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Secretoneurin, substance P and neuropeptide Y in the oxygen-induced retinopathy in C57Bl/6N mice

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ABSTRACT

In this study, we investigated whether the proangiogenic neuropeptides secretoneurin (SN), substance P (SP), and neuropeptide Y (NPY) contribute to the development of abnormal neovascularization in the oxygen-induced retinopathy (OIR) model in mice. By exposing litters of C57Bl/6N mice to 75% oxygen from postnatal day 7 (P7) until postnatal day 11 (P11) and then returning them to normoxic conditions, retinal ischemia and subsequent neovascularization on the retinal surface were induced. Retinae were dissected on P9, P11, P12–P14, P16 and P20, and the concentrations of SN, SP, NPY and VEGF determined by radioimmunoassay or ELISA. The levels of SN and SP increased in controls from P9 until P16 and from P9 until P14, respectively, whereas the levels of NPY were high at P9 and decreased thereafter until P20, suggesting that NPY may participate in the development of the retina. However, dipeptidyl peptidase IV (DPPIV) and the NPY-Y2 receptor were not detectable in the immature retina indicating that NPY is not involved in the physiological vascularization in the retina. Compared to controls, OIR had no effect on the levels of SN, whereas levels of both SP and NPY slightly decreased during hyperoxia. Normalization of the levels of SP, and to a more pronounced extent of NPY, was significantly delayed during relative hypoxia. This clearly indicates that these three neuropeptides are not involved in the pathogenesis of neovascularization in OIR. Moreover, since there were no differences in the expression of two vessel markers in the retina of NPY knockout mice versus controls at P14, NPY is also not involved in the delayed development of the intermediate and deep vascular plexus in the retina in this animal model.

1. Introduction

Neuropeptides including secretoneurin (SN), substance P (SP) and neuropeptide Y (NPY) are synthesized in nerve cells and stored in large dense core vesicles in nerve endings. They are released into the synaptic cleft together with classical neurotransmitters by Ca^{2+}-dependent exocytosis and exert biological effects on target cells via specific receptors. SN is generated *in vivo* by proteolytic processing of secretogranin II, which belongs to the family of chromogranins [21]. SN is distinctly distributed throughout the central and peripheral nervous system and exerts potent chemotactic and angiogenic effects on various tissues including endothelial cells [48]. SP belongs to the family of tachykinins which is characterized by the common C-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2 [38]. Apart from SP, there are two main members of the tachykinin family, neurokinin A (NKA) and neurokinin B (NKB). SP and NKA are encoded by the preprotachykinin (PPT) I gene whereas NKB is encoded by the PPT II gene [29,31]. Tachykinins exert their effects by interacting with specific neurokinin (NK) receptors. Three NK receptors, namely NK-1, NK-2 and NK-3 have been identified that all belong to the superfamily of G-protein-coupled receptors. The tachykinins SP, NKA and NKB act preferentially rather than selectively on these NK-1, NK-2 and NK-3 receptors, respectively [31]. NPY belongs to the pancreatic polypeptide family and was first isolated from porcine brain [45]. It exerts its biological actions through the specific G-protein coupled receptors Y1–Y5 where Y1 is the predominant receptor on vessels that mediates vasoconstriction [40].

Various neuropeptides are expressed in the retina. In general, neuropeptides are found in amacrine cells (ACs) in the proximal inner nuclear layer (INL) and in displaced ACs in the ganglion cell layer (GCL). They represent interneurons with dendrites

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ramifying into the inner plexiform layer (IPL) where specific laminae are formed. Five laminae have been identified in the IPL, and the dendrites of these interneurons ramify into these laminae with differing neuropeptides expressed. In particular, SP has been detected in laminae 1, 3 and 5 [5,27,46], SN in laminae 1, 3 and 5 [32] and NPY in lamina 1 [46] of the human retina. SP is present in some ganglion cells in the rabbit [3], rat [4,44] and chicken retina [8]. The presence and distribution of these peptides in the retina has been extensively studied both in rodents and primates; however, their function is not yet fully understood. SP has been found to modulate the excitability of inner retinal neurons [6,15,51], but for the neuropeptides SN and NPY no biological effects have been described in the retina so far.

There is overwhelming evidence that vascular endothelial growth factor (VEGF) is a main stimulus in the pathogenesis of the neovascularization process. The neuropeptides SN, SP and NPY have also been shown to be pro-angiogenic with a potency similar to VEGF. Thus, they might also contribute to abnormal neovascularization of ocular structures. In particular, SN promoted both vasculogenesis – by mobilization, migration and incorporation of endothelial progenitor cells [19] – and angiogenesis in a corneal pocket model *in vivo* [20] and a murine stroke model [39]. SP has been found to stimulate migration [52] and proliferation of endothelial cells [54], induce neovascularization *in vivo* [54], to stimulate endothelial cell differentiation into capillary-like structures [49] and to promote proliferation of coronary venular endothelial cells [55]. These effects are mediated via the NK-1 receptor [54] by nitric oxide [53] and could be useful for therapeutic angiogenesis in regenerative medicine [23]. NPY is a potent growth factor for vascular smooth muscle cells [57] and endothelial cells [58]. The angiogenic activity of NPY is mediated by either NPY-Y2 [9,24,25], NPY-Y5 or both receptors [57], and involves processing of NPY to the Y2/Y5-selective agonist NPY_{3-36} [14]. Conversion of NPY to a Y2/Y5 agonist occurs endogenously via cleavage of two N-terminal amino acids by endothelial dipeptidyl peptidase IV (DPPIV) [30]. NPY has also been found to exert potent angiogenic effects *in vivo* in the mouse corneal micropocket and the chick chorioallantoic membrane assays [9]. In addition, NPY provokes angiogenesis in aortic sprouting assays in mice [22].

Oxygen-induced retinopathy (OIR) in mice is a well-defined and established animal model for abnormal retinal neovascularization [41]. In this model, mice are exposed to high oxygen levels from postnatal days P7–P11 and then returned to room air inducing episodes of relative hypoxia. During the phase of hyperoxia, vasoobliteration and vessel loss develop in the superficial, intermediate and deep vascular plexus in the retina whereas in the phase of relative hypoxia, there is development of abnormal neovascularization on the retinal surface, which peaks at P17 and regresses thereafter [42]. Furthermore, maturation of the intermediate and deep vascular plexus in the retina is delayed in this animal model. In this study, we used the OIR animal model to investigate whether the proangiogenic peptides SN, SP and NPY are involved in the pathogenesis of abnormal vessel formation. In addition, VEGF levels were determined since this signal protein has been reported to be involved in the development of OIR [34].

2. Materials and methods

2.1. Animal experiments

Animal experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ministry of Science in Austria. The OIR model of ischemia-induced retinal neovascularization in mice has been described in detail [10,41]. In brief, on postnatal day 7 (P7), litters of C57BL/6N mice pups with their mothers were exposed to 75% ± 2% oxygen (hyperoxia) for 5 days (P7–P11) and were then returned to room air for several days (P12–P20) producing retinal ischemia. Control mice of the same strain and age were kept in room air (normoxia). Eight mice of each group were sacrificed at P9, P11–P14, P16 and P20, the eyes were enucleated, the retina dissected, weighed and stored at −80 °C. The retina of the left eye was used to analyze levels of VEGF by ELISA, the retina of the right eye to detect the levels of the neuropeptides SN, SP and NPY by radioimmunoassay (RIA).

NPY k.o. mice were generated by transfecting a 10-kb *Eco*RI fragment from a 129VJ BAC clone containing a 6-kb 5′-flanking sequence as well as exons 1 and 2 of the NPY gene and an 11-kb *Sac*I fragment containing exons 2–4 into ES cells, as described in detail elsewhere [18,47]. Two positive clones were injected into C57Bl/6 blastocysts and chimeric mice were used to generate heterozygous and homozygous NPY-CreGFP knock-in mice. Subsequently mice were backcrossed for at least 10 generations to a C57Bl/6NCrl background (Charles River, Sulzfeld, Germany).

2.2. Determination of VEGF in the retina in controls and OIR by ELISA

Retinal proteins were extracted by sonication in 500 μl PBS buffer. VEGF concentrations were determined using a mouse VEGF ELISA kit from R&D according to the protocol of the manufacturer (R&D Systems Europe, Abingdon, OX14 3NB, UK). In brief, 50 μl of assay diluents RD1N were added to each well of 96-well polystyrene microplates, then 50 μl of standard, control or samples were added to each well, mixed by gently tapping the plate frame for 1 min and incubated for 2 h at room temperature. Thereafter, washing with wash buffer (400 μl) was performed for five times followed by addition of 100 μl of mouse VEGF conjugate to each well, incubation for 2 h at room temperature and washing again with wash buffer for five times. Subsequently, 100 μl of substrate solution was added to each well, incubated for 30 min at room temperature. Finally, 100 μl of stop solution was added to each well and the concentration was determined with an ELISA reader at 450 nm.

2.3. Determination of SN, SP and NPY in the retina in controls and OIR by RIA

Neuropeptides were extracted by sonication in 1 ml of 2 M acetic acid; the supernatant was lyophilized, reconstituted in assay buffer and analyzed by RIA. SN (Neosystems, Strasbourg, France) was iodinated with the chloramines-T method to a specific activity of 7.7×10^4 cpm/ng. SN and ^{125}I-labeled SN were used as a standard and a tracer, respectively. Samples and standards were incubated with the antiserum (dilution 1:18,000) and tracer (10^4 cpm) for 48 h at 4 °C. All dilutions were made with RIA buffer (150 mM NaH_2PO_4 [pH 7.4], 15 mM NaCl, 0.02% NaN_3, 0.0006% phenol red, 0.1% bovine serum albumin and 0.06% gelatine). Bound and free activity was separated by adding 1 ml of dextran-coated charcoal. After 15-min incubation at 4 °C, samples were centrifuged for 15 min at 3200 × *g* followed by counting of the supernatant in a γ-spectrophotometer. The antiserum against synthetic rat SN (SgII 154–186) coupled to keyhole limpet hemocyanin was raised in Chinchilla bastard rabbits with a standard immunization protocol. It reacts equally well with the free peptide and all larger proteins containing the SN sequence including SgII equally well. The SP-RIA was performed with a specific antiserum (diluted 1:10,000) kindly provided by Elvar Theodorsson (Department of Clinical Chemistry, University Hospital, Linkoping, Sweden). The antiserum does not cross-react with other tachykinins or other neuropeptides. Nonradioactive SP was purchased from Peninsula Laboratories (Peninsula Laboratories, San Carlos, CA) and [^{125}I]Bolton-Hunter SP from Amersham

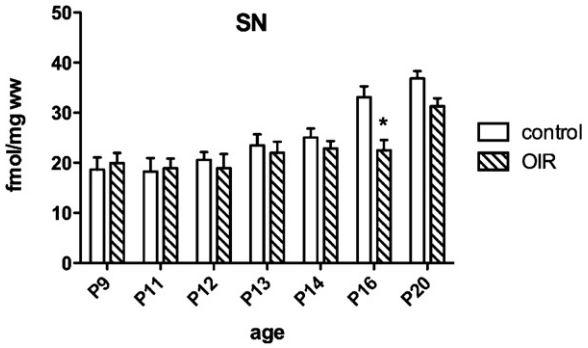

Fig. 1. Effect of OIR on secretoneurin (SN) levels in mice retinae. Concentrations (fmol/mg wet weight) are given as means ± S.E.M. ($n = 8$). Mann–Whitney U test revealed a significant decrease in OIR only at P16 ($^{*}p < 0.05$).

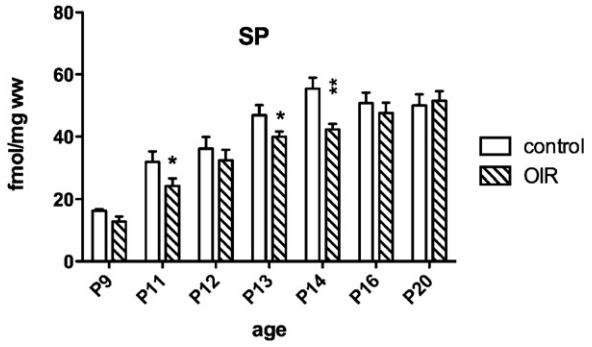

Fig. 2. Effect of OIR on substance P (SP) levels in mice retinae. Concentrations (fmol/mg wet weight) are given as means ± S.E.M. ($n = 8$). Mann–Whitney U test revealed significant decreases of SP in OIR at P11, P13 and P14 ($^{*}p < 0.05$; $^{**}p < 0.01$).

(Amersham Biosciences, Freiburg, Germany). Incubation was performed for 48 h without and a further 48 h with the tracers added (approximately 6000 cpm). Separation of bound and free radioactivity was again performed with dextran-coated charcoal and bound activity was counted by a γ-counter. For NPY, the lyophilized tissue extracts were reconstituted in 550 μl assay buffer (150 mM sodium phosphate buffer containing 150 mM NaCl, 0.06% gelatine, 0.02% bacitracin, 0.02% NaN_3, 0.1% bovine serum albumin (Sigma–Aldrich, MO, USA), and 0.006% phenol red, pH 7.4). The RIA was performed in duplicates (200 μl) as described previously [2]. A highly sensitive NPY antiserum donated by Dr. Elvar Theodorsson, Linköping, Sweden, was used at a dilution of 1:20,000. Standard curves were obtained by including synthetic NPY (NeoMPS, Strasbourg, France) in 1–80 fmol concentrations.

2.4. Quantification and expression of mRNA by RT-PCR

Retinal samples were dissected and stored at −80 °C. Tissues were homogenized after adding 1 ml of RNA-Bee (RNA-Bee CS-105B, Tel-Test Inc.,) using an IKA T-10 basic Ultra Turrax. RNA was extracted by chloroform and isopropanol. DPPIV and NPY-receptor 2 mRNAs were measured from two retinae each at an age of 9 days and 6 weeks by semi-quantitative PCR. Flk-1 and CD31 mRNA concentrations were determined from 14 retinae both of NPY knockout mice and wild-type controls using real-time PCR (CFX TM Real Time System, C1000 TM Thermal Cycler, BioRad). The following primers were used: NPYYR2 fwd GAACGCGCAAGAGTCAATAC, rev CAGTTTGGTGCTGTCTATGAGC; DPPIV fwd GCACGGATCATAAAGAGCTG; rev TCAAAATGAGGGGGCAAG; CD31 fwd CAAGCAAAGCAGTGAAGCTG; rev CTAACTTCGGCTTGGGAAAC; Flk-1 fwd CTGTGGCGAAGATGTTTTTG, rev TTCATCCCACTACCGAAAGC; actin fwd TGGCATTGTTACCAACTGGGACGAC, rev ATCCACATCTGCTGGAAGGTGGACA.

2.5. Statistics

Statistical differences were calculated using the Mann–Whitney U test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3. Results

3.1. SN, SP and NPY

The levels of SN, SP and NPY in the retina in controls and in OIR are given in Figs. 1–3.

The levels of SN in controls were similar at P9 and P11 (18.63 ± 2.45 and 18.29 ± 2.63 fmol/mg wet weight), respectively. The levels raised thereafter in controls to 36.87 ± 1.45 fmol/mg wet weight at P20. In OIR retinae, no difference was seen relative to controls at P9 and P11; during ischemia at P12–P14 and P20, however, levels were slightly decreased. Significance was observed only at P16, when SN levels were 31.96% ± 9.01 lower than in untreated animals (Fig. 1).

The SP concentrations in controls were 16.31 ± 0.54 fmol/mg wet weight at P9 and reached a peak of 55.44 ± 3.55 fmol/mg wet weight at P14 with a plateau thereafter at P16 and P20. In OIR retinae, SP levels were lower than in controls at all time points; statistical significance was reached at P11 (21.46% ± 12.72), P13 (14.79% ± 4.15) and P14 (23.68% ± 4.18) (Fig. 2).

In contrast, levels of NPY in control mice decreased during development. At P9 77.62 ± 5.97 fmol/mg wet weight of NPY were found in controls. The concentration decreased thereafter steadily to 7.78 ± 1.34 fmol/mg wet weight at P20. In OIR retinae, a similar decrease was found. At P12, OIR retinae expressed 24.75% ± 7.51, at P13 34.44% ± 8.49, at P14 41.6% ± 5.21 and at P16 28.82% ± 7.24 less NPY compared to controls (Fig. 3).

3.2. VEGF

VEGF levels of controls remained constant during development ranging from 99.98 ± 4.94 pg/mg at P9 to 61,43 ± 5.03 pg/mg at P20. In OIR, VEGF levels showed a distinct pattern; during the period of relative ischemia (at P12–P16 and P20) VEGF levels were significantly upregulated two to three-fold with a peak at P12 followed by a decrease to P16. In contrast, in the period of hyperoxia (P9, P11) VEGF was decreased by 50% (Fig. 4).

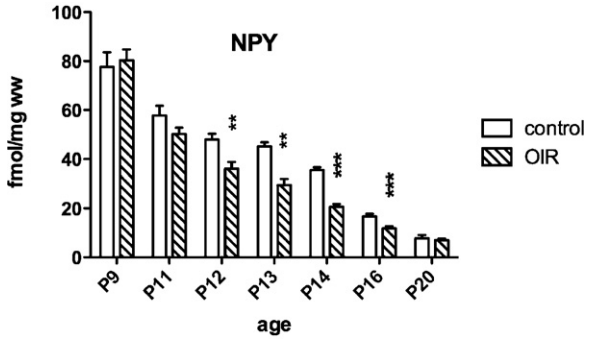

Fig. 3. Effect of OIR on neuropeptide Y (NPY) levels in mice retinae. Concentrations (fmol/mg wet weight) are given as means ± S.E.M. ($n = 8$). Mann–Whitney U test revealed significant decreases in OIR at P12, P13, P14 and P16 ($^{**}p < 0.01$; $^{***}p < 0.001$).

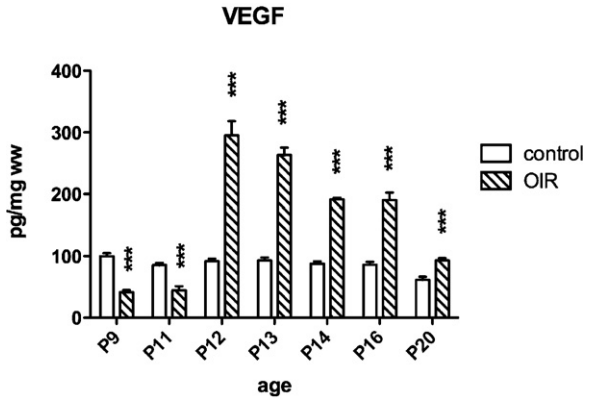

Fig. 4. Effect of OIR on VEGF levels in mice retinae. Concentrations (pg/mg wet weight) are given as means ± S.E.M. ($n = 8$). Mann–Whitney U test revealed high significances both for the decrease at P9 and P11 and the increase thereafter ($^{***}p < 0.001$).

3.3. Expression of NPY-Y2 receptor and DPPIV mRNA in the retina

The expression of the NPY-Y2-receptor and DPPIV was studied in the immature and mature retina of C57Bl/6N mice by RT-PCR (Fig. 5). For Y2-receptor mRNA, neither in the immature retina at P9 nor in the mature retina at an age of 6 weeks, a band was visible. For DPPIV, no mRNA was detected at P9 whereas a very weak band of 151 bp was observed in the mature retina at an age of 6 weeks.

3.4. Real-time PCR for CD31 and Flk-1

To assess the density of retinal endothelial cells, real-time PCR for two endothelial markers was performed, namely CD31 and Flk-1 (Fig. 6). There was no difference in endothelial markers between wild-type and NPY knockout mice (CD31: Ctr 1.0 ± 0.1, NPY knockout 1.0 ± 0.11; Flk-1: Ctr 1.0 ± 0.05, NPY knockout 1.2 ± 0.12). These data indicate that NPY knockout mice show the same retinal vascular density as wild-type mice.

4. Discussion

This study was performed to investigate whether the neuropeptides SN, SP and NPY are involved in the process of retinal neovascularization in the OIR model of the mouse. VEGF

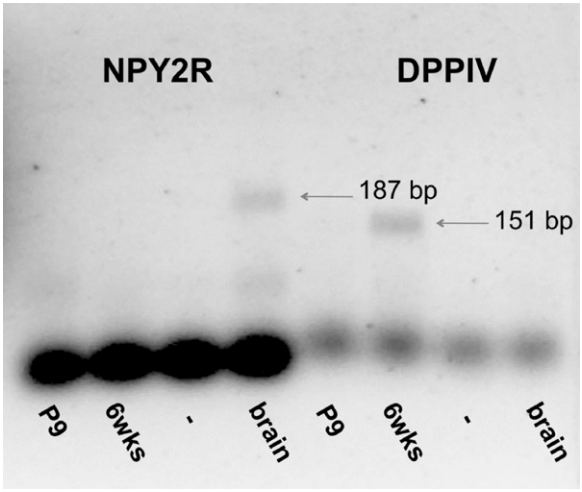

Fig. 5. Expression of the NPY-Y2 receptor (NPY2R) and of DPPIV mRNA in the immature retina at an age of 9 days (P9) and 6 weeks (6wks) by semiquantitative PCR. For the NPY-Y2 receptor no staining is visible at either time points. For DPPIV only a very weak staining was observed at 151 bp at 6 weeks.

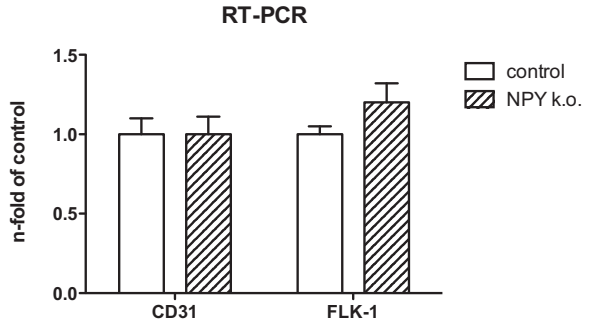

Fig. 6. Levels of mRNA of two endothelial markers, CD31 and Flk-1 in the retina of wild-type and NPY knockout mice by real-time PCR. No differences between controls and knockout mice were found in the expression of the two markers.

levels served as a control for the valid application of this model. In our study, VEGF levels were found to be significantly decreased during hyperoxia and to be significantly increased under relative hypoxic conditions. This is consistent with published data showing that retinal VEGF expression is significantly suppressed during the hyperoxic phase of OIR contributing to vessel loss and that VEGF becomes upregulated during the subsequent hypoxic phase causing pathologic neovascularization [1,12,13,35,43]. Thus, OIR was successfully established in our study.

In contrast to VEGF, OIR had no pronounced effects on SN, SP and NPY levels. Compared to controls, SP as well as NPY levels were decreased during hyperoxia and showed a delayed normalization during relative hypoxia. This effect was more pronounced for NPY than for SP. There are two possible explanations for this phenomenon. Abnormally high oxygen concentrations could damage peptide-expressing cells in the retina. If this was the case, neuropeptides levels would have remained low; however, neuropeptides levels in our model returned to normal values at P20 indicating that cells were still viable. More likely, hyperoxia induces a transient reduction of the expression of SP and NPY. SP, SN and NPY are present in ACs in the proximal INL and in displaced ACs in the GCL. The differential expression of SP, SN and NPY in OIR can be explained by two mechanisms: (i) the peptides are expressed in different cells or (ii) the peptides are colocalized but exhibit different expression patterns following relative ischemia.

The levels of SN and SP continue to increase after eye opening at P11 indicating that these peptides are continuously expressed during retinal cell maturation. NPY levels were high at P9 in the immature retina and decreased thereafter until P20. Since NPY possesses potent proangiogenic properties [9,22], high levels of NPY suggested the possibility that NPY may be involved in the physiologic vascularization in the retina. To test this hypothesis, we determined the expression of DPPIV and the NPY-Y2 receptor both in immature and mature controls. However, neither DPPIV nor the NPY-Y2 receptor could be detected in the immature retina at P9, which is a strong argument against a possible role for NPY in the physiological vascularization in the mouse retina. Moreover, high NPY levels in the immature retina could indicate that this peptide is involved in the development of the retina, in particular in the development of inner-retinal circuits. In fact, NPY is thought to play an important role in neurodevelopmental processes in the nervous system [16]. The delayed normalization of NPY levels under relative hypoxia runs parallel with the delayed development of the intermediate and deep vascular plexus in the retina in the OIR model in mice [42]. This suggests that the decrease of NPY contributes to a delayed vascularization. However, the expression of two vessel markers CD31 and Flk-1 in the retina of controls and

NPY knockout mice at P14 was comparable, which would argue against a participation of this peptide in the delayed development of the intermediate and deep vascular plexus.

If the neuropeptides SN, SP and NPY are involved in the pathogenesis of neovascularization in the OIR model in mice, an increase of the tissue levels of these peptides during relative hypoxia similar to VEGF can be expected. This, however, was clearly not the case. In contrast to the finding of increased expression of both NPY and NPY-Y2 receptor in OIR [50], we found decreased levels of NPY and SP. This discrepancy might be explained by the different detection methods used. In contrast to Yoon et al. [50] who measured mRNA levels by quantitative RT-PCR, we directly measured protein levels by RIA.

Each of these peptides has been found to induce neovascularization in various tissues apart from the eye. SP induces NK-1 receptor-mediated new vessel formation both in neurogenic inflammation in the rat knee synovium [37] and in the bone marrow [33], and it produces an intense angiogenic response synergistically with IL-1 in a rat sponge model [17]. NPY is an endogenous ischemia-induced angiogenic factor that stimulates ischemic angiogenesis in the rat hindlimb model by activating Y2/Y5 receptors and NO-dependent pathways, in part mediated by release of VEGF [26]. NPY also induces aortic sprouting ex vivo [26], it is involved in the delay of skin wound healing with reduced neovascularization in mice [9] and NPY and NPY-Y2-receptor are involved in the development of diabetic retinopathy and retinal neovascularization in mice and rats [24]. Furthermore, NPY stimulates growth of neuroblastomas by an autocrine mitogenic effect on tumor cells and by its angiogenic activity [22]. Both processes are mediated by the NPY-Y2 receptor and blocking of the NPY-Y2 receptor inhibits neuroblastoma growth *in vivo* [28]. SN has been demonstrated to be up-regulated by hypoxia tissue-specific in muscle cells both *in vitro* and *in vivo*; furthermore, inhibition of SN by neutralizing antibody impaired the angiogenic response in the hindlimb ischemia model, indicating a role for SN in physiological angiogenesis [7]. Furthermore, gene therapy with SN induces therapeutic angiogenesis, arteriogenesis and vasculogenesis in the hindlimb ischemia model by a nitric oxide-dependent mechanism [36]. It must be emphasized that vessel sprouting stimulated by VEGF resemble abnormal, leaky, and engorged vessels characteristic of pathological angiogenesis such as in tumors [11], while both SN [19] and NPY [56] stimulate formation of long, normal-looking sprouts. This raises the possibility that SN and NPY are mediators of physiological rather than pathological angiogenesis providing a possible explanation for the missing participation of SN and NPY in abnormal vessel formation in OIR. Furthermore, the missing participation of the three peptides in neovascularization in OIR model compared to new vessel formation in other tissues might be due to different tissue-specific sensitivity and/or might depend on a specific kind of stimulus evoking angiogenesis: it was inflammation for SP [37] and true hypoxia for NPY [26] or SN [7] rather than relative hypoxia in OIR that induces neovascularization.

In summary, our results clearly demonstrate that SN, SP and NPY are not involved in the pathogenesis of neovascularization in the OIR model in mice despite possessing potent pro-angiogenic properties. However, the presence of high levels of NPY in the immature retina suggests a possible participation of this peptide in the development of the retina.

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