the hippocampus (see also Fig. V.1.2 and V.1.3), the thalamus, the hypothalamus, the cortex, the striatum, the preoptic area, the midbrain, and the pons were also labeled for PEDF (Fig. V.2.7-V.2.13). Besides neuronal cells the pons also contained other PEDF positive cells that did not appear to be vessels (Fig. V.2.13). In the olfactory bulb, only a small number of neurons with relatively large somata were double labeled for NeuN and PEDF, while the smaller granule cells did not stain for PEDF (Fig. V.2.14 and V.2.15). The pituitary gland and the optic chiasm also contained PEDF positive cells that were not labeled with the neuronal marker NeuN or GFAP (Fig. V.16 and V.2.17), suggesting that the

labeled cells in the pituitary might be of endocrine origin. Furthermore, PEDF strongly stained the meninges (see Fig. V.1.2), as well as ependymal cells lining the ventricles and the choroid plexus (Fig. V.2.18). In fluorescent stainings, it could not be determined if cerebellar granule neurons stained for PEDF due to the small cytoplasmic content and faint signal (Fig. V. 2.19), although DAB stainings suggested a faint labeling of cerebellar granule cells for PEDF (Fig. V. 2.20).

For a better overview figure V.2.21 shows a schematic diagram of cells that were labeled for PEDF in the brain.

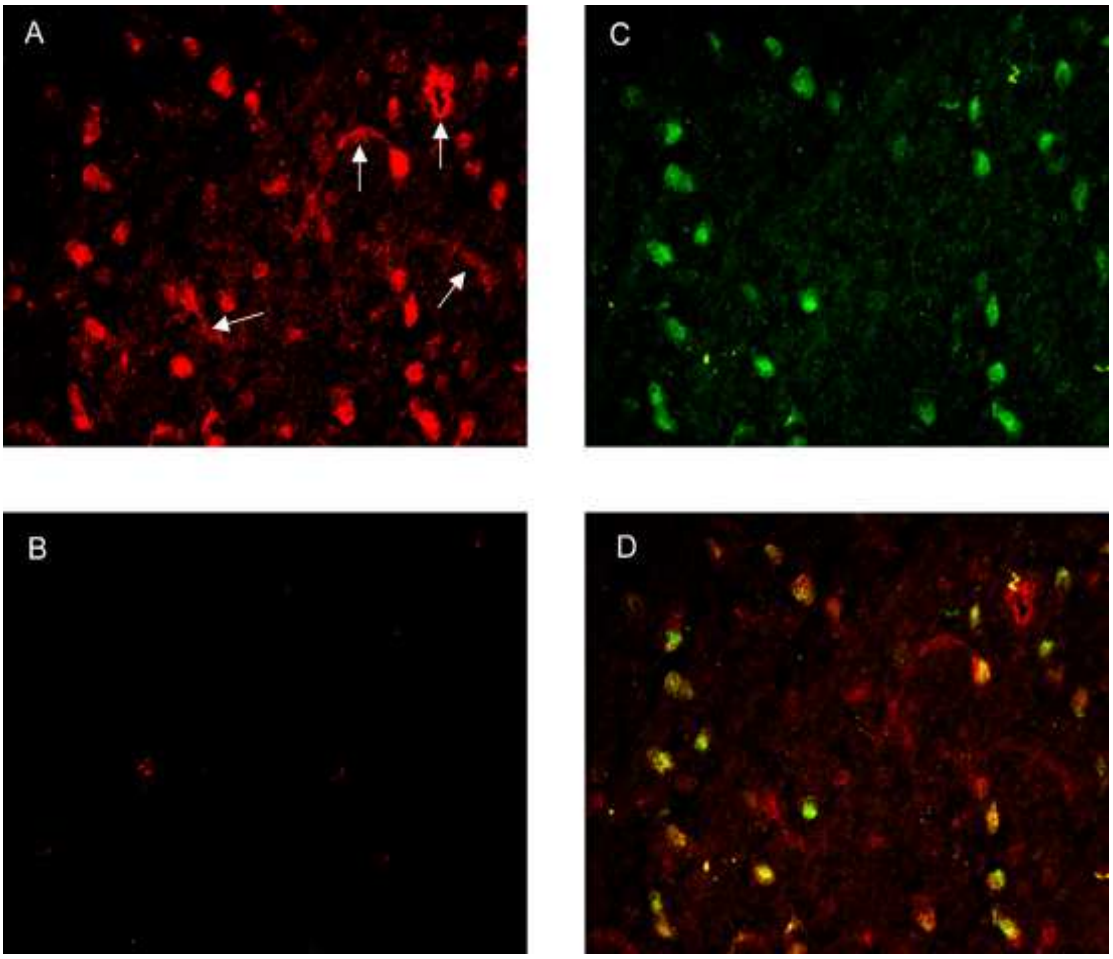

Fig. V.2.7) Fluorescent double labeling of the thalamus of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and the neuronal marker NeuN were super imposed to show double labeling of both antibodies (D). In the figure, double labeled cells are yellowish in appearance. An IgG control for PEDF is shown in the same brain area (B). The data show that in the thalamus all neurons stained positively for PEDF in addition to other PEDF positive structures such as vessels (arrows).

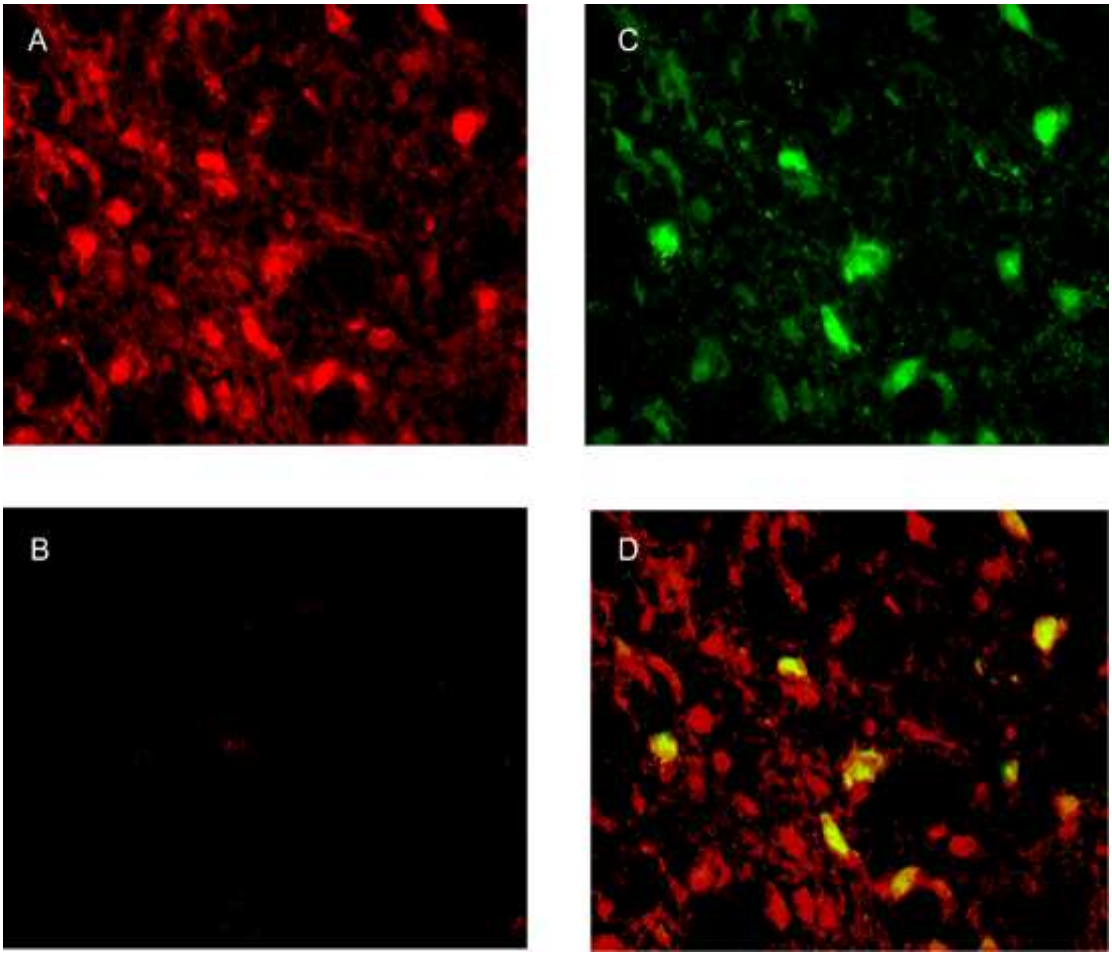

Fig. V.2.8) Fluorescent double labeling of the hypothalamus of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and NeuN were super imposed as shown in (D). Cells that are double labeled for PEDF and the neuronal marker NeuN appear yellowish. An IgG control for PEDF is shown in the same brain area (B). The data show that all neurons in the hypothalamus stained for PEDF and NeuN in addition to other PEDF positive structures including vessels.

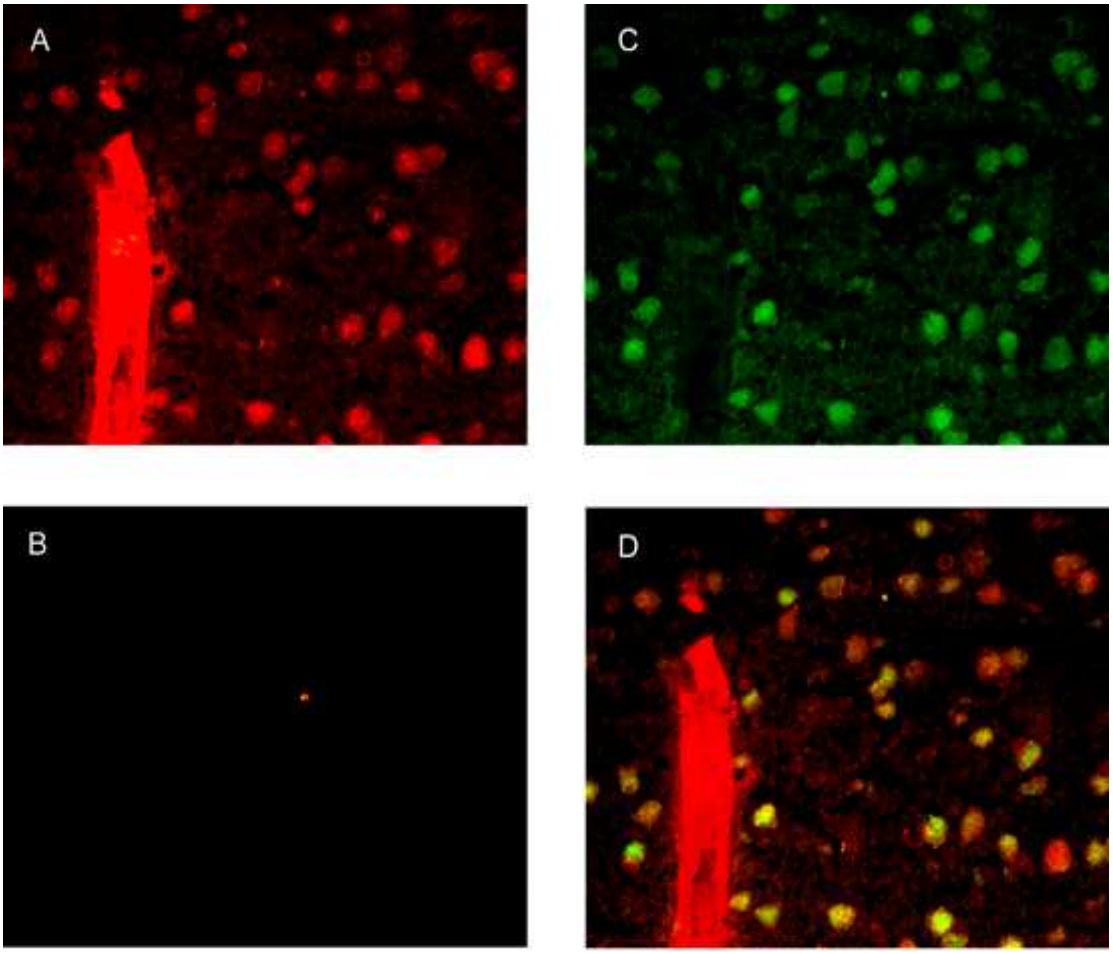

Fig. V.2.9) Fluorescent double labeling of the cortex of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and NeuN were super imposed as shown in (D). Cells labeled with both antibodies appear yellowish. An IgG control for PEDF in the same brain area shows lack of staining for the neurons (B). The figure shows that all neurons in the cortex stained positively for PEDF in addition to a large vessel.

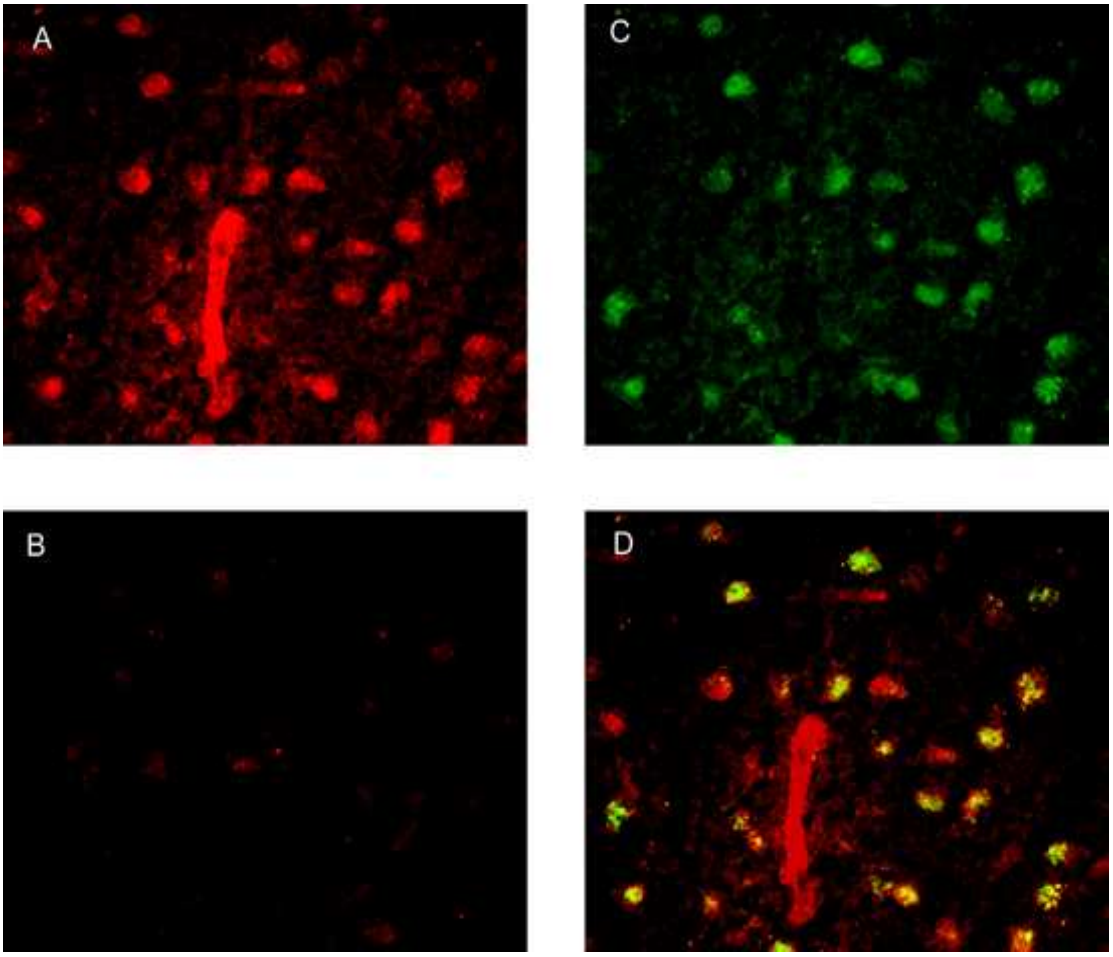

Fig. V.2.10) Fluorescent double labeling of the striatum of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and NeuN were super imposed to show double labeling of both antibodies (D). Double labeled cells appear yellowish. (B) The IgG control for PEDF in the same brain shows no signal indicating the specificity of the staining. In the striatum all neurons stained positively for PEDF in addition to vessels.

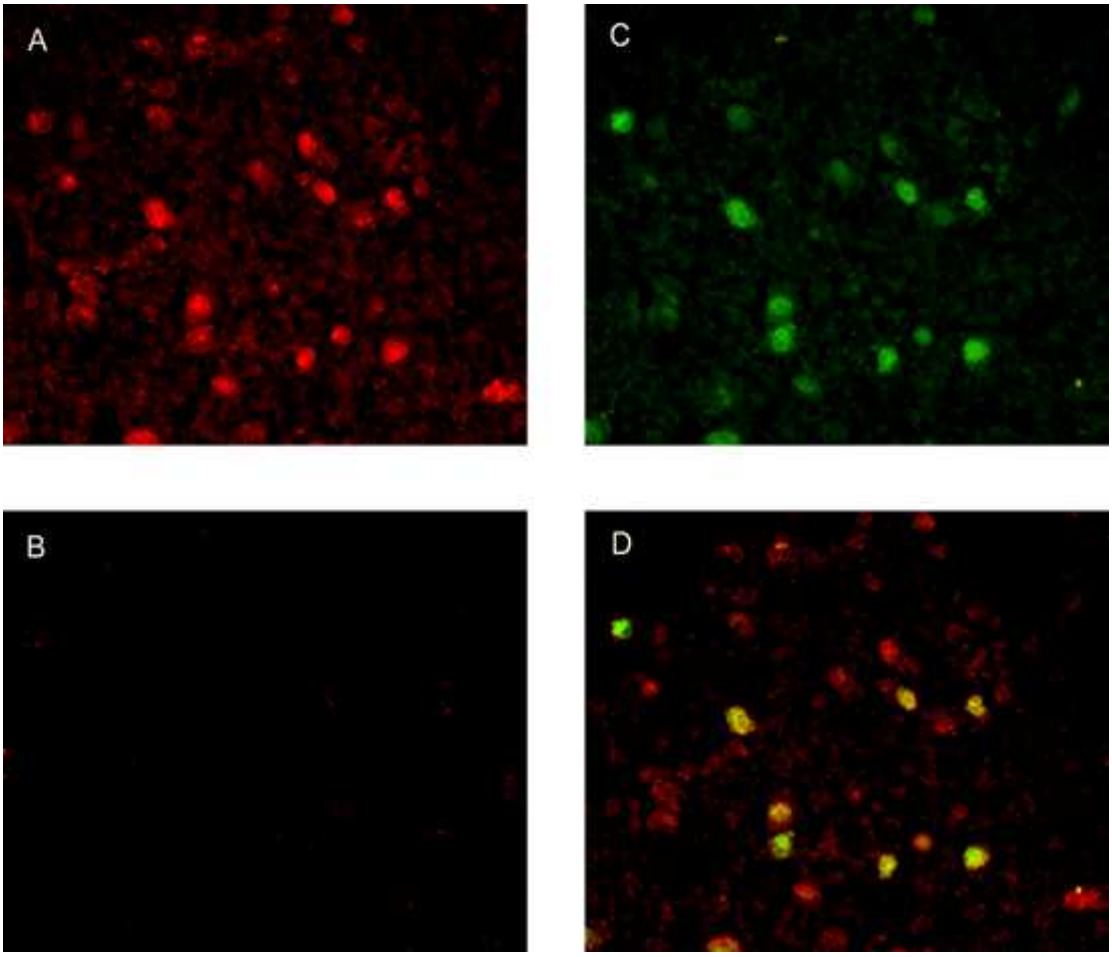

Fig. V.2.11) Fluorescent double labeling of the preoptic area of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody(C). Images for PEDF and NeuN were super imposed to show that all neurons in the preoptic area stained positively for PEDF in addition to other PEDF positive structures (D). Double labeled cells appear yellowish. An IgG control for PEDF in the same brain area shows no staining (B).

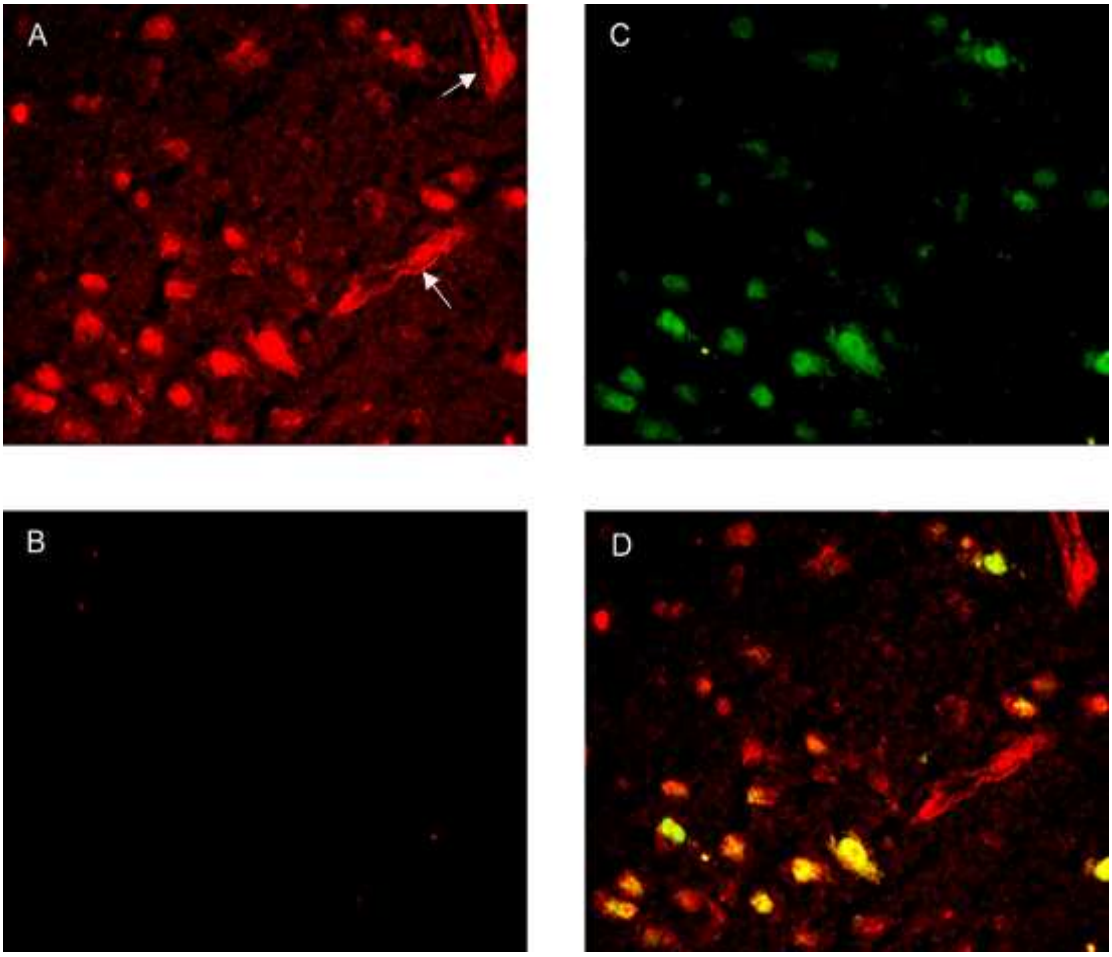

Fig. V.2.12) Fluorescent double labeling of the midbrain of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and NeuN were super imposed to show that all neurons in the midbrain stained positively for PEDF in addition to PEDF positive vessels (arrows) (D). Double labeled cells appear yellowish. The IgG control for PEDF in the same brain area shows negative staining (B).

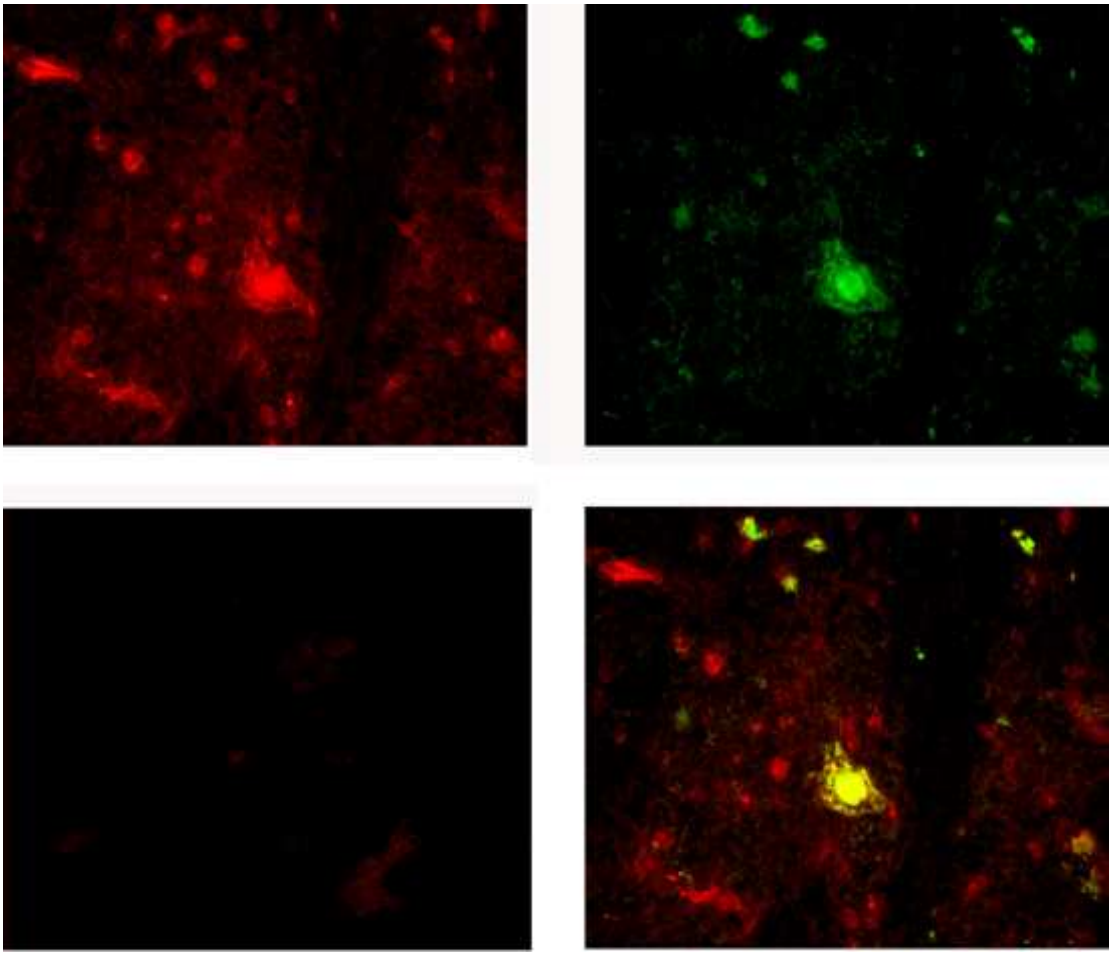

Fig. V.2.13) Fluorescent double labeling of the pons of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and NeuN were super imposed and show that basically all neurons stained positively for PEDF in addition to many other PEDF positive structures (D). Double labeled cells appear yellowish. (B) The IgG control for PEDF in the same brain area shows some unspecific background in this area of the brain. This was however significantly fainter than PEDF labeled structures.

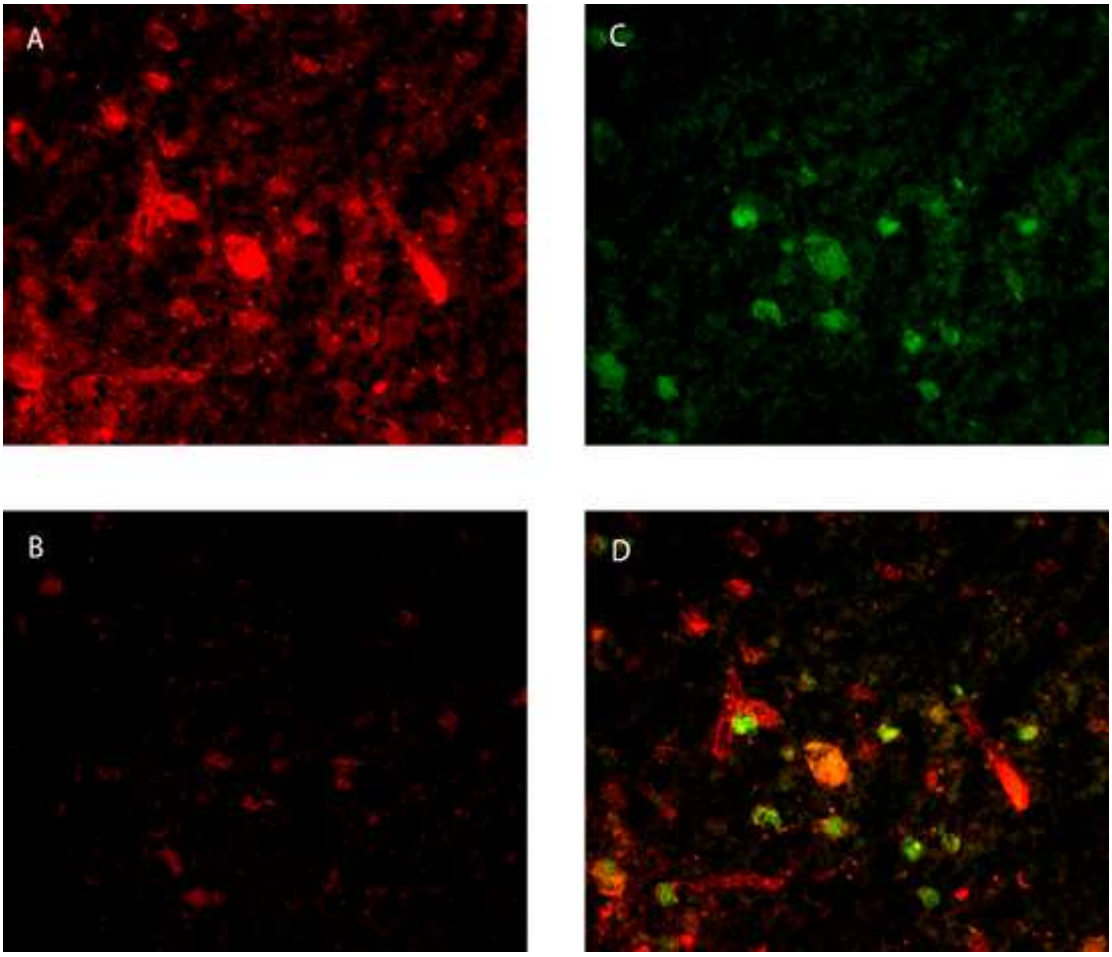

Fig. V.2.14) Fluorescent double labeling of the olfactory bulb of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (B). Images for PEDF and NeuN were super imposed to show double labeling (D). (B) The IgG control in the same brain area shows some unspecific background which was however significantly fainter than PEDF labeled structures. The figure shows that larger neuronal cells of the olfactory bulb positively stained for PEDF in addition to other PEDF labeled structures.

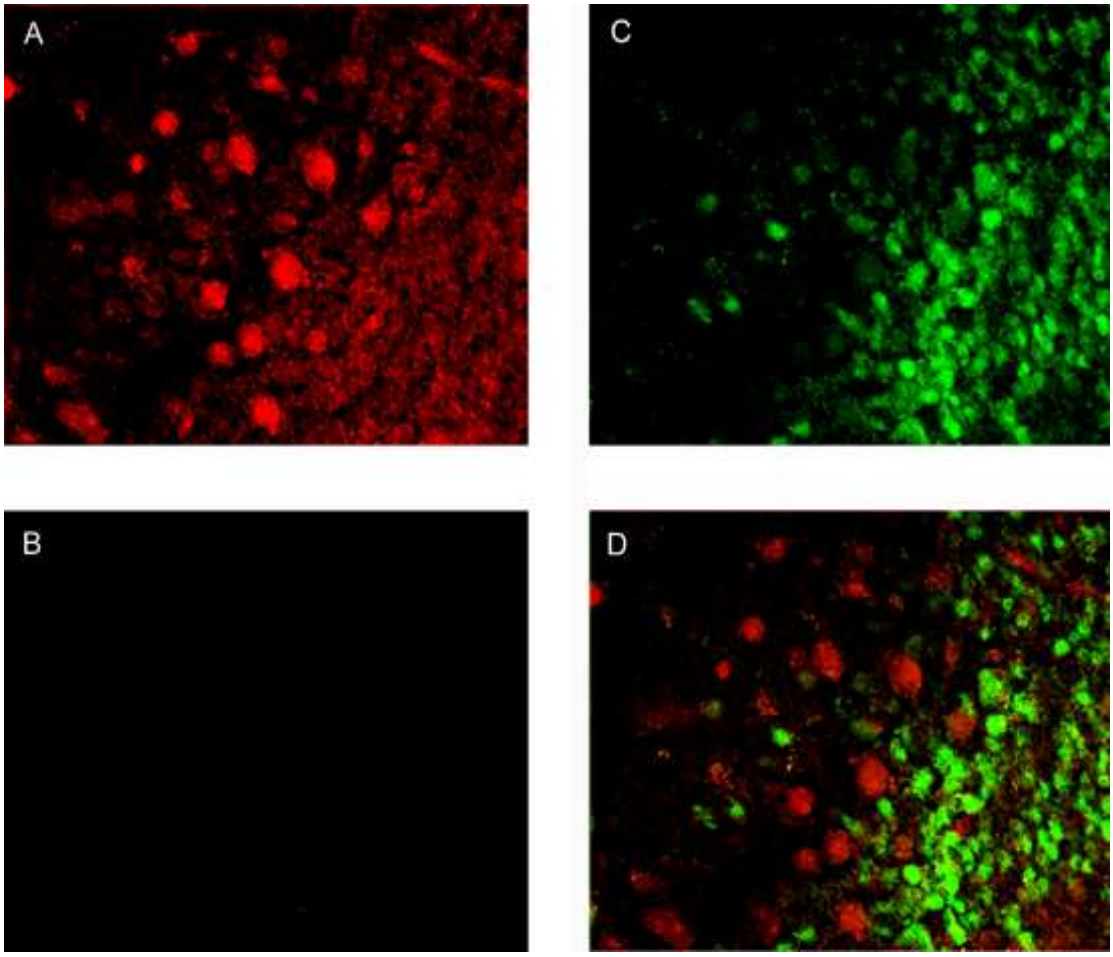

Fig. V.2.15) Fluorescent double labeling of the olfactory bulb of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and NeuN were super imposed to show that in this area of the olfactory bulb there was no colocalization of PEDF and NeuN (D). The IgG control for PEDF in the same area of the olfactory bulb shows completely negative staining (B). The fluorescent data suggests that granular cells of the olfactory bulb are not PEDF positive.

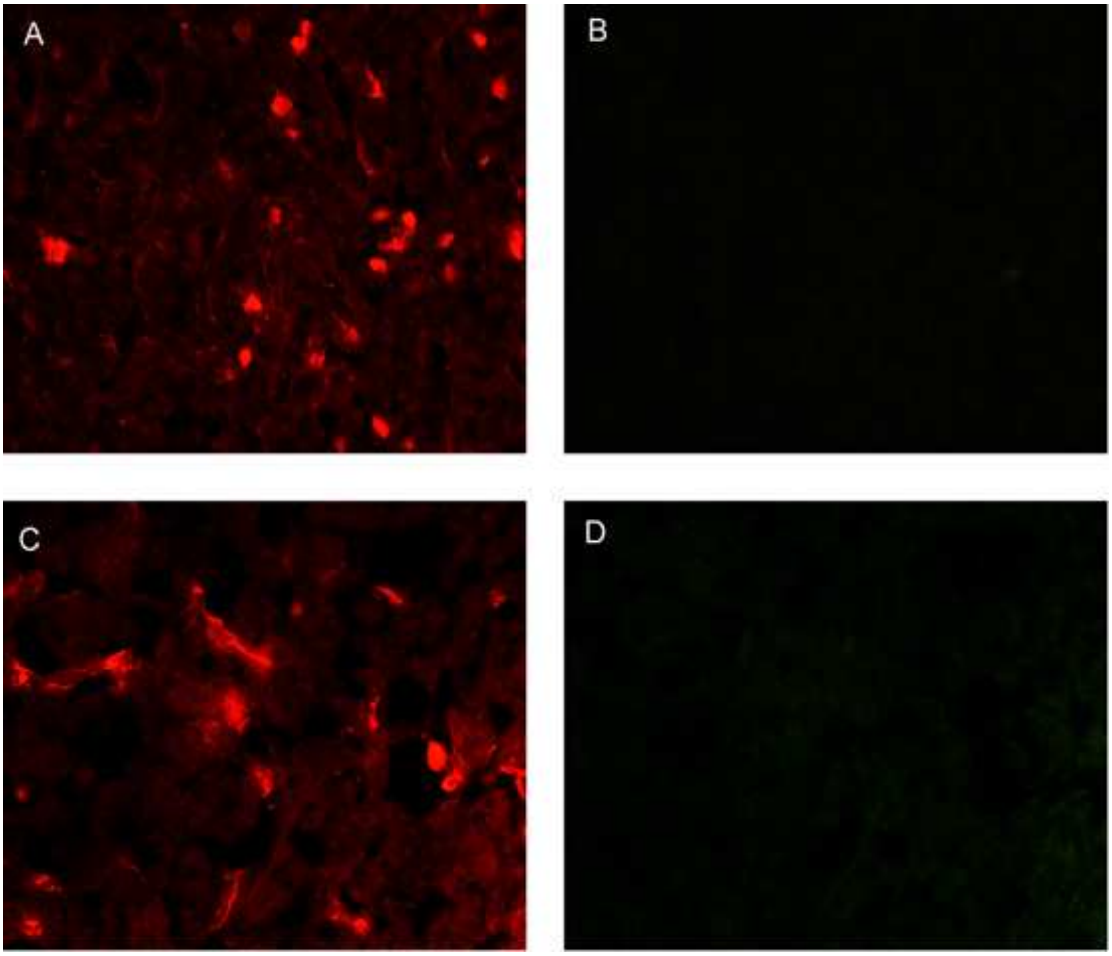

Fig. V.2.16) Fluorescent double labeling of the pituitary gland of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (B), as well as with PEDF (C) and GFAP antibody (D). The PEDF positive cells in the pituitary gland are likely to be of non-neuronal and non-astrocytic origin because they did not react positively with the NeuN or GFAP antibody suggesting that the PEDF stained cells could be of glandular origin.

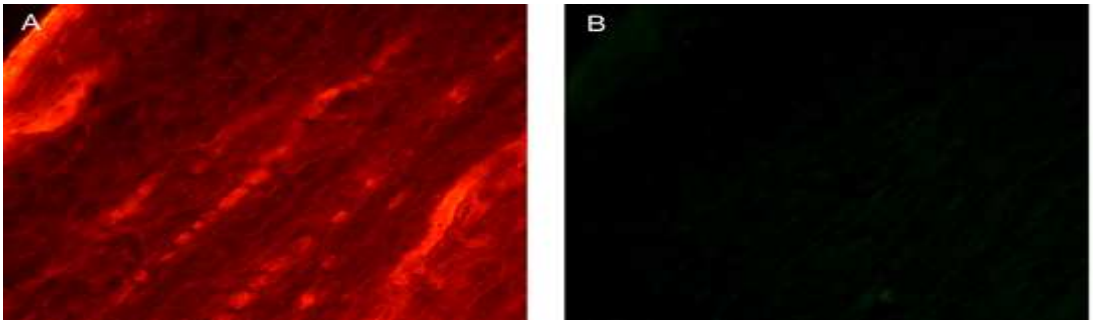

Fig. V.2.17) Fluorescent double labeling of the optic chiasm of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (B). The PEDF positive cells in the optic chiasm are likely to be of non-neuronal origin because they did not react positively with the NeuN antibody.

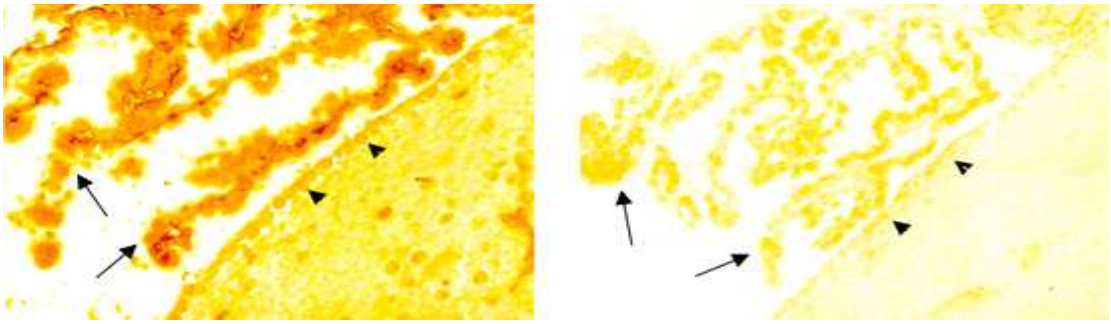

Fig. V.2.18) DAB staining of the third ventricle of a 3 month old Long Evans rat brain with PEDF antibody (A) and the appropriate IgG control (B). There is strong PEDF staining of ependymal cells lining the ventricle walls (arrow heads) and of the choroid plexus (arrows) with the PEDF antibody suggesting that PEDF may be produced in these cells and be the source of PEDF in the CSF.

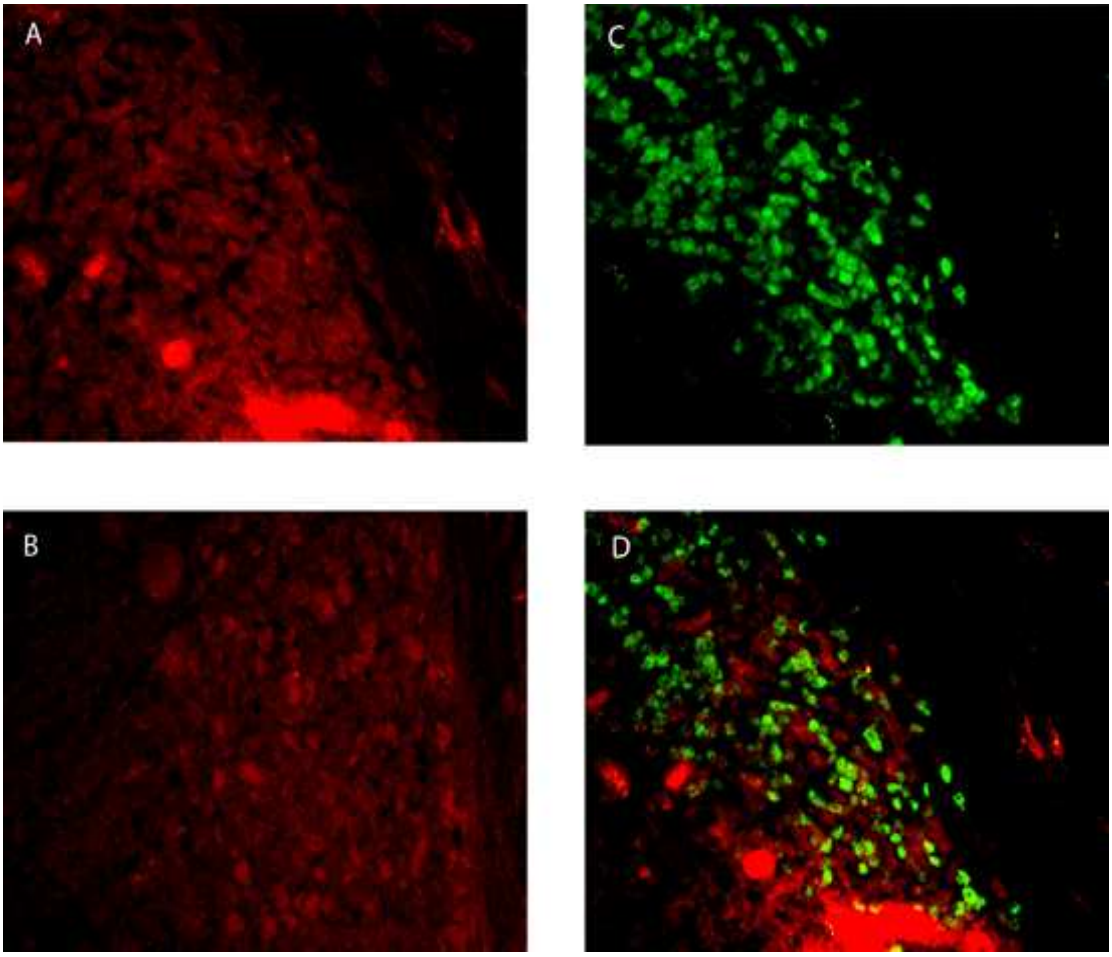

Fig. V.2.19) Fluorescent double labeling of the cerebellum of a 3 month old Long Evans rat brain with PEDF (A) and NeuN antibody (C). Images for PEDF and NeuN were super imposed and show that there was no colocalization of PEDF in the smaller granular neurons of the cerebellum (D). The IgG control for PEDF in the same brain area shows a relatively high general background staining that is comparable to that of the PEDF antibody staining, suggesting that the fainter PEDF signal of smaller structures in the cerebellum may be unspecific (B).

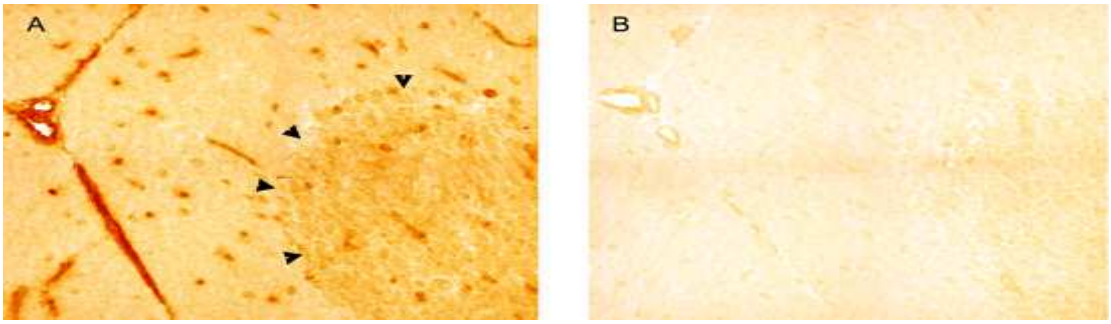

Fig. V.2.20) DAB staining of the cerebellum of a 3 month old Long Evans rat brain with PEDF antibody (A) and the appropriate IgG control (B). Arrow heads mark the location of cerebellar granule neurons. The data shows that cerebellar granule neurons were weakly labeled for PEDF with this technique, while endothelial cells intensely stained for PEDF.

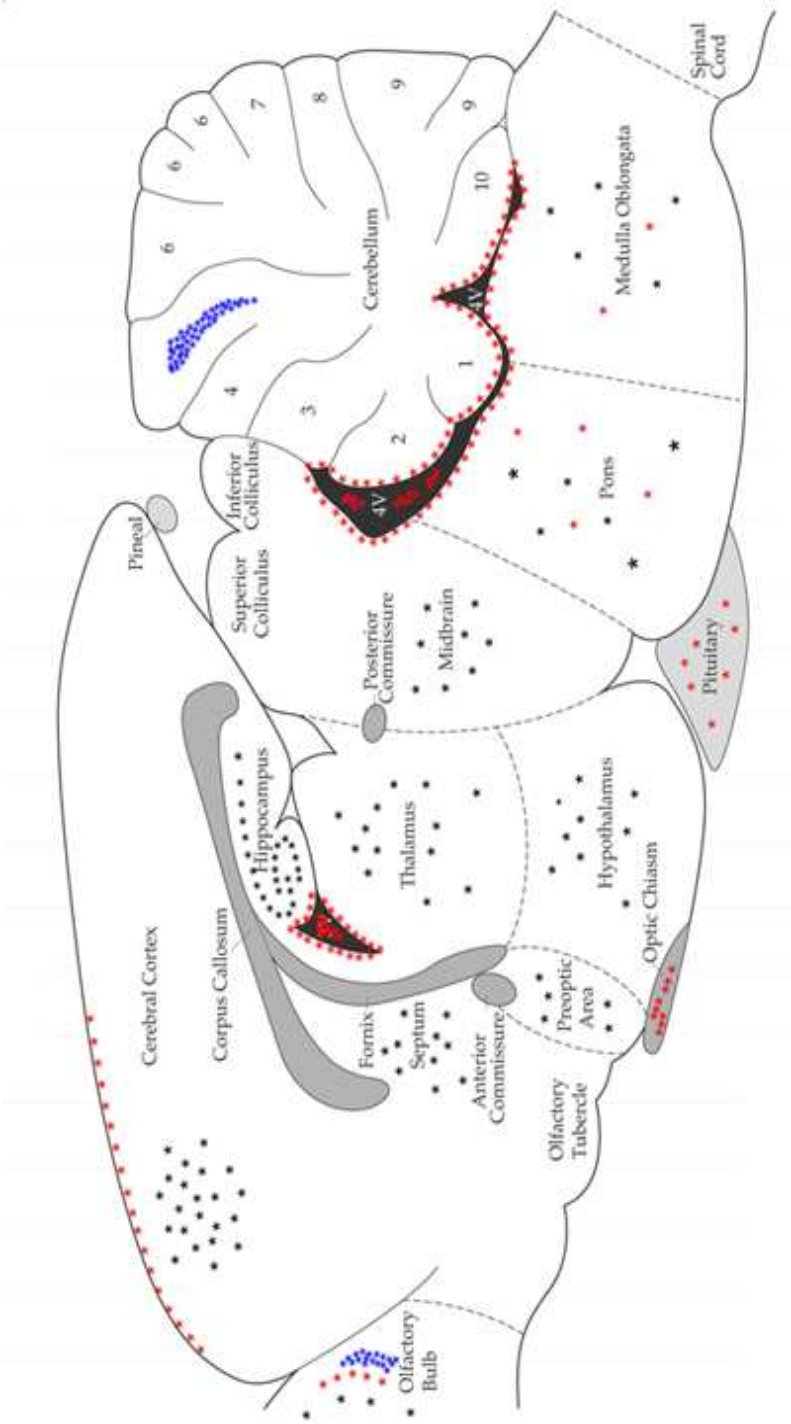

Fig. V.2.21) Schematic overview of PEDF labeled cells in the adult rat brain. Black stars indicate neuronal cells that stained for PEDF while blue stars show granular neurons that either did not label for PEDF or it could not be determined for sure if they were PEDF positive. PEDF labeled cells that were of non-neuronal origin and were not glial cells are indicated by red stars. Since PEDF stained all endothelial cells in the entire brain, they are not shown in this schema.

Discussion:

PEDF immunohistochemistry and cell specific double labeling indicate that all endothelial cells lining the vascular walls and most neurons were positive for PEDF suggesting that vessels and neurons of most brain areas might produce and/or bind PEDF, while oligodendrocytes were not positive for PEDF. In the majority of brain areas it was evident that PEDF also did not label astrocytes as indicated by PEDF/GFAP double labeling. In certain areas including the corpus collosum and ventricles, PEDF and GFAP labeled structures were in so close vicinity that it could not be excluded for sure that some GFAP positive cells were also labeled for PEDF. To examine this possibility in more detail, electron microscopy would have to be performed.

Since the rather fast secretion of PEDF from the cell has caused difficulties in the visualization of the protein in the cell in some cases (Kozaki et al., 1998), it cannot be determined with this method whether these cells just bind PEDF or also produce PEDF. Bilak and colleges (1999) have shown however that PEDF positive motor neurons and ependymal cells in the spinal cord also express the transcript, indicating that PEDF may act as an autocrine factor in these cells. It is therefore possible that the stained cells that were observed in this study might also produce PEDF. The fact that PEDF positive endothelial cells are also known to produce PEDF (Aparicio et al., 2005; Matsuoka et al., 2004) furthermore suggests that other PEDF labeled structures might produce PEDF as well. To answer this question for sure *in situ* hybridization would have to be performed.

Location of PEDF staining in the brain:

Since PEDF has been shown to have protective effects on various neuronal cell types, promotes neurite outgrowth of some neuronal types, and has been shown to modulate vessel growth, it is not surprising that neuronal and endothelial cells stained for PEDF and is in support of the known effects of PEDF. The presented data are also in line with previous reports that have shown PEDF gene expression and protein labeling of endothelial cells (Aparicio et al., 2005; Matsuoka et al., 2004) and neurons (Bilak et al., 1999 and 2002, Houenou et al., 1999; Yamagishi et al., 2004). In the majority of the investigated brain areas, PEDF seemed to label all neurons and potentially also other cells that were not of neuronal, astrocytic or oligodendrocytic origin. In the olfactory bulb (and possibly also the cerebellum), granule neurons did not obviously stain for PEDF and only some neuronal cells of larger size were labeled for PEDF. PEDF however also labeled other non-neuronal cells that were not of endothelial origin, suggesting that PEDF may possibly have additional functions in these brain areas.

In most brain areas it was clear that PEDF did not label astrocytes, nor was there a double labeling with oligodendroglia anywhere in the brain. Thus far, there are no indications that PEDF is produced by astrocytes in the brain and Bilak and colleagues (1999) also could not detect any PEDF mRNA expression in glial cells of the spinal cord. However, PEDF production has been reported by Schwann cells of neuroblastoma (Crawford et al., 2001) and by Muller glia of the retina (Eichler et al., 2004a and b). PEDF has also been shown to affect the

metabolism of microglia (Takanohashi et al., 2005; Sugita et al., 1997) which can indirectly affect the proliferation of astroglia (Sugita et al., 1997). PEDF, however, appears to be able to directly induce proinflammatory genes in neonatal astrocytes through the activation of NF-kappaB and CREB, but has very little effect on adult astrocytes (Yabe et al., 2005). Thus far, a preliminary report has shown that PEDF immunoreactivity of astrocytes could only be detected in patients with Alzheimer's disease (Yamagishi et al., 2004). All these data suggest that astrocytes might be able to react to PEDF only under certain conditions, but might not be able to produce PEDF themselves. Thus far there is no proof yet that under normal physiological conditions PEDF has any effects on astrocytes in the adult brain and that astrocytes produce PEDF. This might explain why astrocytes were not visualized by immunohistochemical means in the adult brain under physiological conditions.

In addition to endothelial cells and neuronal cells, PEDF also stained ependymal cells. Ependymal cells line the ventricles of the brain, the choroid plexus and the central canal of the spinal cord. PEDF immunoreactivity as well as *in situ* hybridization of ependymal cells have been shown previously (Bilak et al., 1999). Since PEDF also exists in the CSF, it is likely that the secretable protein is derived from ependymal cells of ventricles and choroid plexus. Additionally, PEDF in the CSF might be also derived from brain tissue, since there is no real barrier between the intercellular spaces of the brain and the CSF. Because PEDF levels of the CSF have been found to be elevated in amyotrophic

lateral sclerosis patients (Kuncl et al., 2002), it would be interesting to know if the transcript is elevated in ependymal cells as well as other brain cells, and if in this condition PEDF is elevated as a neuroprotective means to counter the degenerative effects.

Apart from endothelial, neuronal and ependymal cells, PEDF also labeled NeuN-negative cells in the olfactory bulb, the pituitary, the optic chiasm, the pons and possibly also in the corpus collosum that were not of glial or endothelial origin as determined by double labeling and typical appearance of cells. PEDF binding has been previously shown in small non-neuronal cells of spinal cord slices (Bilak et al., 2002). Thus, it appears likely that PEDF could be produced by and/or affect further cell types besides neuronal, endothelial and ependymal cells. Further experiments including triple labeling will have to be performed in order to find out which type of cells these could be.

Function of PEDF in the brain:

The wide distribution of PEDF protein in neurons of different brain areas suggests that many different neuronal phenotypes seem to have a PEDF receptor, and that it is likely that PEDF has neurotrophic and/or neuroprotective effects on all these neurons. Thus far, it has been shown that PEDF has neuroprotective effects on immature hippocampal neurons. This study shows however that neurons in the adult hippocampus stained for PEDF, suggesting that PEDF might also have some kind of neuroprotective effect on mature

hippocampal neurons as well. If staining of neurons for PEDF in fact indicates that PEDF might have some kind of neuroprotective or neurotrophic effect, or might keep neurons in a differentiated state, then most neurons in the brain would be responsive to PEDF in some kind of way. Thus far, this has been shown for at least hippocampal and motor neurons, and possibly also cerebellar granule neurons (see also chapter IV question 4). Depending on the neuronal origin, there seem to be however slight differences against the type of insult that PEDF protects these cells from, suggesting that PEDF may activate different cellular mechanisms in different neuronal phenotypes and during different stages of development.

The finding that PEDF protein can be located in two neurogenic regions of the brain, the dentate gyrus and the subventricular zone, suggests that PEDF might potentially also affect neurogenesis. This idea is supported by preliminary unpublished data from our laboratory that suggest that PEDF might influence the VEGF-induced replication of neuronal stem cells of the subventricular zone *in vitro* and *in vivo*. This finding is in line with previous reports of PEDF as a differentiating factor. Since PEDF has been also positively linked to cells entering the G0 state, PEDF might work by reducing the number of cells that enter the S-phase of the cell cycle, thereby interrupting proliferation and inducing the cell's differentiation. It is therefore likely that PEDF supports the maintenance of neurons and may keep them in a differentiated phenotype.

In some cases, PEDF seemed to stain the nucleolus of neurons. Previously, immunohistochemical data have shown that PEDF appears to be located not only in the cytoplasmic region, but also in the perinuclear region of rat neurons (Bilak et al., 1999) and is taken up and transported retrogradely by injured motor axons (Houenou et al., 1999). Furthermore, PEDF was observed in the nucleus of postnatal P8 rat motor neurons after incubation of motor neurons slices with fluorescein labeled PEDF. Incubation of isolated embryonic motor neurons with PEDF however did not label the nucleus, although PEDF increased their survival (Bilak et al., 2002). Also, PEDF has been described to be localized in association with the nucleus of RPE cells (Tombran-Tink et al., 1995) which was also observed in this study (see Fig. IV.2.4). The presented data therefore suggest that PEDF might be also taken up by other neuronal phenotypes than motor neurons and possibly function as a transcription factor in the nucleus.

PEDF has been shown to play a potential role in degenerative diseases of the CNS. For instance, PEDF has been found elevated in the CSF of patients with amyotrophic lateral sclerosis where it has been suggested to have an autoprotective function (Kuncl et al., 2002). Furthermore, a preliminary study has found increased immunoreactivity of cortical neurons and astrocytes in the CNS of patients with Alzheimer's disease, where it has been proposed to function as a compensation mechanism to fight against neuronal cell injury (Yamagishi et al., 2004). It has been suggested that PEDF may preferentially act on "abnormal" or

“sick” cells (Bilak et al., 2002). The finding that PEDF induces the differentiation of tumor cells *in vitro* and *in vivo* (Crawford et al., 2001), and has neuroprotective effects on various neuronal phenotypes would be in line with this idea.

The labeling of endothelial cells for PEDF suggests that PEDF probably also has antiangiogenic function in the brain as has been shown in other organs before. This idea is supported by the finding that the loss of PEDF gene expression does not only correlate with the degree of glioma progression in the brain and in prostate cancer (Guan et al., 2003; Halin et al., 2004), but that PEDF expression also inversely correlates with the degree of vascularisation (Halin et al., 2004; Uehara et al., 2004). Consequently, PEDF overexpression has been shown to cause the inhibition of tumor growth by suppressing tumor angiogenesis and induction of apoptosis of tumor cells in the brain and other tissues (Matsumoto et al., 2004; Guan et al 2004; Abe et al., 2004; Wang et al., 2003; Doll et al., 2003). Thus, PEDF appears to have a dual effect on tumors by either inducing their differentiation or apoptosis and by suppressing tumor angiogenesis.

Furthermore, there is evidence that PEDF may function as an antivasopermeability factor that counteracts the effects of VEGF (Liu et al., 2004). Since PEDF labeled all vessels in the brain and colocalized with the blood brain barrier marker Glut1, it would be interesting to investigate if PEDF expression of endothelial cells decreases in conditions with vascular leakage and

breakdown of the blood brain barrier. In fact, the permeability modulating effect of PEDF during development and pathological conditions is currently being investigated in our laboratory.

In summary, the wide distribution of PEDF in neuronal and endothelial cells in the brain is in line with the hypothesis that PEDF may have important functions as a neurotrophic and/or neuroprotective factor, as well as a modulator of vessel growth. Further research will be needed in order to find out which additional cells types were immunolabeled for PEDF that were not of neuronal, astrocytic or oligodendrocytic origin. These unidentified cells, together with the localization of PEDF-positive neurons in many specific –so far not described- areas of the adult brain, including neurogenic regions, open the possibility to find other important effects of this factor in the CNS.

V.3. Do Cerebellar granule cell cultures produce PEDF *in vitro*?

It has been previously shown that cerebellar granule neurons (CGN) respond to PEDF (Araki et al., 1998; Yabe at al, 2001) and appear to have a PEDF binding protein (Alberdi et al., 1999). Interestingly, the cerebellum was one of the two brain regions of the adult human brain that did not show a transcript for PEDF (Tombran-Tink et al., 1996), suggesting that in this brain region PEDF may not be produced. Since the present study could not clearly determine whether CGN's were PEDF immunopositive, and in order to test if CGN's might produce PEDF, CGN cultures from 7 day old Wistar rat pubs were established, stained for PEDF and their conditioned media tested for PEDF.

Results:

CGN cultures were established as described in chapter III, grown for 5 days in culture and then homogenized or stained for PEDF. Figure V.3.1 shows a clear PEDF signal in the conditioned medium of CGN cultures, while the control medium is negative for PEDF, indicating that CGN cultures produce PEDF. Although the same amount of protein was loaded on the Western gel as for eye and brain homogenates as indicated by the strong signal for ß-actin, no signal for PEDF could be detected in CGN cell lysates.

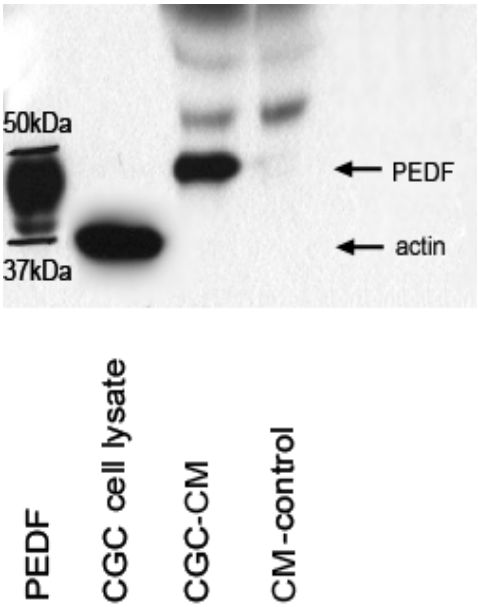

Fig. V.3.1) PEDF production of cerebellar granule neuron cultures.
50 µg of a cell lysate of a CGN culture (lane 2) that was grown for 5 days in culture and 35 µl of its conditioned medium (CM, lane 3) were separated by SDS/Page and stained for PEDF. PEDF (lane 1) and growth medium served as control (CM-control, lane 4). As an internal control for loading, Western blots were stripped and incubated with b-actin which was detected at about 37 kDa.

Contrary to immunohistochemical stainings of the cerebellum of the adult rat brain, in culture CGN's showed a strong reaction to PEDF (Fig. V.3.2). IgG control stainings were completely negative indicating the specificity of the staining. Although cultures also contained astrocytes, they did not stain for PEDF (data not shown) and only CGN's and a few fibroblasts stained for PEDF. CGN's were identified by their typical appearance and small round soma, as well as by double labeling with NeuN (data not shown).

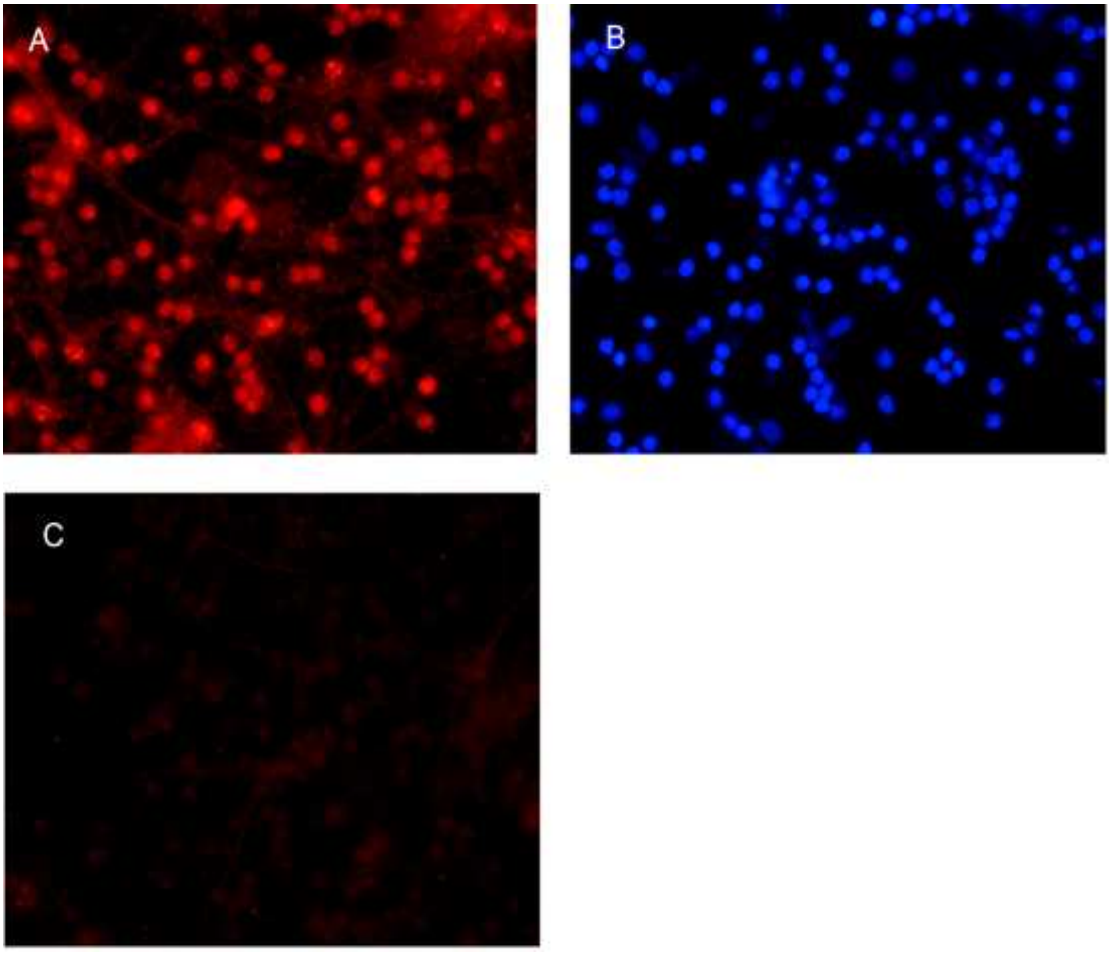

Fig. V.3.2 Fluorescent staining of a CGN culture with PEDF antibody (A). The appropriate IgG control shows completely negative staining (C). A nuclear staining with Hoechst of the same area as shown in (A) shows that the majority of cells in culture were CGN's (B). CGN's were identified by their typical small round appearance. The data shows that nearly all cells in culture stained with PEDF antibody.

Discussion:

Western results support the hypothesis that CGN cultures not only produce, but also secrete PEDF, since PEDF could be detected in the conditioned medium while the control medium was negative for PEDF. The finding that cell lysates did not contain measurable amounts of PEDF, although a high amount of protein was loaded on the gel, indicates that PEDF does not

accumulate in these cells to a large extend. Similarly, PEDF could only be localized in some cells after inhibition of its secretion (Kozaki et al., 1998).

It is not sure if PEDF in CGN cultures might have been also derived from a small amount of fibroblasts in culture, since it has been shown that fibroblasts are able to produce PEDF (Pignolo et al., 1993; Palmieri et al., 1999). In order to answer this question *in situ* hybridization would have to be performed, or cell cultures produced that are 100% free of fibroblasts.

However, PEDF strongly labeled CGN's *in vitro,* suggesting that CGN's might possibly produce PEDF. This idea is supported by the fact that PEDF mRNA is expressed in nearly all cells of the retina (Behling et al., 2002; Karakousis et al., 2001), as well as motor neurons (Bilak et al., 1999) which also immunohistochemically stain for PEDF. Alternatively, CGN's might only bind PEDF. This hypothesis is supported by the finding that contrary to most other brain regions the PEDF transcript could not be detected in the human cerebellum, suggesting that PEDF may not be produced in this part of the brain (Tombran-Tink et al., 1996) and only bind to CGN's. PEDF would then have to be transported into the cerebellum via the CSF or other cellular connections in order to affect CGN function. The lack of PEDF gene expression in the cerebellum, and therefore a potential low PEDF content in this part of the brain, might explain why it was not possible to determine for sure whether CGN's stained for PEDF in brain slices. Alternatively, the PEDF production in the cerebellum under physiological conditions might be too low in order to be visualized by immunohistochemical means. A low PEDF content in the cerebellum would also

explain why CGN's in culture were strongly labeled for PEDF while CGN's in brain slices were only weakly stained for PEDF or not at all.

In summary, although it could not be determined for sure whether CGN's produce PEDF themselves, the data indicate that CGN's *in vitro* and in brain slices bind PEDF. The low PEDF signal in brain slices is likely due to the presence of a low PEDF content in this part of the brain.

V.4. PEDF immunostaining in the rat brain during postnatal brain development.

PEDF has been shown to have different effects on mature versus immature neurons (Taniwaki et al., 1997; Araki et al., 1998; Bilak et al., 1999; Houenou et al., 1999) and has been found to be elevated in brain diseases like amyotrophic lateral sclerosis and Alzheimer's disease (Kuncl et al., 2002; Yamagishi et al., 2004). To test if the same staining pattern of PEDF could be observed at early postnatal ages and very old ages compared to the young adult brain, brains from Long Evans rats were stained for PEDF and analyzed at birth, 2 weeks, 1 month, 3 and 17 months of age.

Results:

At birth, only vessels intensely stained for PEDF, as indicated by double labeling with Glut-1, although there was a relatively strong general background of the tissue (Fig. V.4.1 and V.4.2). Although staining intensities were not measured, it was obvious that the PEDF labeling of vessels was the strongest at the earliest age tested and vessel staining significantly decreased already by two weeks of age after which it did not appear to change any more (Fig. V.4.2). Neuronal labeling of PEDF only started to appear around one month of age, as determined by double labeling with NeuN (data not shown), typical appearance of stained structures and location in the brain (Fig. V.4.2 and Fig. V.4.3). Neurons still showed prominent staining at old age, although only at this age some unspecific granular staining could be observed as well (Fig. V.4.2F and V.4.3F).

In general, neurons were labeled stronger in adulthood than at young age and endothelial staining was the strongest at birth.

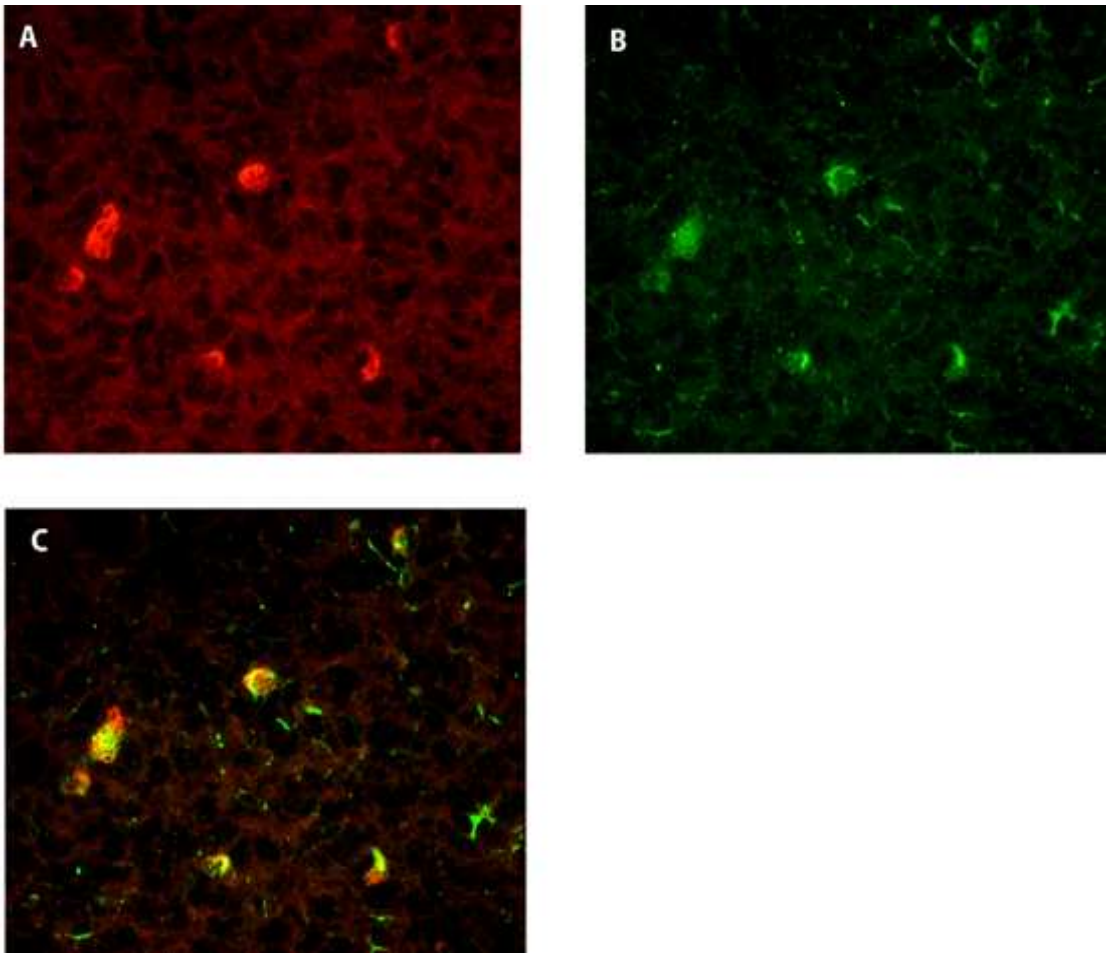

Fig. V.4.1) Fluorescent double labeling of a Long Evans brain at birth with PEDF (A) and Glut-1 antibody (B). Images were super imposed and show that all PEDF positively stained structures also stained for the vessel marker Glut-1 (C), indicating that at birth PEDF can be only localized in vessels.

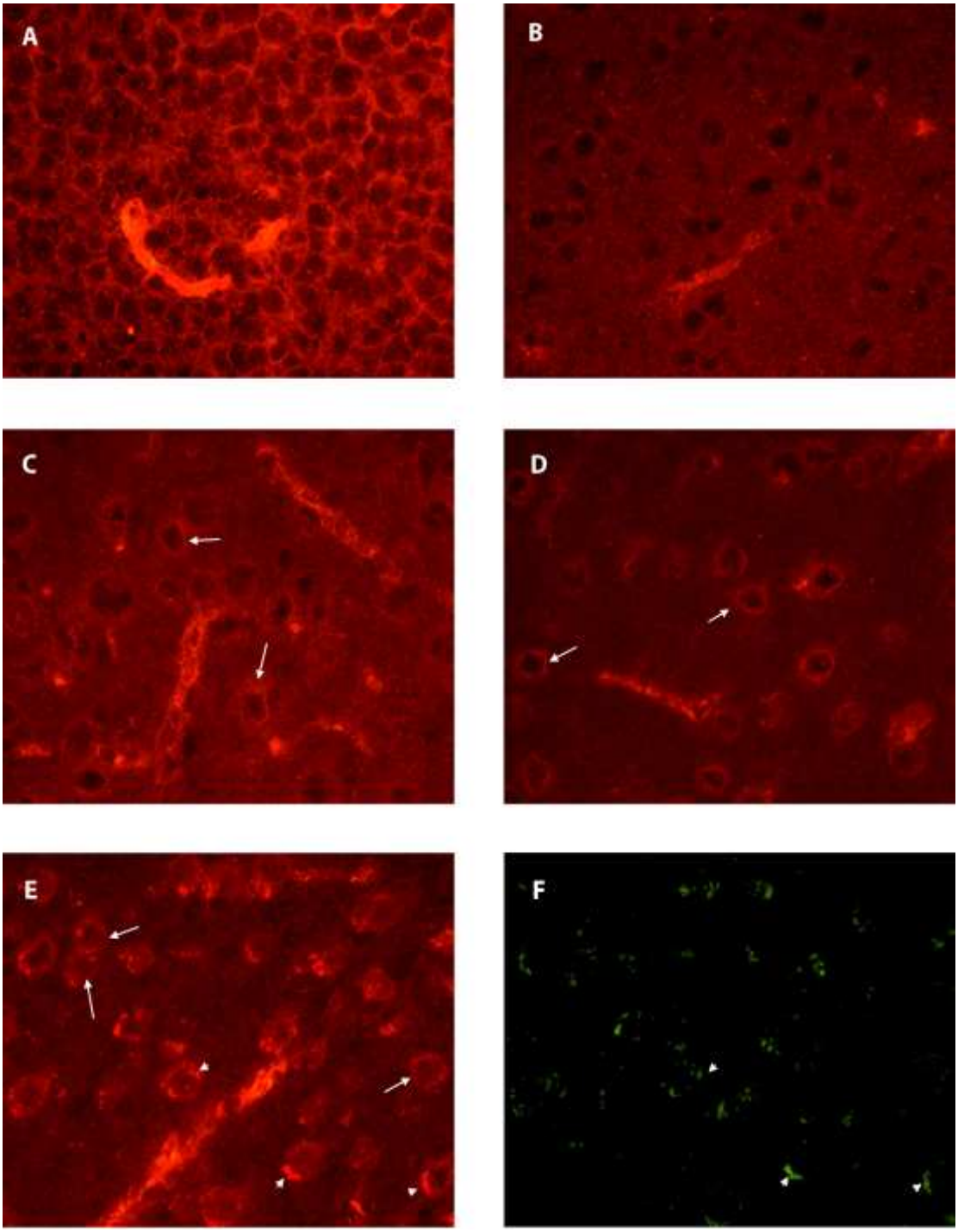

Fig. V.4.2) Fluorescent staining of the cortex of Long Evans brains with PEDF antibody at birth (A), 2 weeks (B), 1 month (C), 3 months (D) and 17 months of age (E). (F) shows the same image in (E) with a green filter, indicating that there is some unspecific staining that is granular in appearance (arrow heads) and only appeared at 17 months of age. The figure shows that typical labeling of neuronal cells with PEDF antibody (arrows) only started to appear at 1 month of age and lasted until high age.

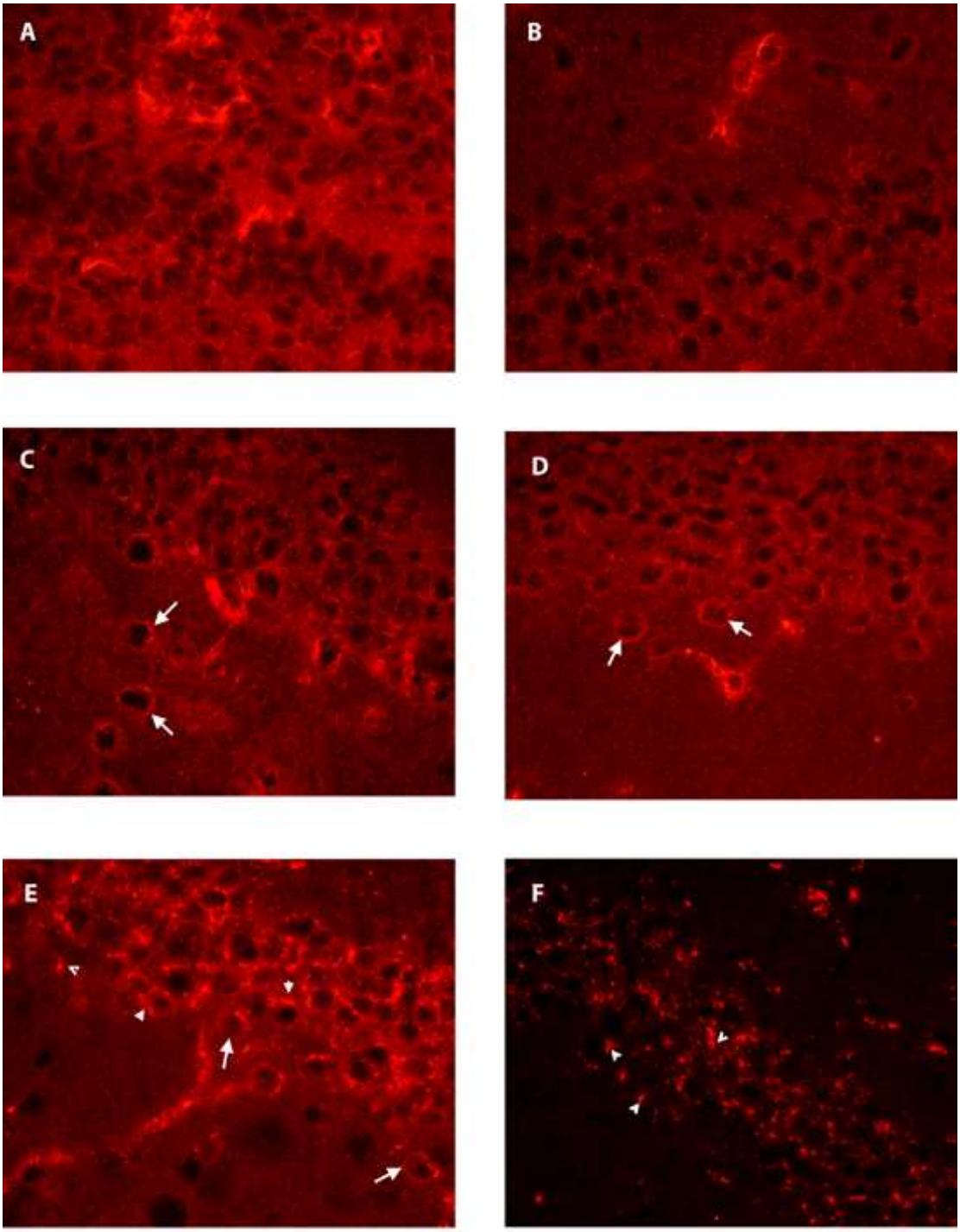

Fig. V.4.3) Fluorescent staining of the dentate gyrus of Long Evans brains with PEDF antibody at birth (A), 2 weeks (B), 1 month (C), 3 months (D) and 17 months of age (E). The IgG control of a 17 month old brain (F) indicates that there is some unspecific staining that is granular in appearance (arrow heads) and only appeared at this age. The figure shows that typical labeling of neuronal cells with PEDF antibody (arrows) only started to appear at 1 month of age and lasted until high age.

Discussion:

PEDF immunohistochemistry of endothelial cells was especially strong at birth and decreased in staining intensity already by two weeks of age, suggesting PEDF's involvement in early brain development and especially in vessel development. The clear labeling of endothelial cells at a time when vessels are still growing suggests that PEDF might not only function as an antiangiogenic agent in the brain at this developmental stage. This idea is supported by a study that shows that PEDF can in fact have opposite effects on endothelial cells of different phenotype (Hutchings et al., 2002). In this study, PEDF stimulated endothelial cell growth if endothelial cells were previously stimulated with VEGF. Another hypothesis that might explain the strong vessel staining early in development would be that PEDF might only play a role in later stages of vessel formation after the sprouting endothelial cells roll up to form tubes and blood circulation begins (Peterson et al., 2003). PEDF might then function as an angiogenesis terminator. It has been also shown in various eye diseases that PEDF induces apoptosis only of activated endothelial cells that participate in the process of neovascularisation, but not of endothelial cells of established vessels, furthermore indicating that not all endothelial cells seem to respond to PEDF in the same way.

In addition to modulating the growth of endothelial cells, in the eye PEDF has been shown to have antivasopermeability function and reduces the VEGF-induced vascular permeability (Liu et al., 2004). Thus, PEDF could be an important regulator of the permeability of the blood brain barrier as well. It is

possible that PEDF confers its different effects on endothelial cells by different receptors on the same cell, or by different posttranslational modifications of the protein that affect its function. For instance, the same non-phosphorylated 44mer (amino acid 78-121) that confers the neurotrophic activity of PEDF also possesses permeability modulating activity for endothelial cells. It furthermore has been shown that the antivasopermeability effect depends on a subset or all four amino acids at position 101, 103, 112 and 115. Interestingly, PEDF in the plasma is a phosphoprotein and Ser114 is the main phosphorylation site of the extracellular located protein kinase CK2 which enhances the antiangiogenic activity of PEDF (Maik-Rachline et al., 2004). Thus, it could be possible that the phosphorylation state of the protein may not only influence the antiangiogenic and neurotrophic function of the protein, but also its antivasopermeability effect due to the close vicinity of amino acids that confer the anti-angiogenic and anti-vasopermeability function to the protein. Another possibility is that PEDF might have different effects on endothelial cells during different stages of development. Further knowledge of the PEDF receptor(s) and the effects of the different posttranslational modifications of PEDF will be needed in order to understand how PEDF exerts its different functions on endothelial cells under normal physiological conditions.

For neuronal cells, the temporal pattern of the PEDF staining intensity was exactly the opposite compared to endothelial cells. At birth and young postnatal age, virtually no or very little PEDF labeling of neuronal cells could be detected.

With increasing maturity of the brain, however, PEDF immunoreactive neurons increased in staining intensity. Due to the fast secretion of PEDF from the cell, it is probable that immunohistochemical staining of cells for PEDF reflects bound or internalized PEDF rather than intracellularly produced PEDF, so that the increased staining could possibly reflect an upregulation of a PEDF receptor. However, PEDF has been shown to affect many different embryonic neurons. Thus far, there are no indications why PEDF staining of neurons only appeared between 2 and 4 weeks after birth. Similarly to the brain, at birth no PEDF staining could be detected in the undifferentiated neuroblastic cell layer of the retina while the differentiated RGC layer was labeled for PEDF, suggesting that only fully mature and differentiated cells may be labeled for PEDF. In line with this idea is the report that no PEDF signal could be observed in the nucleus of isolated embryonic cultures upon incubation with labeled PEDF, while in postnatal day 8 slice cultures of motor neurons PEDF could be localized in the nucleus (Bilak et al., 2002). Bilak and colleges have therefore suggested that PEDF might have different signaling pathways at different developmental stages (such as autocrine versus paracrine).

In fact, it has been shown that PEDF can have different effects on the same cell type depending on its state of maturity. Thus, PEDF protects developing but not mature hippocampal neurons against glutamate-induced toxicity (DeCoster et al., 1999). PEDF also protects immature CGN's, but not mature CGN's against natural apoptotic cell death (Araki et al., 1998), and protects mature CGN's from glutamate-induced toxicity (Yabe at al, 2001). It

furthermore has been shown that the different effects of PEDF on mature versus immature CGN's appear to be caused by a differential activation of genes. For instance, PEDF led to a long lasting induction of genes for nerve growth factor, brain derived neurotrophic factor and glial cell derived neurotrophic factor in mature neurons, while immature neurons upregulated anti-apoptotic genes for Bcl-2, Bcl-x and manganese superoxide dismutase upon PEDF exposure (Yabe at al, 2001). Similarly, PEDF was shown to activate proinflammatory genes in neonatal astrocytes, but not in adult astrocytes (Yabe et al., 2005). Thus, the state of differentiation appears to control the intracellular cascades and gene expression that are activated by PEDF.

In summary, the data of this work are in support of the hypothesis that PEDF plays an important role during early development of the brain and in particular in vessel growth and maintenance. The stronger immunohistochemical staining of neurons for PEDF with increasing maturity of the brain suggests that the function or signaling pathway of PEDF in the brain may change with age. The findings of this study are also in agreement with previously published reports which suggest that PEDF may affect immature neurons by inducing their differentiation and by preventing their natural apoptotic death, while promoting protection against acute toxicity and slow glutamate-mediated neurodegeneration of mature neurons in later postnatal life.

V.5. Overall PEDF content during brain development.

There is evidence that PEDF protects immature, as well as some mature neurons against various types of cell death. However, PEDF could only be localized in neurons later in postnatal life. Contrary to that, staining of vessels was the strongest just after birth. Therefore, the overall PEDF content was measured during late embryonic brain development until old postnatal age in order to determine the overall PEDF content during brain development. For that purpose, brains of Long Evans and Wistar rats were collected at various time points between embryonic day 15 and 17 months of age and their PEDF content measured by Western blot analysis.

Results:

Western blot results indicate that there was no overall difference in PEDF content of brains between Long Evans and Wistar rats as determined by a Kruskal Wallis test ($P=0.355$, $n=119$; data not shown)**,** indicating that there are no strain specific differences in the PEDF content of the brain. Therefore results were combined.

As can be seen in figure V. 5.1, the PEDF content of the entire brain was the highest during embryogenesis with 402±4.2 pg/µg protein at E15, and decreased already by about 43% at birth. With increasing postnatal age, the PEDF content decreased even further, but not to the same degree as during embryogenesis and reached a low level of 105.4±1.9 pg/µg protein at 3 months of age. A Spearman rank correlation test showed that after 3 months there was

no significant change any more in the PEDF content with age (n=30, r=-0.247; P=0.189). Overall however, a Spearman rank correlation indicated that the PEDF content of rat brains significantly decreased with increasing age (n=120, r=-0.899; P<0.000).

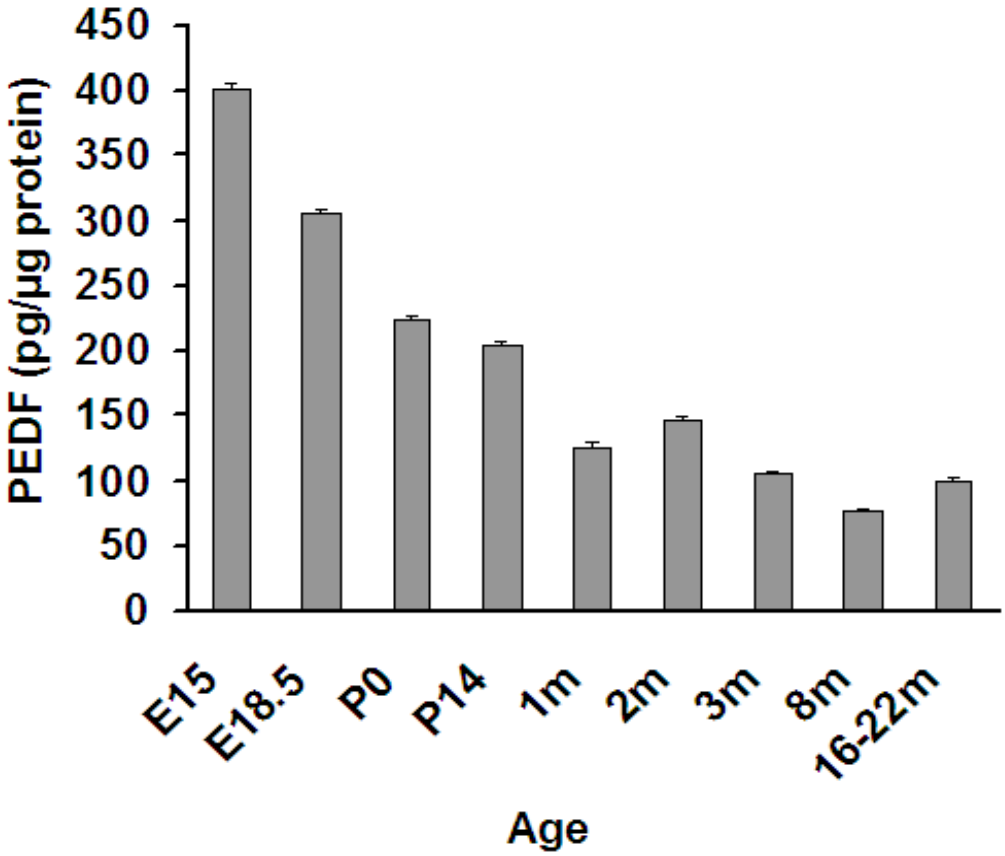

Fig. V. 5.1) PEDF content of rats brains from E15 until high age.
50 µg of complete male Long Evans and Wistar brain homogenates between E15 and 22 months of age were analyzed by Western blot analysis. PEDF was normalized to the average actin signal and then compared to a standard curve of PEDF (15-60 ng, not shown). Data are shown as mean ± SEM. A Spearman rank test showed that overall there was a significant decrease of PEDF with increasing age (n=119, r=-0.927, P<0.000). After 3 months of age, however, there was no correlation between the PEDF content of brains and age any more (Spearman rank, n=30, r=-0.247, P=0.189).

In order to find out whether the measured PEDF content of the brain was mainly derived from cells in the brain or from blood, the PEDF content of serum from animals between birth and 17 months was tested. As can be seen in figure V.5.2, the PEDF content in the serum significantly increased between birth and 2 months (P<0.002, n=20) from 2.96±0.31 µg/ml to 9.2±0.43 µg/ml. Serum levels stayed the same between 2 and 3 months (P=0.762, n=10), but significantly decreased afterwards to levels between 5.89±0.4 and 6.99±0.53 µg/ml (3 versus 8 months: P=0.029, n=8). There was no significant change in PEDF serum levels after 8 months of age (P= 0.257, n=10).

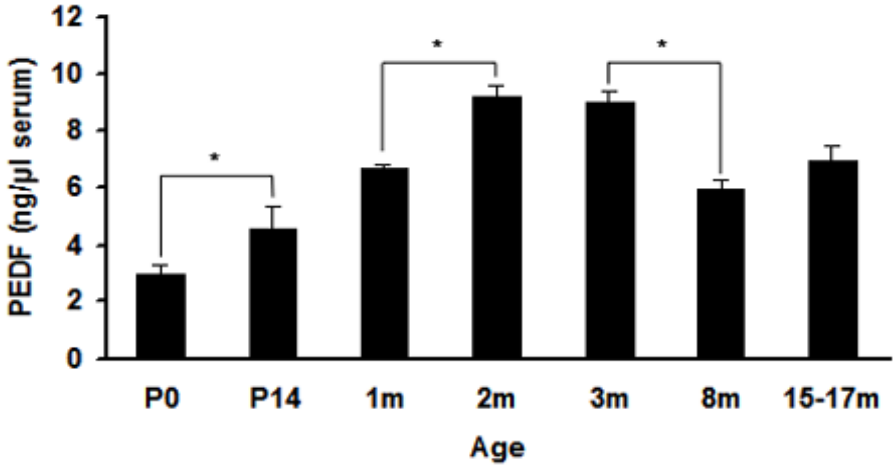

Fig. V.5.2) The PEDF content of blood serum from birth until old age.
0.5 µl of blood serum from Long Evans rats between P0 and 17 months of age was separated by SDS/Page, stained for PEDF and compared to a standard curve of PEDF (1-10 ng, not shown). Data are shown as mean ± SEM. A Kruskal Wallis test showed that overall there was a significant change of PEDF over age (P<0.000, n=34) and a Mann-Whitney-U test showed that there was a significant difference in the PEDF levels between P0 and P14 (P=0.038, n=10), 1 and 2 months (P=0.01, n=10), as well as 3 and 8 months of age (P=0.029, n=8).

Discussion:

Peterson and colleges (2003) have previously reported the adult human PEDF plasma concentration to be approximately 5 µg/ml which is similar to rat serum levels of this study which ranged from 3-9.2 µg/ml. Contrary to PEDF levels in the brain which decreased with increasing age, the serum concentrations of PEDF more than tripled between birth and young adulthood, but significantly decreased again by 34-22% at 8-17 months compared to levels at 3 months.

The mean capillary distance can be considered as an indicator for neuronal activity in the pericapillary environment and is an indicator for vessel growth (Jucker et al., 1990). It has been shown that in the rat visual cortex the capillary volume and density increases before birth and between 10 and 20 days after birth, with no subsequent change thereafter (Keep and Jones, 1990). Similarly, Tieman and colleges (2004) have shown that there is an increase in the blood vessel density between 1 and 6 weeks of age in the cat's visual cortex. At old age however, the capillary density decreases again in various areas of the brain (Jucker et al., 1990; Lynch et al 1999). There are also indications that PEDF in the blood functions as an antiangiogenic factor (Maik-Rachline et al., 2004). This would be in agreement with data of this study that show that PEDF in the serum is low early in postnatal life, at a time when brain development and vessel growth are still ongoing, and high during adulthood when normally there is no change in vascular density.

The decrease of PEDF in the serum at old age, at a time when the capillary density also decreases, suggests that PEDF may be required for vessel maintenance. Likewise, the plasma levels of insulin-like growth factor 1, which has an important role in vascular maintenance and remodeling, have been shown to be correlated with the decreasing vascular density with increasing age (Sonntag et al., 1997).

In the brain, the most dramatic decrease of the total PEDF content occurred between embryonic day 15 and 3 months of age. Afterwards, there was no significant change any more in the PEDF content of the brain. In comparison to that, vessel volume and density increase until about 3 weeks in the rat's visual cortex and PEDF serum concentrations reach their maximum at 8 weeks after birth. Therefore, one has to conclude that the PEDF production in brain tissue itself is the highest during embryogenesis and decreases already early in postnatal life. Because PEDF levels in complete brain homogenates remained fairly constant after 3 months of age, but PEDF serum concentrations decreased and potentially also the vessel density as well, it may be possible that PEDF production increases slightly again in old brain tissue compared to young adult brain. This idea is supported by the relatively strong staining of neurons as well as endothelial cells at old age. Furthermore, since the PEDF content was the highest at a time when practically only endothelial cells intensely stained for PEDF, it could be assumed that at least early during brain development the majority of the PEDF in the brain might be derived from endothelial cells and not

from neurons. However, further investigations such as *in situ* hybridization will be needed in order to verify these hypotheses.

PEDF is also known as early population doubling level complementary DNA-1 or EPC-1 and in RPE cells and fibroblasts PEDF production has been shown to be decreased with the exhaustion of the cell cycle in replicative senescence (Pignolo et al., 2003; Tombran-Tink et al., 1995). The protein has also been suggested to play a role in the entry of early passage cells into a Go state or in the maintenance of such a state, since low PEDF levels increase the number of cells entering DNA synthesis (Pignolo et al., 2003). PEDF may thus function as a negative controller of the cell cycle and as a differentiation factor, as has been shown previously for motor neurons and retinoblastoma cells (Houenou et al., 1999; Tombran-Tink and Johnson, 1989; Tombran-Tink et al., 1991). This would be in line with the finding of this study that PEDF levels decreased rapidly during embryogenesis and early postnatal life once cells are differentiated. Preliminary unpublished *in vitro* and *in vivo* data of our laboratory have also shown that PEDF inhibits the VEGF-induced replication of stem cells in the subventricular zone, furthermore supporting the idea of PEDF as a negative controller of the cell cycle.

Previously cited reports have shown that in immature neurons PEDF appears to have mainly survival and differentiating effects, while it mainly protects mature neurons against various kinds of insults. A high concentration of PEDF in the brain during embryogenesis and decreasing levels of PEDF with

age would be in accordance with this idea and suggest that the main function of PEDF may be during embryogenesis and early postnatal development of the brain. In support of this idea is the suggestion of Kozaki and colleges (1998) that PEDF may play an important role in organogenesis and morphogenesis due to the location of PEDF in relation to the formation of bone, cartilage, teeth and basement membrane in the mouse embryo.

In conclusion, the finding that PEDF levels were the highest at E15 and decreased fast during embryogenesis suggests an important role of PEDF during early brain development and are in line with the idea of PEDF as a differentiation factor. The fact that PEDF levels in the entire brain remained constant in adulthood, although PEDF serum levels decreased, suggests that PEDF may have also significant functions in the brain during adulthood. The finding of this work that serum PEDF concentrations changed with age, and that blood cells are able to express the PEDF gene could possibly help in the early detection of diseases in which serum PEDF might play a role.

V.6. Final conclusions of chapter V.

- The high PEDF content in the brain during embryogenesis and early postnatal age, and the strong labeling of vessels at young age suggest that PEDF may play an important role early during brain development and in particular in vessel growth and their maintenance.
- The low PEDF concentrations in the blood at a time when vessel growth continues in the brain is in support of the idea that PEDF is an antiangiogenic agent in the blood.
- The labeling of neurons in many different brain regions until old age and the low, but constant PEDF levels in the entire brain from 3 months on indicate that PEDF may have also important functions in the brain even at high age and support the idea of PEDF as a neurotrophic and neuroprotective factor.
- The strong immunoreaction of cells in the ventricular zone and dentate gyrus suggests that PEDF may be even involved in neurogenesis. This hypothesis is supported by preliminary *in vitro* and *in vivo* data of our laboratory that suggest that PEDF modulates stem cell replication.

In conclusion, the data of this study are in line with the hypothesis that during postnatal development of the brain PEDF functions as a neurotrophin, differentiating and/or neuroprotective factor, as well as an important regulator of vessel growth, maintenance and vessel permeability.

VI. Differences in posttranslational glycosylation of PEDF are tissue specific and developmentally regulated.

VI.1. Introduction:

There is only one known gene for PEDF that is rather conserved among different species (Singh et al., 1998; Tombran-Tink et al., 1996). The gene contains 8 exons and 7 introns and there are no alternative splice forms known. PEDF is a monomeric glycoprotein with a globular structure that has one protease-sensitive exposed loop. Since there is no known protease target for inhibition, no conformational change and no impairment of its neurotrophic function upon cleavage of the protease-sensitive loop, PEDF belongs to the non-inhibitory serpins (Becerra et al., 1995).

The human 418 amino acid large protein is secreted from the cell upon production and has been reported to have a size of 46-50 kDa. It has been shown that residue position 78-121 confers neurotrophic function to the protein (Alberdi et al., 1999; Bilak et al., 2002) and has antivasopermeability function (Liu et al., 2004), while the phosphorylation of PEDF at Ser 24 and mainly 114 is related to its angiogenic activity (Maik-Rachline et al., 2004).

Although there are indications that the effects of PEDF as angiogenic inhibitor and neuroprotective agent may be mediated by different types of receptors of different size (Alberdi et al., 1999; Yamagishi et al., 2002), until now no receptor has been characterized in detail. Apart from phosphorylation at three different amino acids, other posttranslational modifications of the PEDF protein

have been reported, such as deamination of the N-terminal and N-linked glycosylation (Peterson et al., 2003; see also Fig. I.2.2, page 6) which might also have an effect on the function of PEDF. The N-terminal of the protein can be deaminated by a posttranslational modification of glutamine to pyroglutamate (pGlu) and is then sensitive to Edman degradation or not sensitive to Edman degradation if only the preceding asparagine is present (Peterson et al., 2003). Although the function of this modification is not known, it has been suggested that removal of pGlu may modify the higher order structure of PEDF (Peterson et al., 2003) and therefore possibly also its function. This is insofar interesting as PEDF protein from bovine interphotoreceptor matrix is unblocked (Wu et al., 1995) and PEDF in bovine vitreous and blood is blocked to Edman degradation (Wu and Becerra, 1996; Petersen at al, 2003).

Most proteins are subject to posttranslational modifications of which glycosylation is the most common form. Glycosylations perform critical biological functions such as protein sorting, immune recognition, receptor binding, inflammation, pathogenicity and many other processes. The majority of proteins that are secreted into the extracellular space are glycoproteins, while very few proteins in the cytosol are glycosylated. There are two major classes of oligosaccharides (glycans) that are attached to proteins in the endoplasmatic reticulum and/or the Golgi apparatus. N-linked glycans are attached to the amide side chain of Asn which form part of the amino acid triplet AsnXaaSer/Tre, where Xaa is any amino acid except Pro. O-linked glycans are added to Ser or Thr residues. The terminal residues on these carbohydrate chains are commonly

sialic acids which affect the charge of the protein. The diversity of oligosaccharide structures attached to proteins often results in heterogeneity of the mass and charge of proteins.

PEDF has one potential N-linked glycosylation site at position Asn 266 and PEDF in the blood has been reported to carry a disialylated biantennary complex type glycan with or without core fucosilation (Petersen et al., 2003; see Fig I.2.2, page 6). Di- monosialylated and asialylated structures without core fucosilation have also been found, so that the calculated molecular mass of PEDF can range from 4197-4926 Da and deglycosylation of blood PEDF with PNGaseF has been previously reported to reduce the molecular weight by 2 kDa as analyzed by Western Blot analysis (Petersen et al., 2003).

To investigate whether PEDF may be differentially glycosylated, PEDF was enzymatically digested with N-Glycosidase F (PNGaseF) or sialidase (neuraminidase) and analyzed by Western blotting**.** PNGaseF removes virtually all N-linked oligosaccharides from glycoproteins that do not contain a fucose, while sialidase removes all sialic acids groups from the oligosaccharide while leaving the glycan chain attached to the protein. To detect any change in molecular mass, the migration pattern of protein samples was analyzed by Western blotting. During Western blot analysis, denaturated proteins migrate in an electrical field and are separated on a SDS/page gel by their different size. The height of the stained band thus corresponds to the molecular weight of the protein and the number of bands indicates differences in the posttranslational modification of the protein that change the protein's mass. Since with this method

it is hard to calculate the absolute weight of the protein, the molecular mass of PEDF was estimated for easier reading of this chapter.

The chapter is divided in three parts in order to address the following questions. First, to test whether age affects the glycosylation status of PEDF, the glycosylation status of PEDF of eye and brain samples of various postnatal ages was investigated. Second, in order to investigate whether the molecular mass of PEDF and thus its posttranslational modifications are tissue specific, several cell cultures were established and their cell lysates and conditioned media tested for PEDF in order to furthermore compare secreted and intracellular PEDF. Third, cell lysates and conditioned media of differentiated and undifferentiated retinal pigment epithelial cells were tested for PEDF in order to investigate whether the cell's differentiation status affects the glycosylation status of PEDF.

VI.2 Results:

VI.2.1 Is the glycosylation status of PEDF related to the age of the organism?

During postnatal development of the eye, the number and molecular weight of PEDF bands changed (Fig. VI.1), indicating a different posttranslational modification of the protein at different developmental ages. Just after birth, PEDF was displayed as three bands of very close molecular weight. However, the middle sized protein of about 47kDa was the most prominent one at this age. Homogenates from young adult eyes clearly contained two prominent PEDF forms of 46 and 48 kDa that were equally presented. At very old age, only the 48 kDa band of PEDF could be consistently detected. When eye homogenates of these developmental ages were deglycosylated with PNGaseF, PEDF of all samples was reduced to one major PEDF form of about 44 kDa and an additional lower band that was rather faint, indicating that PEDF of all eye samples was N-glycosylated.

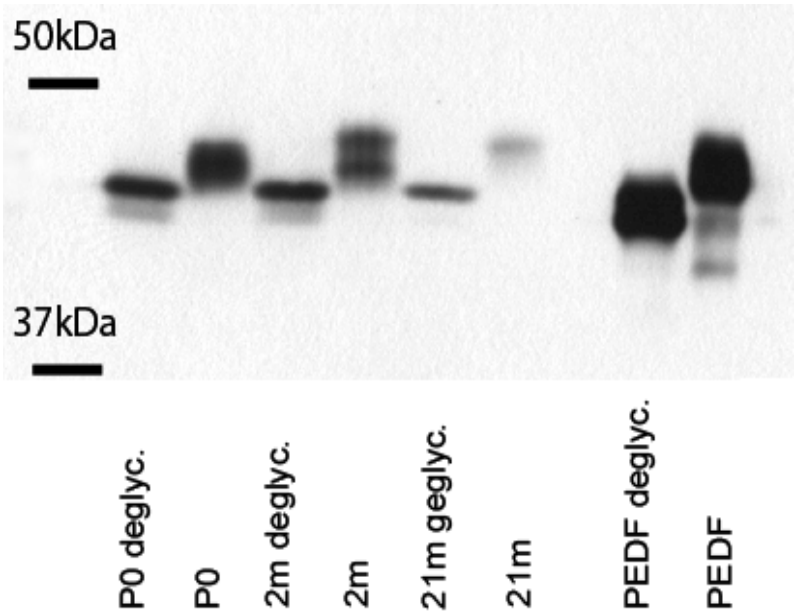

Fig. VI.1) Changes in posttranslational N-linked glycosylation of PEDF during postnatal development of the eye.
50 µg protein of eye homogenates from Long Evans rats at P0 (lane 2), 2 months (lane 4) and 21 months (lane 6) were deglycosylated with PNGaseF, separated by SDS/Page and stained with PEDF antibody. Human recombinant PEDF served as control (lane 8). Deglycosylated proteins are always shown on the left compared to native proteins.

Similar to PEDF from complete eye homogenates, PEDF derived from brain homogenates of different ages also showed differences in the size and number of PEDF bands as revealed by Western blot analysis (Fig VI.3a). At birth, PEDF was displayed as one major band of about 46 kDa, while PEDF from adult brains was always detected as a double band of 46 and 48 kDa. Similar to old eye homogenates, only the 48 kDa band could be detected in very old brain homogenates. Digestion of a P8 brain homogenate with sialidase reduced PEDF to one major molecular weight form of about 44 kDa and a smaller faint band (Fig VI.3b), indicating that PEDF in the brain is a glycoprotein as well. Furthermore, PEDF of the digested brain homogenate appeared to migrate at similar height as

PEDF from the digested eye homogenate at 2 months of age. The data provide evidence that in the eye and the brain PEDF is a glycoprotein and that the degree of glycosylation is age dependent.

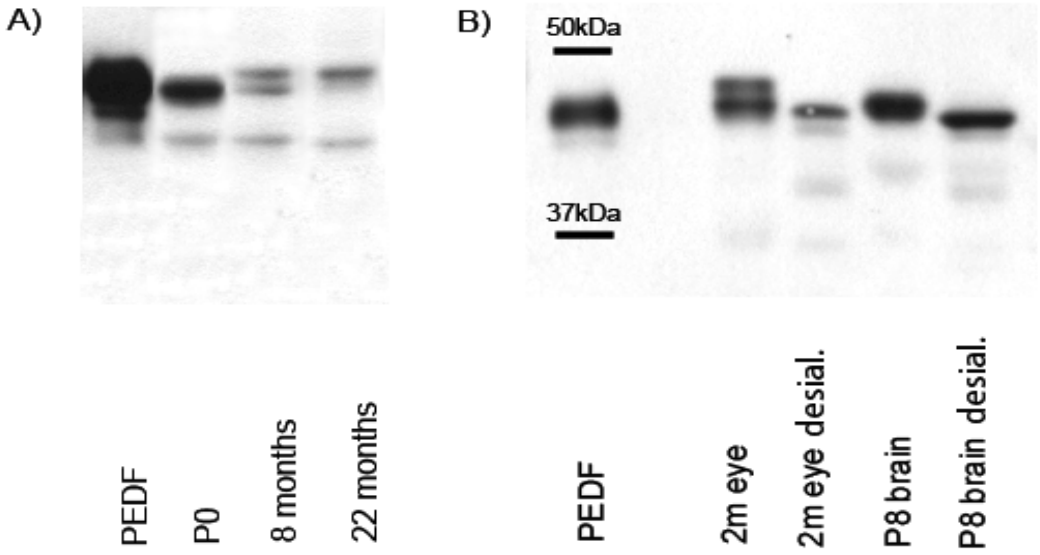

Fig. VI. 3) PEDF in the brain is a glycoprotein.

A) 50 µg from rat brain homogenates from animals at P0 (lane 2), 8 months (lane 3) and 22 months (lane 4) were separated by SDS/Page and stained with PEDF antibody. PEDF at P0 migrated as one major band, while PEDF at 8 months migrated as a double band of larger size. At 22 months, only one band of higher molecular size was detected. Lane 1 shows a positive control of recombinant human PEDF.

B) 50 µg of a homogenate from a 2 month old Long Evans eye (lane 2) and an 8 day old rat brain (lane 4) were enzymatically treated with sialidase (lane 3 and 5), separated by SDS/Page and stained with PEDF antibody. Human recombinant PEDF served as positive control (lane 1). PEDF from both sources were decreased in molecular weight upon cleavage of sialic acid residues.

The following table gives an overview of the effect of deglycosylation on the molecular weight of PEDF of eye and brain homogenates (table VI.1).

Tissue	age	native PEDF		deglycosylated PEDF	
		number of bands	molecular size [kDa]	number of bands	molecular size [kDa]
Eye	postnatal	1 main band	47	1 main band	44
	adult	2	46, 48	1 main band	44
	old	1	48	1 main band	44
Brain	postnatal	1	46	n/a	
	adult	2	46, 48	n/a	
	old	1	48	n/a	

Table VI.1. Estimated molecular weight and number of native and deglycosylated PEDF bands of eye and brain tissue during postnatal development as shown in Western blots.

VI.2.2 Does the molecular mass and the glycosylation status of intracellular and secreted PEDF from different cell types change?

PEDF derived from tissue homogenates contains both the secreted and the intracellular protein, as well as PEDF derived from blood serum. To find out whether there are any differences in protein size between the intra- and extracellular form of the protein, cell cultures were established of rat retinal pigment epithelial cells, rat fibroblasts, rat CGN's and human hepatocytes. Intracellular PEDF derived from RPE cell lysates was detected as a double band of about 46 and 44 kDa (Fig. VI. 2a). The secreted 46 kDa protein in the conditioned medium was however detected as a single band.

As for RPE cultures, PEDF from the conditioned medium of CGN cultures contained only one homogeneous form of PEDF as indicated by the existence of one prominent band of about 46 kDa (see Fig V.3.1). However, intracellular PEDF from CGN culture cell lysates could not be detected, indicating that the intracellular amount of PEDF in CGN's was not high enough in order to be

detected by Western blot analysis. Comparison of PEDF from conditioned media of CGN's, RPE- and fibroblast cultures showed that all conditioned media contained a single size protein of 46 kDa (Fig VI.4). Furthermore, RPE and fibroblast cell lysates both displayed a double band of about 44 and 46 kDa, indicating a large amount of intracellular PEDF in order to be detected by Western blot analysis. The data of these experiments show that the molecular mass of PEDF and therefore also its posttranslational modification differs between intracellular and extracellular derived PEDF of three different rat cell types.

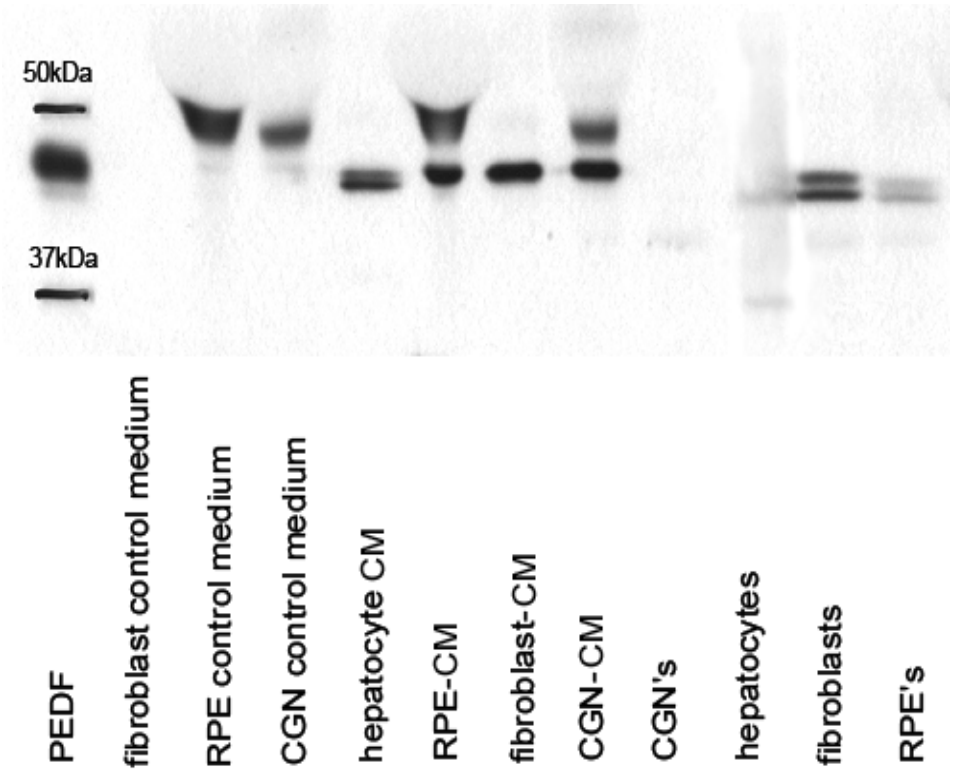

Fig. VI.4) Intracellular and secreted PEDF from various types of cell cultures.
25 µl of conditioned media (CM) from human hepatocytes (lane 5), undifferentiated rat RPE cells (lane 6), rat fibroblasts (lane 7) and rat CGN's (lane 8) were separated by SDS/Page and stained with PEDF antibody. Equal amounts of growth medium for fibroblasts (lane 2), RPE cells (lane 3) and CGN's (lane 4) served as controls and showed no or neglectable amounts of PEDF. Lanes 9 to 12 show signals from cell lysates of CGN's (90 µg), hepatocytes (200 µg), fibroblasts (about 100 µg) and RPE cells (3.75 µg). Lane 1 shows a positive control of human recombinant PEDF. Except for hepatocytes, secreted PEDF consisted of one molecular weight form, while PEDF from fibroblast and RPE cell lysates migrated as a double band.

To confirm the cell type which was used for cell lysates, cultured cells were stained immunohistochemically. Rat RPE cells have been previously described to stain for nestin under the employed culture conditions (Engelhardt et al., 2005) and are known to stain for PEDF. The typical morphological appearance and the labeling for both PEDF and nestin indicated that cultures in fact contained RPE cells (Fig. VI.5). Fibroblasts have been previously described to also produce PEDF (Pignolo et al., 1993 and 2003). Therefore, the employed skin fibroblasts from neonatal transgenic Sprague Dawley rats which were overexpressing green fluorescent protein (GFP) were immunostained for PEDF (Fig. VI.5). The typical appearance and staining for PEDF indicated that cultures contained fibroblasts. Interestingly, the nucleus of RPE cells, but not of fibroblasts stained for PEDF.

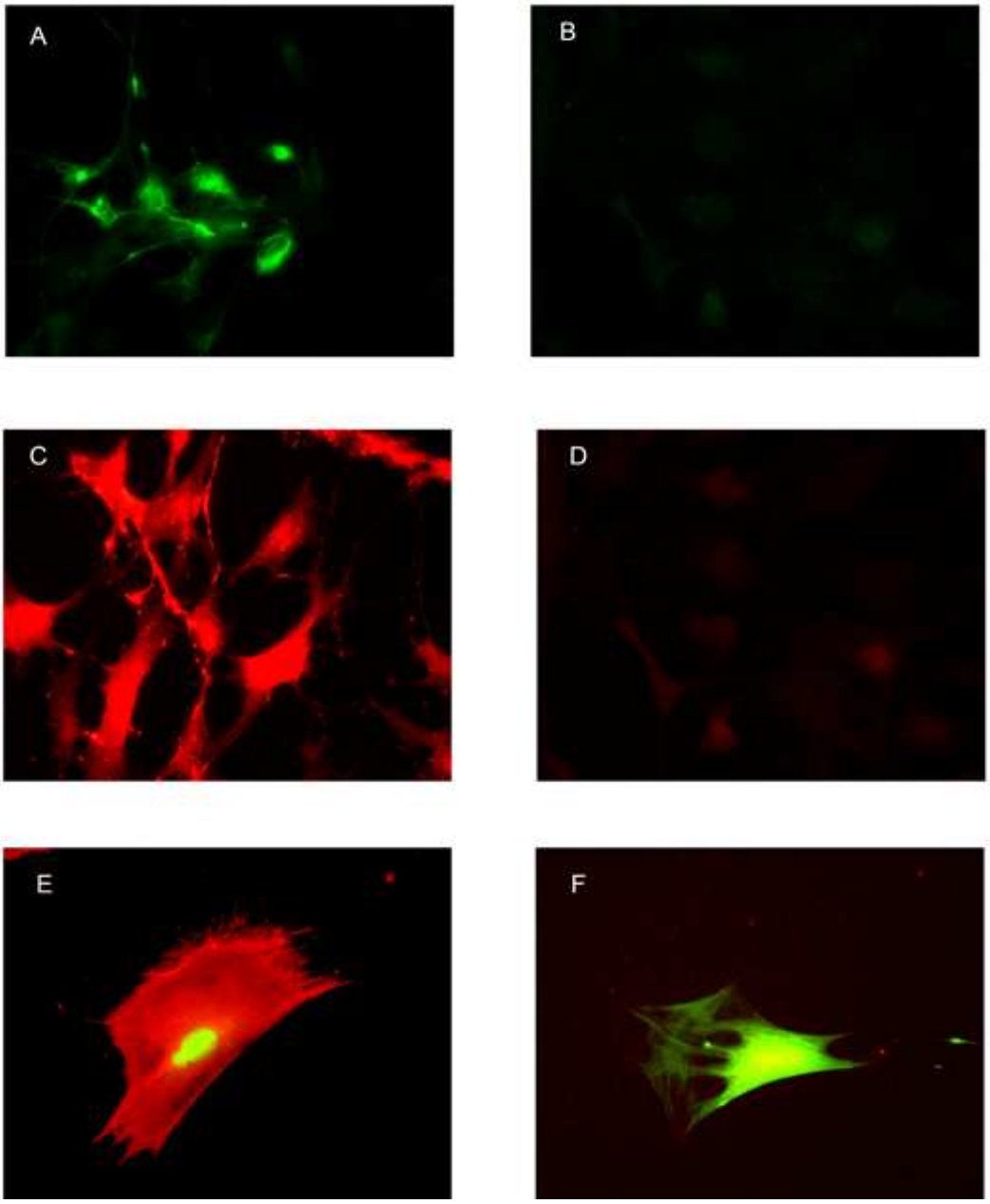

Fig. VI.5) Staining of differentiated rat RPE cells (C) and green autofluorescent skin fibroblasts from GFP expressing transgenic rats (E) with PEDF antibody. RPE cells were also nestin positive (A). Appropriate IgG controls are always shown on the right (B, D, F) which showed no staining. The figure shows that rat RPE cells and skin fibroblasts are stained positively for PEDF.

PEDF has been shown to be expressed in large amounts in the liver (Singh et al., 1998; Tombran-Tink et al., 1996). Proteins of the plasma are mainly

produced in the liver and PEDF exists at a physiologically relevant concentration in the blood (Fig. V.5.2; Peterson et al., 2003). To investigate the posttranslational processing of PEDF derived from this nonneuronal tissue, human hepatocyte culture lysates and their conditioned media were digested and tested for PEDF. Interestingly, the protein pattern was the opposite compared to that found in RPE, fibroblast and CGN cultures, in that the intracellular PEDF was displayed as a single band of about 42 kDa, while the secreted protein migrated as a double band of about 44 and 46 kDa (Fig. VI.6 and VI.7). This indicates that both extracellular forms are modified upon secretion of the cell, since both extracellular PEDF forms migrated higher than the intracellular PEDF of hepatocytes. Digestion of the conditioned medium of hepatocytes with sialidase reduced the upper 46 kDa band, indicating that the difference in protein size of the secreted protein was due to differences in the amount of sialic acid residues that are attached to the N-glycan (Fig. VI.6). Upon enzymatic deglycosylation of the secreted PEDF, both bands seemed to be reduced to one molecular weight of around 42-43 kDa, indicating that the two secreted PEDF forms differ in the glycan residue (Fig. VI.6). Removal of glycan chains and sialic acid residues from the intracellular protein had no effect on the migration pattern of PEDF from hepatocyte cell lysates, suggesting that intracellular PEDF is not N-glycosylated (Fig. VI.6). The results show that in hepatocytes the glycolysation status of intracellular and extracellular derived PEDF differs and that the posttranslational modification varies from rat fibroblast, RPE and CGN cultures.

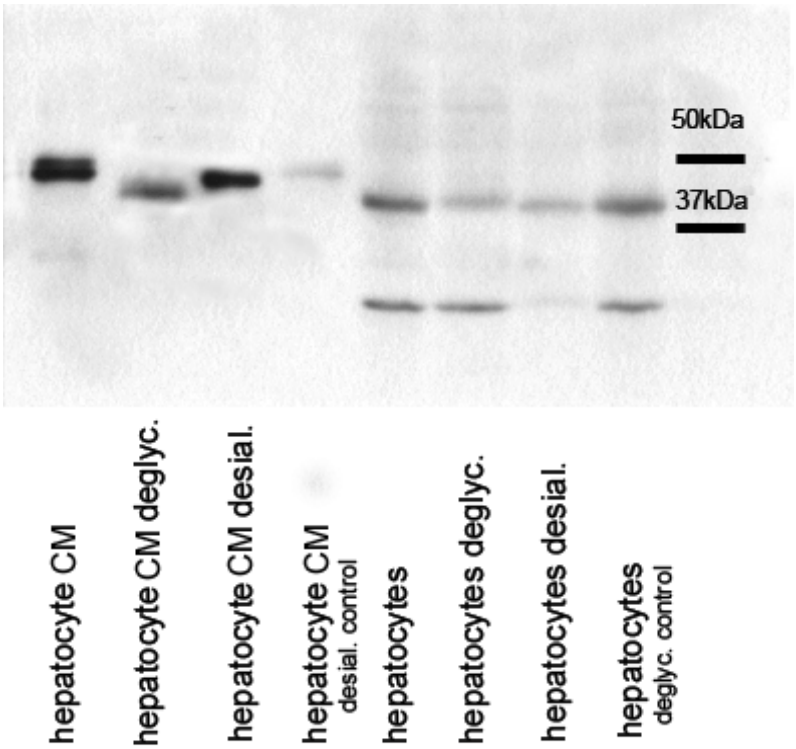

Fig. VI. 6) Only the secreted PEDF protein from hepatocytes is a glycoprotein. 20 µl conditioned medium of hepatocytes (lane 1) were either digested with PGNaseF (lane 2) or sialidase (lane 3), which decreased the molecular weight of PEDF as analyzed by Western blotting and staining with PEDF antibody. Treatment of 100 µg hepatocyte cell lysate (lane 5) with PGNaseF (lane 6) or with sialidase (lane 7) did not change the migration pattern of PEDF. A control for the enzymatic digestion with sialidase (lane 4) or PGNaseF (lane 8) was treated the same, but without enzyme, and did not change the molecular weight of PEDF from conditioned medium and cell lysate, respectively.

Since hepatocytes are the main source of proteins in the blood, PEDF from conditioned medium of heptocytes was compared to PEDF of blood serum. As can be seen in figure VI.7, PEDF from both sources consists of double bands of about 44 and 46 kDa that migrate at the same height. Furthermore, upon digestion with sialidase, the upper 46 kDa band of both sources became reduced, indicating that PEDF from both sources is partially sialylated. This

suggests that PEDF in the blood may be at least in part derived from hepatocytes, since PEDF from both sources had similar molecular weight and reacted in the same way to treatment with sialidase.

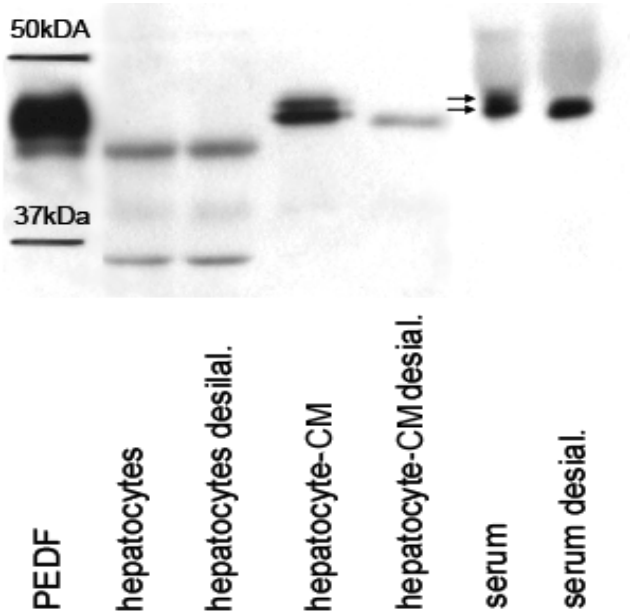

Fig. VI.7) PEDF from serum and hepatocytes are similar in molecular size and both are partially sialylated.
100 µg hepatocyte cell lysate (lane 2), 20 µl conditioned medium of hepatocytes (lane 4) and 30 µg human serum (lane 6) were treated with sialidase (lane 3, 5 and 7, respectively), separated by SDS/Page and stained with PEDF antibody. Human recombinant PEDF (lane 1) served as control. While the migration pattern did not change in the hepatocyte cell lysate, the upper band of the double band of both conditioned medium of hepatocytes and serum was reduced.

The following tables give an overview of the effect of deglycosylation on the molecular weight of PEDF of various cell lysates (table VI.2) and their conditioned media (table VI.2).

Cell lysates	native PEDF		deglycosylated PEDF	
	number of bands	molecular size [kDa]	number of bands	molecular size [kDa]
CGN's	0		n/a	
RPE's	2	44, 46	n/a	
fibroblasts	2	44, 46	n/a	
hepatocytes	1	42	1	42

Table VI.2. Estimated molecular weight and number of native and deglycosylated PEDF bands of various cell lysates as shown in Western blots.

Conditioned medium	native PEDF		deglycosylated PEDF	
	number of bands	molecular size [kDa]	number of bands	molecular size [kDa]
CGN's	1	46	n/a	
RPE's	1	46	n/a	
fibroblasts	1	46	n/a	
hepatocytes	2	44, 46	1	44
blood	2	44, 46	1	44

Table VI.3. Estimated molecular weight and number of native and deglycosylated PEDF bands of conditioned media of various cell lysates as shown in Western blots.

VI.2.3 Is the cell's differentiation status related to the molecular mass of PEDF?

To answer this question differentiated and undifferentiated rat RPE cell cultures were established. Differentiation of cells was achieved by removal of growth factors and addition of serum (for more details, see Methods, page 32-34). Intracellular PEDF derived from both differentiated and undifferentiated RPE cell lysates was detected as a double band of about 46 and 44 kDa (Fig. VI. 2a), suggesting that in these cells the differentiation status has no effect on the posttranslational processing of PEDF that influences the protein's mass.

Similarly, the secreted 46 kDa protein of both conditioned media was detected as a single band. Treatment of undifferentiated RPE lysates with sialidase reduced the upper of the two bands to one 44 kDa band (Fig. VI.2b), suggesting that the two molecular weight forms differ in the amount of sialic acid residues. Deglycosylation of N-linked glycans of the undifferentiated RPE cell lysate (which removes the entire attached oligosaccharide) however equally reduced both bands to the same degree, suggesting the presence of yet another type of modification (Fig. VI.2b). The data of these experiments therefore suggest, that the differentiation status has no effect on the molecular mass of PEDF and that PEDF from RPE cultures is a glycoprotein.

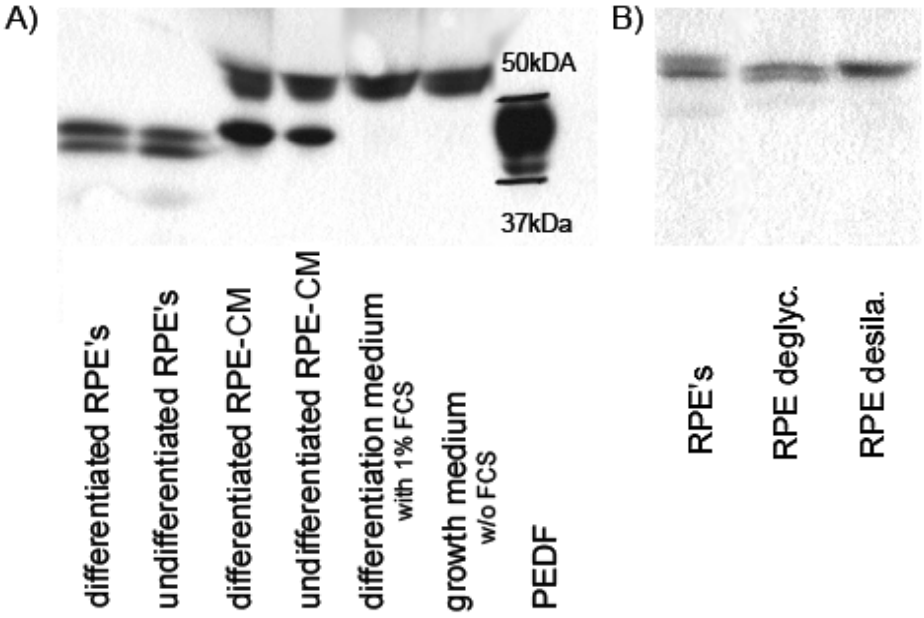

Fig. VI. 2) Differentiated and undifferentiated RPE cell cultures produce PEDF.

A) Seven µg of cell lysates from differentiated (lane 1) and undifferentiated RPE cell cultures (lane 2) and 35 µl of their conditioned media (CM, lane 3 and 4) were separated by SDS/Page and stained with PEDF antibody. PEDF from RPE cell lysates was detected as a double band and PEDF from conditioned media consisted of one band that migrated at the same height as human recombinant PEDF (lane 7). Differentiation medium containing 1% FCS (lane 5) and normal growth medium devoid of FCS (lane 6) were used as controls for conditioned media of differentiated and undifferentiated RPE cell cultures, respectively, and showed no signal.

B) About 3.75 µg of cell lysates from undifferentiated RPE cell cultures (lane 1) were either treated with PGNaseF (lane 2) or with sialidase (lane 3) over night, separated by SDS/Page and stained with PEDF antibody. Deglycosylated PEDF migrated as a double band of lower size compared to the untreated sample, while desialylated PEDF was detected as a single band.

VI.3 Discussion:

VI.3.1 The degree of glycosylation of PEDF is age dependent.

Eye homogenates of various postnatal ages contained one to three forms of PEDF that differed in molecular weight (46-48 kDa) as indicated by their different migration pattern on Western blots. However, all different molecular weight forms of PEDF became reduced to one prominent band of same size. This finding indicates that the differences of PEDF in molecular size at different ages are mainly due to differences in the degree of glycosylation. While all forms of PEDF of different developmental ages were glycosylated, the degree of glycosylation changed during postnatal development and increased with age. Further digestion with sialidase would indicate if this change is due to an increase in the size of the glycan chain or more likely to an addition of sialic acid residues. The answer to this question is insofar interesting since sialic acid residues change the charge of the protein to a more negative one, which might have an influence on the binding and the function of PEDF. This hypothesis is supported by the finding that binding of the neuroprotective and differentiating serpin protease nexin-1/glia-derived nexin to extracellular matrix components changes its function (Bock, 1990; Donovan et al., 1994).

As in the eye, PEDF in brain homogenates is also a glycoprotein. Since PEDF from eye and brain showed the same change in migration pattern during postnatal development, and desialylated PEDF samples from eye and brain also showed a similar migration pattern, it can be assumed that in the brain differences in molecular size of PEDF at different ages are also likely due to

differences in the degree of glycosylation. This, however, will have to be verified by further analysis.

Thus far the significance of the differences in glycosylation and sialylation patterns of PEDF during development is not known. It has been previously shown that the type of glycosylic linkage in the overall brain changes during development of the CNS and the amount of terminal sialic acid residues increases in the adult CNS compared to younger ages (Vallejo et al., 2000). Since alterations in the N-glycan pattern of proteins are known to take place in pathophysiological conditions, such as in inflammation and diabetes mellitus (Higai et al., 2003), the changes in glycosylation during postnatal development are likely relevant to the function of PEDF. For instance, it has been shown that the sialylation status of human erythropoietin affects its activity *in vivo* (Higuchi et al., 1992) and is related to the circulatory clearance rate of some proteins in the bloodstream (Chitlaru et al., 1998; Kronman et al., 1995). Furthermore, the phosphorylation status of PEDF changes its biological function (Maik-Rachline et al., 2004). Thus, depending on the cell's specific glycosylation status of PEDF, as well as the presence of extracellular enzymes that are able to posttranslationally modify PEDF, such as matrix proteases and serine proteases, the function of PEDF may change. That implies that there can be different functional working forms of PEDF present at the same time and space.

VI.3.2 The molecular mass differs between intracellular and secreted PEDF of different cell types.

The presence of only one 46 kDa band of PEDF in conditioned media of RPE, fibroblast and CGN cultures (Fig. VI.4) suggests that these cells may modify PEDF in the same way regarding their glycosylation status. Moreover, there was no difference in the gross amount and migration pattern of PEDF produced by differentiated and undifferentiated RPE cells under the used culture conditions, indicating that the differentiation status has no influence on the production and posttranslational modification of PEDF in RPE cells as analyzed by Western blot analysis. PEDF has been shown to be downregulated with increased passaging of the cell culture (Tombran-Tink et al., 1995). Interestingly, PEDF could be readily detected in passaged RPE and fibroblast culture cell lysates, while in primary CGN culture cell lysates it was below the detection limit of Western blot analysis, suggesting that in RPE cells and fibroblasts PEDF appears to accumulate more than in CGN's. In addition to the 46 kDa band, RPE and fibroblast cells also contained a smaller 44 kDa PEDF band.

Treatment of RPE cell lysates with sialidase indicated that the two molecular weight forms only differed in the degree of sialic acid residues. However, deglycosylation of the RPE homogenate equally reduced both bands to the same degree, which suggests that there must be yet another type of modification in which the two forms differ. Since PNGaseF only removes N-linked glycans, this seemingly contradiction might be explained by the presence of O-linked glycosylations that can also carry up to three sialic acid residues. However, so far there is only evidence of a N-linked glycosylation in human blood PEDF (Peterson et al., 2003). Further digestion experiments and a better

resolution of the different sized proteins will be needed in order to answer this question.

It is not clear, why intracellular PEDF from RPE cultures consisted of a double band of about 44 and 46 kDa, while the extracellular PEDF showed only one 46 kDa form. Deglycosylation experiments suggest that both intracellular PEDF forms may be glycosylated. Since sugars are usually added on secreted proteins as they are made, it is unlikely that the smaller intracellular PEDF represents an immature protein. One explanation could be that the cell takes up some of the secreted protein that is bound to the surface and strip off some sugars or amino acids during this process. This scenario is insofar possible as RPE cells react to PEDF (Malchiodi-Albedi et al., 1998), their nucleus stains for PEDF and it has been shown that PEDF is taken up by axotomized motor neurons where it is transported towards the nucleus (Houenou et al., 1999). Blockage of the uptake of proteins by inhibition of endocytosis might help to answer this question.

VI.3.3 PEDF from blood and hepatocytes.

The liver is a major source of PEDF production (Kozaki et al., 1998; Tombran-Tink et al., 1996) and the main source of serum proteins in the blood. Digestion analysis indicated that only the secreted PEDF of hepatocytes was glycosylated and partially sialylated. The finding that PEDF from serum and conditioned medium of hepatocytes have similar migration patterns and react the same way to treatment with sialidase suggests that PEDF in the serum might be

at least in part derived from hepatocytes, in addition to PEDF that can be produced by blood cells themselves (see Fig V.1.1). The finding that hepatocytes contained an intracellular smaller form of PEDF of about 42 kDa, whose size could not be modified by deglycosylation or desialylation, suggests that hepatocytes may produce a PEDF form that does not get secreted since secreted proteins are normally glycosylated. It is likely that in these cells there is a separate biosynthetic pathway for intracellular PEDF which would not involve the Golgi apparatus that normally attaches sugars to proteins. Alternatively, but less likely, all posttranslational modifications would have to be stripped off during the take up of the protein from the extracellular space. Furthermore, the protein pattern of hepatocyte cultures was the opposite compared to that found in RPE cultures regarding the number of PEDF forms in cell lysates and conditioned media. This suggests that PEDF derived from RPE and hepatocyte cultures may have different functions. This idea is supported by the finding that PEDF in the blood has antiangiogenic function due to phosphorylation of several amino acids (Maik-Rachline et al., 2004), while PEDF production by the RPE layer has neurotrophic and neuroprotective activity (Jablonski et al., 2000 and 2001).

VI.4 Final conclusions of chapter VI.

In conclusion, the data of this study indicate that:

- The degree of glycosylation changes with age in the eye and most likely also in the brain which might affect the binding and the function of PEDF.
- Depending on the type of cell PEDF is produced in there are differences in the degree of posttranslational glycosylation.
- In most cell types the molecular mass differs between intracellular and secreted PEDF.
- The cell's differentiation status does not influence the degree of glycosylation of PEDF in RPE cells.
- Since PEDF forms of both hepatocytes and blood serum have the same molecular mass and are both partially sialylated, it is likely that PEDF in the blood is in part derived from hepatocytes.

In summary, a better understanding of the different posttranslational modifications of PEDF during postnatal development by different cell types and their biological functions will help in the understanding how PEDF experts its many different effects in the entire organism which might be of benefit in future clinical applications.

VII. Summary

The data that were collected during the research for this thesis suggest that:

- The prominent role of PEDF in the CNS may be during embryogenesis and early postnatal development since overall PEDF levels decreased with increasing age in both eye and brain.

- This decrease of PEDF is a natural phenomenon that appeared to be associated with the exhaustion of the cell cycle and was independent of pathological conditions.

- The changing glycosylation patterns of PEDF during postnatal development of the eye and most likely also of the brain suggest a changing function of PEDF in the CNS with age.

- The different posttranslational modifications of PEDF from various cell types suggest that depending on the cell type the protein is produced in PEDF may have different functions.

- There was no significant difference in between albino and pigmented eyes regarding the PEDF content of complete eyes, as well as immunohistochemical localization of PEDF within the retina that could be associated with the lack of pigmentation in Wistar rats.

- The data suggest that a decrease of PEDF production within the retina -as indicated by a reduced staining of RPE cells and preliminary Western blot

data of retinas- in combination with overall declining PEDF levels in the eye might contribute to the observed retinal degeneration at old age.

- The results indicate that a shift in the VEGF expressing cells within the retina, which causes an uncoupling of PEDF and VEGF protein expression at old age, might be related to pathological neovascularisation.

- The data of this study therefore support the idea that declining PEDF levels in organs at old age could easily tip the balance between angiogenic stimulators and inhibitors, so that the lack of PEDF as a differentiating factor and angiogenic inhibitor could contribute to the increase in cancer rates in the elderly.

- The strong expression of PEDF especially in outgrowing, but also established vessels of both eye and brain suggest not only an important role of PEDF during physiological angiogenesis early in postnatal life, but also in the maintenance of vessels during adulthood.

- The finding that overall VEGF/PEDF ratios, as well as the expression patterns of VEGF and PEDF within the retina differed between pathological neovascularisation and vessel growth after birth, suggests that neovascularisation and normal occurring angiogenesis may be regulated differently.

- The data of this study are furthermore in agreement with the hypothesis that PEDF does not always act on endothelial cells as antiangiogenic factor per se and that the effect of PEDF depends on the cell's phenotype.

- The finding that PEDF serum concentrations changed with age and that blood cells are able to produce PEDF as well could possibly help in the early detection of diseases in which PEDF serum levels vary.

- This study demonstrates for the first time that with increasing maturity of the brain, PEDF is localized in neurons of most brain areas until old age, suggesting a neuroprotective role for PEDF even at high age.

- Interestingly, PEDF was located in two different neurogenic zones of the brain, suggesting that PEDF might be able to also modulate neurogenesis.

- The finding that apart from neuronal and endothelial cells additional cell types were immunopositive for PEDF -that were not of astrocytic and oligodendrocytic origin-, together with the localization of PEDF in many specific –so far not described- areas of the adult brain, open the possibility to find other important effects of this factor in the CNS.

We are only starting to understand the complex, multifunctional aspects of PEDF. The protein is clearly an interesting, multifaceted factor with promising therapeutic functions in degenerative diseases of the eye and potentially also the brain, as well as in cancer therapy due to its unique

combination of neuroprotective, neurotrophic, differentiating and anti-angiogenic effects on cells during normal and pathological conditions.

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VIV. Publications and Presentations.

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Piña A.L, **Van Wagenen S**., Stoerr EM, Wachs FP., Aigner L., Winkler J.,Brawanski A. Partial Recovery of Conditioned Taste Aversion after Stem Cell Transplantation in Insular Cortex Lesioned Rats. Third International Symposium on Neuroprotection and Neurorepair. Magdeburg, Germany. May 7-11, 2003

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