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Title: Emerging novel roles of Neuropeptide Y in the retina: from neuromodulation to neuroprotection

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Title: Emerging novel roles of Neuropeptide Y in the retina: from neuromodulation to neuroprotection.

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Abstract

Neuropeptide Y (NPY) and NPY receptors are widely expressed in the central nervous system, including the retina. Retinal cells, in particular neurons, astrocytes, and Müller, microglial and endothelial cells express this peptide and its receptors (Y₁, Y₂, Y₄ and/or Y₅). Several studies have shown that NPY is expressed in the retina of various mammalian and non-mammalian species. However, studies analyzing the distribution of NPY receptors in the retina are still scarce. Although the physiological roles of NPY in the retina have not been completely elucidated, its early expression strongly suggests that NPY may be involved in the development of retinal circuitry. NPY inhibits the increase in [Ca²⁺], triggered by elevated KCl in retinal neurons, protects retinal neural cells against toxic insults and induces the proliferation of retinal progenitor cells. In this review, we will focus on the roles of NPY in the retina, specifically proliferation, neuromodulation and neuroprotection. Alterations in the NPY system in the retina might contribute to the pathogenesis of retinal degenerative diseases, such as diabetic retinopathy and glaucoma, and NPY and its receptors might be viewed as potentially novel therapeutic targets.

Key words

Neuropeptide Y; NPY receptors; Retina; Retinal neural cells; Neuroprotection; Neuromodulation.
Contents

1. The Retina ............................................................................................... 4
    1.1. Visual pathways in the retina ................................................................. 4
    1.2. Neurotransmitters in the retina .............................................................. 4
    1.3. Neuropeptides in the retina .................................................................... 5

2. Neuropeptide Y (NPY) and NPY receptors in the retina .................. 6
    2.1. Localization of NPY and NPY receptors in the retina ......................... 8
    2.2. NPY and retinal development ............................................................... 11

3. Modulatory effects of NPY in the retina .............................................. 12

4. Potential role of NPY in cell proliferation, differentiation and
   neuroprotection in the retina ................................................................. 14

5. NPY involvement in retinal pathologies ............................................. 17

6. Conclusions .......................................................................................... 18

Acknowledgments ...................................................................................... 19
1. The Retina

1.1. Visual pathways in the retina

The vertebrate retina, like other regions of the central nervous system (CNS), is nervous tissue derived embryologically from the neural tube (Yang, 2004). The retina is composed of four main groups of cells: neurons, glial cells (astrocytes, Müller and microglial cells), epithelial cells (retinal pigment epithelium) and vascular cells. There are five basic types of neurons in the retina: photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. These cells are organized into clearly distinct layers, namely three layers of nerve cell bodies: outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL), and two layers of synapses: the outer plexiform layer (OPL) and the inner plexiform layer (IPL). The ONL contains cell bodies of rods and cones, the INL contains cell bodies of bipolar, horizontal and amacrine cells, while the GCL contains cell bodies of ganglion cells and displaced amacrine cells. In the OPL there are connections between rods and cones, with vertically running bipolar, and horizontally oriented horizontal cells. The second synaptic area is the IPL. It works simultaneously as a relay station for the vertical-information-carrying nerve cells, the bipolar cells, to connect to ganglion cells, and as a station for information processing which is mainly carried out by amacrine cells. It is at the end of all this neural processing in the IPL that the message concerning the visual image is transmitted to the brain along the optic nerve (Figure 1).

1.2. Neurotransmitters in the retina

Chemical transmission mediated by neurotransmitters is predominant in the neural circuitry of the retina. Although the retina contains a variety of neurotransmitters, glutamate and γ-
aminobutyric acid (GABA) are the major excitatory and inhibitory neurotransmitters, respectively. Glutamate is responsible for the radial flow of the visual signal in the retina, and both photoreceptors (rods and cones) and bipolar cells release glutamate, which induces and/or alters the activity of the post-synaptic neurons (horizontal and bipolar cells for photoreceptors in the outer retina; amacrine and ganglion cells for bipolar cells in the inner retina) by directly changing membrane permeability to ions or by activating intracellular systems through ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs) (Yang, 2004). There is also a lateral or indirect pathway in the retina. This pathway is mainly mediated by GABA, which is used by numerous horizontal and amacrine cells, modulating synaptic transmission in both synaptic layers. In the OPL, horizontal cells receive direct input from photoreceptors and reply with a negative feedback to cone photoreceptors. Horizontal cells mediate the responses of the surrounding receptive field of bipolar cells. The inputs to bipolar cells are from both photoreceptors and horizontal cells. In the IPL, reciprocal synapses connect bipolar and amacrine cells, and both types of cells send input to ganglion cells. Amacrine cells are involved in spatial and temporal integration of visual signals in the IPL (Yang, 2004).

Although glutamate and GABA are the main neurotransmitters in the retina, other neurotransmitters are present, such as glycine, acetylcholine (Lindeman, 1947), dopamine (Haeggendal and Malmfors, 1963), serotonin (Kojima et al., 1961), ATP and adenosine (De Berardinis and Auricchio, 1951). Retina has also several neuropeptides, which we describe below.

1.3. Neuropeptides in the retina

Neuropeptides are widely distributed, both in the central and peripheral nervous systems. Functionally, neuropeptides act as neurotransmitters and/or neuromodulators through the activation of specific receptors to modulate the functional properties of neurons, such as their
membrane excitability or their signal transduction pathways (Bagnoli et al., 2003). Over the last decades, several neuropeptides, which were highly conserved during evolution, have been discovered in the eye. Substance P was the first peptide described in the retina and is also present in peripherally innervated tissues of the eye (Duner et al., 1954; Stone et al., 1987). The interest was extended to investigate the presence and distribution of other neuropeptides including calcitonin gene-related peptide (CGRP) (Kiyama et al., 1985), vasoactive intestinal polypeptide (VIP) (Loren et al., 1980), pituitary adenylate cyclase-activating polypeptide (PACAP) (Onali and Olianas, 1994), cholecystokinin (CCK) (Yamada et al., 1981), somatostatin (Rorstad et al., 1979), galanin (Hokfelt et al., 1992), neurokinin A and B (Schmid et al., 2006), corticotrophin-releasing factor (CRF) (Kiyama et al., 1984), angiotensin II (Senanayake et al., 2007), secretoneurin (Overdick et al., 1996), and neuropeptide Y (NPY) (Bruun et al., 1984). In this review we will focus on the NPY system and its role in the retina.

2. Neuropeptide Y (NPY) and NPY receptors in the retina

NPY is a member of a peptide family named NPY family or “PP-fold” family that also includes peptide YY (PYY) and pancreatic polypeptide (PP) (Michel et al., 1998). NPY is a 36-amino acid peptide that possesses an amidated C-terminal residue and a large number of tyrosine residues (which are abbreviated by the letter Y) included in both ends of the molecule. NPY was first isolated from the pig brain in 1982 by Tatemoto (Tatemoto et al., 1982). NPY is one of the neuropeptides with the highest degree of phylogenetic preservation, while the PP differs considerably between species (Larhammar et al., 1992). The NPY gene is located on human chromosome 7 at the locus 7p15.1 (Cerda-Reverter and Larhammar, 2000). In mouse, it is located in chromosome 6, locus 6 B3; 6 26.0 cM while in rat it is localized in chromosome 4, locus 4q24 (Pruitt et al., 2012). The prepro-NPY generated after translation is directed into the endoplasmic reticulum, where a 28 amino acid peptide is removed and Pro-NPY produced.
This NPY precursor, Pro-NPY, is a 69 amino acid peptide formed by NPY\textsubscript{1-39} where the carboxylic group is flanked by a group of 33 amino acids called the C-flanking peptide of NPY (CPON). The following processing step is the cleavage of the precursor Pro-NPY at a dibasic site by prohormone convertases, which generates NPY\textsubscript{1-39} and CPON. Then, a truncation at the C-terminal end by a carboxypeptidase B (CPB) generates NPY\textsubscript{1-37}, which is a substrate for the enzyme peptidylglycine alpha-amidating monooxygenase (PAM) and leads to the biologically active amidated NPY\textsubscript{1-36} (NPY) (Medeiros Mdos and Turner, 1996). NPY can be further cleaved by two enzymes, dipeptidyl peptidase IV (DPP-IV) and aminopeptidase P (AmP) (Medeiros and Turner, 1994; Medeiros Mdos and Turner, 1996).

All known NPY receptors belong to the large super-family of G-protein-coupled, heptahelical receptors (Michel et al., 1998). The NPY family receptors are the same for all members of the NPY family (NPY, PP, PYY) and are comprised of the receptor subtypes NPY Y\textsubscript{1}, Y\textsubscript{2}, Y\textsubscript{4}, Y\textsubscript{5} and y\textsubscript{6} (Silva et al., 2005; Xapelli et al., 2008).

Generally, NPY receptors use similar signal transduction pathways, acting via pertussis toxin-sensitive G-proteins, i.e., members of the Gi and Go family. Thus, inhibition of adenylyl cyclase upon NPY receptor activation is found in almost every tissue and cell type investigated (Michel, 1991; Olasmaa and Terenius, 1986). However, the inhibition of adenylyl cyclase cannot probably explain all functional responses observed upon stimulation of NPY receptors (Michel et al., 1998). Additional signaling responses that are restricted to certain cell types include modulation of the Ca\textsuperscript{2+} or K\textsuperscript{+} channels conductance (Gammon et al., 1990; Michel and Rascher, 1995; Millar et al., 1991; Xiong and Cheung, 1995). Moreover, there is also evidence to suggest that NPY may be associated with the activation of phospholipase A2 (Martin and Patterson, 1989), mitogen-activated protein kinases (MAPK) (Alvaro et al., 2008a; Keffel et al., 1999; Rosmaninho-Salgado et al., 2009; Thiriet et al., 2011), protein Kinase C (PKC) (Chen et al., 2008; Pons et al., 2008; Rosmaninho-Salgado et al., 2007; Rosmaninho-Salgado et al.,
phosphatidylinositol 3-kinase (PI3K) (Zhou et al., 2008), guanylyl cyclase (Rosmaninho-Salgado et al., 2007), nitric oxide (NO) synthesis (Ferreira et al., 2010; Hodges et al., 2009; Rosmaninho-Salgado et al., 2009), or protein kinase A (PKA) (Pons et al., 2008; Rosmaninho-Salgado et al., 2009).

2.1. Localization of NPY and NPY receptors in the retina

The presence of NPY in the retina was first described in guinea pig in the early eighties (Bruun et al., 1984). Later studies demonstrated that NPY is also present in the retina of several non-mammalian vertebrates, as described in Table 1, and NPY-IR localizations in the retina of several mammalian species are depicted in Figure 2.

In rat and mouse retinas, NPY-IR is present in the inner retina being localized in cell bodies in INL and GCL, mainly in amacrine cells and displaced amacrine cells, respectively, and also in processes located in the IPL (Oh et al., 2002; Sinclair and Nirenberg, 2001). All NPY-immunoreactive amacrine cells are also GABAergic, containing GABA or the GABA synthesizing enzyme, glutamic acid decarboxylase 65 (GAD65), or even GAT-1, a vesicular GABA transporter (VGAT) (Kang et al., 2001; Oh et al., 2002; Sinclair and Nirenberg, 2001). Additionally, in rat retinal cells in culture, NPY-IR is present not only in retinal neurons, but also in Müller and microglial cells (Alvaro et al., 2007; Santos-Carvalho et al., 2012). The NPY-IR is also present in processes that ramify in the IPL in species such as pigeon, chicken, pig and baboon (Bruun et al., 1986; Oh et al., 2002; Sinclair and Nirenberg, 2001). A study in dolphin and dog retina shows that NPY-IR appears in polygonal and oval medium to large ganglion cells in the GCL which processes extended to IPL but only a few cells in the INL, IPL, OPL or ONL are NPY-immunoreactive (Chen et al, 1999). Furthermore, in cat retina the NPY-IR is localized in processes in the IPL, amacrine cells at INL and in ganglion cells in GCL (Hutsler and Chalupa, 1994, 1995), while only small amounts of NPY-IR are found in rabbit retina (Osborne et al., 1985). Moreover, immunoreactivity to NPY is also detected in
bovine retinal pigment epithelium (RPE) (Ammar et al., 1998). Finally NPY-IR is detected in RPE, amacrine cells at INL and in ganglion cells in GCL (Ammar et al., 1998; Jen et al., 1994; Straznicky and Hiscock, 1989). In the case of non-mammalian retinas, in trout, carp, goldfish and killifish retinas, NPY-IR is found in cell bodies of amacrine cells (middle and innermost INL) and its processes constituting distinct sub-layers in the IPL (Bruun et al., 1986; Osborne et al., 1985; Subhedar et al., 1996). Goldfish retina also has dense fiber plexus with NPY-IR in layers 1, 3 and 5 of IPL (Muske et al., 1987). Zebrafish and gilthead seabream (Sparus aurata L.) retina presents NPY-IR in amacrine cells (Mathieu et al., 2002; Pirone et al., 2008). In skates (Raja clavata, Raja radiate and Raja oscellata), NPY-IR is localized in amacrine cells in the innermost part of INL while NPY-immunoreactive fibers are found in IPL (Bruun et al., 1985). In river lamprey (Lampetra japonica), NPY-IR is present in a subclass of pyriform amacrine cells (Negishi et al., 1986). No NPY-IR is found in squid retina (Osborne et al., 1986).

Retina of anuran species, such as frogs (ex. Bufo marinus and Xenopus laevis), has the highest NPY-IR levels among other species (such as pigs, cats, rabbits, chickens) and is characterized by seasonal variations (Bruun et al., 1991; Hiscock and Straznicky, 1989). NPY-IR is located in a small population of amacrine cell bodies in INL co-localizing with GABA (Bruun et al., 1986; Hiscock and Straznicky, 1990; Osborne et al., 1985; Zhu and Gibbins, 1995, 1996), in bipolar-like cell bodies sparsely in the middle of INL, in GCL with ovoid shape, in Müller cells within the INL (Zhu and Gibbins, 1996) and in IPL processes (Bruun et al., 1986).

In reptiles, such as lizards (Pogona vitticeps and Varanus gouldii), NPY-IR is present in the retina in two classes of amacrine cells: type A (large cell body) and type B (small cell body) located in INL and, occasionally, in displaced amacrine cell bodies at GCL (Straznicky and Hiscock, 1994). In contrast, in turtles the retina presents NPY-IR in bipolar cells and in three types of amacrine cells: type A, with large cell body located at INL and occasionally at GCL,
and processes at IPL and; type B, with smaller cell body at INL and processes at IPL; type C, amacrine cells located at the periphery of retina (Isayama and Eldred, 1988). NPY-IR is also located in cytoplasm and within large vesicles of amacrine and bipolar cells in turtle retina (Isayama et al., 1988; Wetzel and Eldred, 1997).

With NPY receptors, there are only a few studies showing their presence and localization in the retina (Table 2). In rat retina, we and others have detected the presence of mRNAs for NPY Y1, Y2, Y4 and Y5 receptors (Alvaro et al., 2007; D'Angelo and Brecha, 2004). The NPY Y1 receptor-IR was found to be localized in horizontal and amacrine cell bodies and processes (D'Angelo et al., 2002).

Others have suggested that NPY Y1 receptors are present in retinal neurons and responsible for the modulation of glutamate release and consequent inhibition of osmotic swelling of Müller cells (Uckermann et al., 2006). A more recent study has revealed that NPY inhibits the swelling of freshly isolated rat Müller cells (Linnertz et al., 2011), suggesting that rat Müller cells express the NPY Y1 receptor. In fact, the presence of NPY Y1 receptor in Müller cells has recently been observed both in rat retinal neural cell cultures and rat purified Müller cell cultures (Santos-Carvalho et al., 2012). Milenkovic and collaborators also suggest the presence of NPY Y1 receptor in guinea pig Müller cells (Milenkovic et al., 2004). Additionally, NPY Y1 and Y2 receptors immunoreactivities are found in photoreceptors (rhodopsin-positive cells), bipolar (PKC α-positive cells), horizontal (calbindin-positive cells), amacrine (parvalbumin- or calretinin-positive cells) and ganglion cells (Brn3a-positive cells), as well as macroglial (GFAP- and vimentin-positive cells) and microglial (CD11b and ED1/CD68-positive cells) cells in rat retinal cultures (Santos-Carvalho et al., 2012). NPY Y1 and Y2 mRNAs were also detected in mouse retina (Sinclair and Nirenberg, 2001; Yoon et al., 2002). In human retinal pigment epithelium (RPE), NPY Y1, Y2 and Y5 receptor mRNAs were detected, while in the bovine RPE only the NPY Y1 and Y2 receptors were detected (Ammar et al., 1998).
2.2. NPY and retinal development

Several studies indicate that NPY plays a role in the beginning of and during retinal development. Chicken NPY-immunoreactive retinal neuroblasts appear at embryonic day 13 in INL. Between that day and day 15 they migrate to their positions in innermost layers. At day 17, these immature cells start to differentiate in amacrine cells establishing connections with ganglion cells. At day 19, NPY-IR appears in few cell bodies of amacrine cells and large cells in GCL (Prada Oliveira et al., 2003). In zebrafish, NPY-IR appears in amacrine cells at embryonic day 15, suggesting its involvement in retinal synaptogenesis during ontogeny (Mathieu et al., 2002).

In *Xenopus laevis* retina, NPY-IR appears early in larval life. The dendritic maturation of NPY–IR amacrine cells occurs later during larval development than in cell bodies, and just before metamorphosis. In the adult retina of this frog, NPY-immunoreactivity (IR) is present in a wide field of amacrine cells in the INL and GCL (Hiscock and Straznicky, 1990). In the retina of blue acara (*Aequidens pulcher*), NPY-immunoreactive amacrine cells appear in IPL around hatching, at day 3-4 (Negishi and Wagner, 1995).

During cat retina development, NPY-IR is detected in central retina within the GCL at embryonic day 46 and amacrine cells within INL at embryonic day 50. Cat NPY-IR in amacrine population reaches adult levels at P7, while NPY-IR in ganglion cell population shows an extended development, with new cells expressing NPY until the third post-natal week (Hutsler and Chalupa, 1995).

Regarding the developing human retina, NPY-immunoreactive amacrine cells are found around 14 weeks of gestation (Jotwani et al., 1994). Another study indicates the presence of round and pear-shaped NPY-immunoreactive amacrine cells in INL after 15 weeks of gestation. NPY positive-ganglion cells were only detected at 17 weeks of gestation. NPY-immunoreactive amacrine and ganglion cells are located in INL and GCL, respectively, at 26-28 weeks of
gestation. Later, by 38-40 weeks of gestation, NPY-immunoreactive cells are present in INL, GCL and IPL (Jen et al., 1994).

In rats, NPY-IR appears in the retina in small quantities in GCL only at E18, and increases over pre- and postnatal development. Subsequently, at eye opening (P13) NPY-IR markedly increases in INL and GCL, but falls during maturation until adult levels forming two subpopulations in INL and GCL. This transient increase at eye opening may have a role in modulating the developing retina circuitry (Ferriero and Sagar, 1989).

In conclusion, several studies report the presence of NPY in undifferentiated retinal cells indicating a putative role of NPY in retinal development (Ferriero and Sagar, 1989; Hiscock and Straznicky, 1990; Hutsler and Chalupa, 1995; Jen et al., 1994; Jotwani et al., 1994; Mathieu et al., 2002; Negishi and Wagner, 1995; Prada Oliveira et al., 2003).

3. Modulatory effects of NPY in the retina

A fine tuning neuromodulator has the capacity to exert subtle influence on synapse activity by changing receptor activation of other neurotransmitters or neuromodulators as well as its own receptors. NPY co-localizes with other neurotransmitters in different areas of the CNS (Allen et al., 1983; Hendry et al., 1984; McDonald, 1996; Silva et al., 2005) and modulates the release of several neurotransmitters (Silva et al., 2005), inhibiting the release of glutamate, aspartate, growth hormone, epinephrine and acetylcholine (Bitran et al., 1999; Bleakman et al., 1992; Greber et al., 1994; Gu et al., 1983; Hastings et al., 2004; Martire et al., 1995; Potter, 1987; Rettori et al., 1990b; Rodi et al., 2003; Schwertfeger et al., 2004; Silva et al., 2001; Silva et al., 2003; Tsuda et al., 1995), and enhancing the release of somatostatin and dopamine and the production of nitric oxide (Ault and Werling, 1999; Bitran et al., 1999; Rettori et al., 1990a).

Therefore, NPY may play a fine-tuning modulator in the nervous system (Grandt et al., 1996;
Magni, 2003; Mazzocchi et al., 1996; Prod'homme et al., 2006). Some studies have also suggested that NPY may be a neuromodulator in the retina. NPY modulates the intracellular calcium concentration ([Ca$_{2+}$]$^i$) in rat retinal neurons. NPY inhibits the depolarization-evoked Ca$_{2+}$ influx into rod bipolar cells through the activation of NPY Y$_2$ receptors (D'Angelo and Brecha, 2004). NPY also inhibits the KCl-evoked increase in [Ca$_{2+}$]$^i$ in cultured rat retinal neurons through the activation of NPY Y$_1$, Y$_4$ and Y$_5$ receptors (Alvaro et al., 2009). On the other hand, when applied exogenously, NPY stimulates the release of [$^3$H]-glycine, [$^3$H]-dopamine, [$^3$H]-5-hydroxytryptamine and [$^3$H]-choline chloride-derived radioactivity in the rabbit retina and of [$^3$H]-GABA, [$^3$H]-5-hydroxytryptamine and [$^3$H]-choline chloride-derived radioactivity in chicken retina (Bruun and Ehinger, 1993). These results and the presence of NPY in amacrine cells (in the INL) and displaced amacrine cells (in the GCL), which may connect with other amacrine cell subtypes and ganglion cells that are not immunoreactive for NPY, suggest that NPY may also play a role as a neuromodulator in the inner retinal layers (D'Angelo et al., 2002; Oh et al., 2002)."

The ablation of NPY-immunoreactive amacrine cells causes alteration of receptive field surround size of ganglion cells, suggesting that NPY-immunoreactive amacrine cells are involved in tuning ganglion cells to low spatial frequencies/large spatial patterns (Sinclair et al., 2004).

In conclusion, NPY may affect neurotransmission between different retinal neurons (photoreceptors, and bipolar, ganglion, horizontal and amacrine cells), which depends on [Ca$_{2+}$]$_i$ regulation, and therefore NPY may exert a relevant fine tuning neuromodulatory role in retinal cells.
4. Potential role of NPY in cell proliferation, differentiation and neuroprotection in the retina

*In vitro* and *in vivo* studies suggest that NPY has pro-neurogenic properties in the olfactory epithelium, subventricular zone (SVZ) and subgranular zone (SGZ) of dentate gyrus (Agasse et al., 2008; Decressac et al., 2011; Hansel et al., 2001b; Howell et al., 2005; Howell et al., 2007; Rodrigo et al., 2010). In addition, in the CNS, NPY induces alterations in the rostral migratory stream, differentiation of progenitor cells into distinct interneuronal subsets in the olfactory bulb (Stanic et al., 2008), migration of newly generated neurons to the striatum and the olfactory bulb and also increases the number of cells in the rostral migratory stream, olfactory bulb and striatum (Decressac et al., 2009). These NPY effects on neural cell proliferation and differentiation are mediated by the NPY Y$_1$-receptor activation (Agasse et al., 2008; Decressac et al., 2011; Hansel et al., 2001b; Howell et al., 2003; Rodrigo et al., 2010; Stanic et al., 2008). The involvement of Y$_2$ receptor in these NPY effects is controversial (Decressac et al., 2011; Stanic et al., 2008). The neurogenic effect of NPY requires ERK1/2 activation (Agasse et al., 2008; Hansel et al., 2001a; Howell et al., 2005) while NPY promoting effect on neuronal differentiation and axonal sprouting is mediated through the activation of the SAPK/JNK pathway (Agasse et al., 2008). These studies suggest that secreted NPY may act locally in an autocrine/paracrine manner, at least in the hippocampus, to stimulate proliferation or neuronal differentiation, either at an equal or even greater level than other trophic/growth factors, such as ciliary neurotrophic factor, vascular endothelial growth factor, and transforming growth factor (Decressac et al., 2011; Emsley and Hagg, 2003; Jin et al., 2002).

In the retina, it has been shown that NPY induces proliferation of retinal glial (Müller) cells mediated by NPY Y$_1$ receptor activation, through ERK 1/2, and partially, p38 pathways (Milenkovic et al., 2004). However, this proliferative effect on Müller cells is biphasic: at lower
concentrations (0.1 ng/mL and 1 ng/mL) NPY decreases the cell proliferation rate, while at higher concentration (100 ng/mL) increases Müller cell proliferation (Milenkovic et al., 2004). It was accepted that the mature mammalian retina lacked regenerative capacity (Tropepe et al., 2000). However, many studies in fish, amphibians, birds, rodents and humans have identified neural progenitors in the adult eye with capacity to generate all retinal cell types (Ahmad, 2001; Cepko et al., 1996; Coles et al., 2004; Johns, 1977; Martinez-Navarrete et al., 2008; Reh and Fischer, 2001; Straznicky and Gaze, 1971; Tropepe et al., 2000; Xu et al., 2007). The identification and characterization of neural progenitors stem cells in the eye may open new avenues for the treatment of several ocular diseases characterized by neuronal death, such as retinitis pigmentosa, age-related macular degeneration, diabetic retinopathy and glaucoma (Ahmad, 2001; Bernardos et al., 2007; Ohta et al., 2008; Ooto et al., 2004; Tropepe et al., 2000).

Some studies suggest that NPY might have an important role on progenitor cells proliferation and/or differentiation in the nervous tissue (Baptista et al., 2012; Doyle et al., 2012; Thiriet et al., 2011). In cultured rat retinal cells, we have shown that NPY stimulates the proliferation of neuronal progenitor cells (BrdU+/nestin+ cells), which means that NPY promotes the proliferation of committed neural immature cells, with this effect being mediated by the activation of the nitric oxide synthase - soluble guanylyl cyclase (NOS–sGC) and ERK 1/2 signaling pathways (Alvaro et al., 2008a). Additionally, NPY, through Y₁ and Y₅ receptor activation, has the potential of maintaining self-renewal and pluripotency of human embryonic stem cells (hESC) (Son et al., 2011). NPY signaling can be useful in the development of defined and xeno-free culture conditions for the large-scale propagation of undifferentiated hESCs (Son et al., 2011). Thus, the NPY system is a putative target to develop new strategies to increase retinal progenitor cells proliferation.
Neuroprotection is an important strategy to prevent cell death occurring in neurological disorders. The neuroprotective effects of NPY against excitotoxicity are well known both in different brain regions, and also in the retina (Alvaro et al., 2008b; Alvaro et al., 2009; Silva et al., 2003; Silva et al., 2005; Smialowska et al., 2009; Xapelli et al., 2006; Xapelli et al., 2007). In rat and mouse organotypic hippocampal cultures, NPY is able to reduce cell death induced by glutamate receptor agonists, through activation of NPY Y₁, Y₂ and/or Y₅ receptors (Silva et al., 2003; Smialowska et al., 2009; Xapelli et al., 2007). NPY also exerts a neuroprotective effect against toxicity (necrosis and apoptosis) induced by 3,4-methylenedioxymethamphetamine (MDMA) in rat retinal neural cell culture (neurons, astrocytes, Müller cells (GFAP-positive cells) and microglial cells) (Alvaro et al., 2008b). However the mechanism underlying this neuroprotective effect of NPY against MDMA toxicity has not yet been clarified (Alvaro et al., 2008b). As mentioned above, we have shown that NPY inhibits the increase in [Ca²⁺]ᵢ in rat retinal neurons through the activation of NPY Y₁, Y₄, and Y₅ receptor subtypes. Since sustained elevated cytosolic [Ca²⁺]ᵢ levels have been linked to cell death, this inhibitory effect of NPY may also contribute to its neuroprotective effect in these cells (Alvaro et al., 2009). More recently, we also showed that NPY has a protective role against glutamate-induced toxicity in rat retinal cells (in vitro and in an animal model) (Santos-Carvalho et al., 2013). In rat retinal neural cell cultures, the activation of NPY Y₂, Y₄ and Y₅ receptors inhibited necrotic cell death, while apoptotic cell death was only prevented by the activation of NPY Y₅ receptor. Moreover, NPY neuroprotective effect was mediated by the activation of PKA and p38K. In the animal model, NPY inhibited the increase in the number of apoptotic cell death induced by glutamate (Santos-Carvalho et al., 2013). In retinal slices, it has also been shown that NPY, through the release of glutamate and ATP, inhibits osmotic swelling of Müller cells. This inhibitory effect was mediated by NPY Y₁, but not NPY Y₂ or Y₅ receptors, expressed in retinal neurons. This glial volume regulation may
contribute to the neuroprotective effects of NPY in the retina (Uckermann et al., 2006).

Subsequently, the same group found that this neuroprotective effect of NPY was also detected in freshly isolated rat Müller cells, which suggests that NPY receptors of rat Müller cells were directly activated (Linnertz et al., 2011), and that rat Müller cells express NPY \( Y_1 \) receptor. Thus, NPY receptor agonists might be viewed as putative therapeutic drugs against neural cell degeneration occurring in several retinal degenerative diseases, such as glaucoma and diabetic retinopathy.

5. NPY involvement in retinal pathologies

A genetic study in a Finnish population has shown that a Leu7Pro polymorphism in the NPY gene (substitution of a leucine to proline in human prepro-NPY) is associated with an increased predisposition to develop diabetic retinopathy (DR) in Type 2 diabetic patients (Koulu et al., 2004; Niskanen et al., 2000), and could be used to predict earlier onset of type 2 diabetes and retinopathy (Jaakkola et al., 2006). In contrast, it has been shown that this polymorphism is not a risk factor for exudative age related macular degeneration (Kaarniranta et al., 2007).

Retinal NPY and NPY \( Y_2 \) receptor expression are increased in oxygen-reared animals compared with room-air reared ones, in the hyperoxic vasoconstrictive phase (P12) and the period of retinal neovascularization (P17) of the development of oxygen-induced retinopathy of this mouse model. Therefore, NPY and NPY \( Y_2 \) receptor could be associated with angiogenesis and vasoconstriction in this mouse model of oxygen-induced retinopathy (Yoon et al., 2002).

In another study, Koulu and collaborators, using \( Y_2^{−/−} \) mice and rats treated with NPY \( Y_2 \) receptor mRNA targeted antisense oligonucleotide, demonstrated that the NPY \( Y_2 \) receptor plays an important role in hyperoxia-induced retinal neovascularization (Koulu et al., 2004). Thus, they corroborate the contribution of NPY \( Y_2 \) receptor in neovascularization processes in
the progression of diabetic retinopathy and the contribution of NPY gene in type 2 diabetes diabetic retinopathy (Koulu et al., 2004).

However, more recently, another group found decreased levels of NPY and NPY Y_2 receptor in a similar model of oxygen-induced retinopathy to that used by Yoon and collaborators. (Schmid et al., 2012). The authors justify this discrepancy by the fact that Yoon et al. measured mRNA levels by quantitative RT-PCR while Schmidt et al measured protein levels by radioimmunoassay. They suggested, therefore, that NPY is a mediator of physiological but not pathological angiogenesis, thus explaining the absence of this peptide in abnormal vessel formation in retinopathy (Schmid et al., 2012).

In humans, retinas of patients with proliferative vitreoretinopathy present NPY Y_1 receptor immunoreactivity in reactive and proliferating Müller cells. This immunoreactivity was not detected in normal human retina. Therefore, the presence of this receptor may be related to the proliferation of Müller cells, the regrowth of proliferative vitreoretinopathy membranes, and the consequent secondary retinal detachments (Canto Soler et al., 2002).

In conclusion, the available data suggests a relevant role of NPY on the development of some retinal disease, but further studies are needed to clarify the mechanisms involved.

6. Conclusions

NPY and NPY receptors are expressed in the retina of several species, in neurons, astrocytes, and Müller and microglial cells. Activation of NPY receptors appears to mediate several, potentially important effects in the retina, including cell proliferation, neurotransmitter modulation and neuroprotection summarized in Table 3. Future studies are likely to uncover several further functions of NPY in retinal physiology and pathophysiology.
Acknowledgments

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References


involved in development of diabetic retinopathy and retinal neovascularization. 


Lindeman, V. F. (1947) The cholinesterase and acetylcholine content of the chick retina, with especial reference to functional activity as indicated by the pupillary constrictor reflex. Am J Physiol 148, 40-44.


**Fig. 1 - The structural organization of the retina.** Diagram illustrating the distribution of retinal neurons (photoreceptors, bipolar cells, ganglion cells, horizontal and amacrine cells), macroglia and microglial cells in organized layers in the retina. Retinal cells are well organized in several layers: retinal pigment epithelium (RPE); photoreceptor outer segment (POS); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL) and ganglion cell layer (GCL). The astrocytes are in the GCL, while the Müller cells extend through the entire thickness of the retina, extending processes from the outer until the inner limiting membrane. Microglial cells are mainly in OPL, IPL and GCL.

**Fig. 2 – Localization of NPY-IR in different mammalian retinas (red depicted).** A - In the human retina, NPY-IR is present in amacrine cell bodies in INL and GCL, amacrine processes in IPL and ganglion cells in GCL. NPY-immunoreactive fibers are found between INL and OPL and also crossing the INL. NPY-IR is also found in RPE; B - In rat retina, NPY-IR is present in amacrine cells in INL and GCL; C – In mouse retina, NPY-IR is found in amacrine cell bodies in INL and GCL, and processes in IPL; D – In baboon and pig retina, NPY-IR is localized in amacrine cell bodies in INL and processes in IPL; E – In cat retina, NPY-IR is found in amacrine cells in INL, in processes in IPL and in gamma-type retinal ganglion cells. F – In bovine retina, NPY-IR is present in RPE cells; G - In guinea-pig retina, NPY–immunoreactive fibers form a single layer in IPL, and NPY-IR cell bodies are in INL. NPY-IR is also present in some bipolar cells; H – In dolphin and dog retina, NPY-IR is located in medium to large ganglion cells with processes extended to IPL, in some areas of the retina. Only a few cells in the INL are NPY-immunoreactive.
Highlights (Review)

- NPY and NPY receptors are present in the retina of several species;
- NPY modulates neurotransmitters release from retinal cells;
- NPY protects retinal neural cells against toxic insults;
- NPY induces proliferation of retinal progenitor cells.
Figure 1, Santos-Carvalho, *Progress in Neurobiology*
Figure 2, Santos-Carvalho, *Progress in Neurobiology*
### Table 1 - NPY-IR localization in the retina of several non-mammalian species

<table>
<thead>
<tr>
<th>Species</th>
<th>NPY-IR localization in the retina</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fishes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue acara (Aequidens pulcher)</td>
<td>amacrine cells and IPL</td>
<td>(Negishi and Wagner, 1995)</td>
</tr>
<tr>
<td>Carp</td>
<td>cell bodies of amacrine cells in INL and processes in the IPL</td>
<td>(Bruun et al., 1986)</td>
</tr>
<tr>
<td>Gilthead seabream (Sparus aurata L.)</td>
<td>amacrine cells</td>
<td>(Pirone et al., 2008)</td>
</tr>
<tr>
<td>Goldfish</td>
<td>cell bodies of amacrine in INL and cell processes in two layers in IPL</td>
<td>(Bruun et al., 1986; Osborne et al., 1985)</td>
</tr>
<tr>
<td>Killifish (Fundulus heteroclitus)</td>
<td>amacrine cell fibers in IPL</td>
<td>(Subhedar et al., 1996)</td>
</tr>
<tr>
<td>Lamprey (Lampreta fluviatilis)</td>
<td>pyriform subclass of amacrine cells</td>
<td>(Negishi et al., 1986; Rawitch et al., 1992)</td>
</tr>
<tr>
<td>Skates (Raja clavata, Raja radiate and Raja oscellata)</td>
<td>amacrine cells in the innermost part of INL and fibers in IPL</td>
<td>(Bruun et al., 1985)</td>
</tr>
<tr>
<td>Squid</td>
<td>no NPY-IR</td>
<td>(Osborne et al., 1986)</td>
</tr>
<tr>
<td>Trout</td>
<td>cell bodies of amacrine cells in INL and processes in IPL</td>
<td>(Bruun et al., 1986)</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>amacrine cells</td>
<td>(Mathieu et al., 2002)</td>
</tr>
<tr>
<td><strong>Non Mammals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frogs (Bufo marinus and Xenopus laevis)</td>
<td>cell bodies of amacrine cells in INL and cell processes in IPL, bipolar-like cell bodies in the middle of INL and sparsely in GCL, processes ramifying in three sublayers in IPL, Müller cells within the INL and processes in IPL, co-localization of GABA in all NPY-IR amacrine cells of anuran retina.</td>
<td>(Bruun et al., 1991; Bruun et al., 1986; Hiscock and Straznicky, 1989, 1990; Osborne et al., 1985; Zhu and Gibbins, 1995, 1996)</td>
</tr>
<tr>
<td>Lizards (Pogona vitticeps and Varanus gouldii)</td>
<td>amacrine cells: type A and type B in INL and displaced at GCL</td>
<td>(Straznicky and Hiscock, 1994)</td>
</tr>
<tr>
<td>Turtle</td>
<td>three types of amacrine cells: type A, at INL, IPL and occasional processes at GCL; type B, at INL and IPL; type C, at the periphery of retina. bipolar cells</td>
<td>(Isayama and Eldred, 1988; Isayama et al., 1988; Wetzel and Eldred, 1997)</td>
</tr>
<tr>
<td><strong>Birds</strong></td>
<td></td>
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</tr>
<tr>
<td>Chicken</td>
<td>cell bodies of amacrine cells in the middle and innermost INL and processes in the IPL</td>
<td>(Bruun et al., 1986)</td>
</tr>
<tr>
<td>Pigeon</td>
<td>cell bodies of amacrine cells in INL and processes in the IPL</td>
<td>(Bruun et al., 1986; Verstappen et al., 1986)</td>
</tr>
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</table>
Table 2 – Localization of NPY receptors in mammalian retina

<table>
<thead>
<tr>
<th>NPY Receptors</th>
<th>Mammal Species</th>
<th>Localization of NPY receptors in mammalian retina</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY Y₁</td>
<td>Mouse</td>
<td>mRNA expression in room air reared mouse retina.</td>
<td>(Yoon et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Immunoreactivity in horizontal cell bodies in INL and processes in OPL, in cholinergic amacrine cell processes in IPL and in all calbindin horizontal cells in rat retina; mRNA in rat retinas and cultured rat retinal cells; Immunoreactivity in photoreceptors, bipolar, horizontal, amacrine and ganglion cells as well as in macroglial and microglial cells of rat retinal cells in culture.</td>
<td>(D'Angelo et al., 2002; Alvaro et al., 2008; Alvaro et al., 2009; Alvaro et al., 2007; Santos-Carvalho et al., 2012)</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>NPY Y₁ receptor in cultured Müller cells.</td>
<td>(Milenkovic et al., 2004)</td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>mRNA in cultured RPE cells.</td>
<td>(Ammar et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>mRNA in human RPE; Immunoreactivity in Müller cells in retina with proliferative vitreoretinopathy.</td>
<td>(Ammar et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>mRNA expression in post-natal oxygen-reared mice.</td>
<td>(Yoon et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>NPY Y₂</td>
<td>Rat</td>
<td>mRNA in intact retinas and cultured rat retinal cells; Immunoreactivity in photoreceptors, bipolar, horizontal, amacrine and ganglion cells as well as macroglial and microglial cells of rat retinal cells in culture.</td>
<td>(Alvaro et al., 2008; Alvaro et al., 2009; Alvaro et al., 2007; D'Angelo and Brecha, 2004; Santos-Carvalho et al., 2012)</td>
</tr>
<tr>
<td>Bovine</td>
<td>mRNA in bovine RPE and cultured RPE cells;</td>
<td>(Ammar et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>mRNA in human RPE.</td>
<td>(Ammar et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>NPY Y₄</td>
<td>Rat</td>
<td>mRNA in rat retina and cultured rat retinal cells.</td>
<td>(Alvaro et al., 2008; Alvaro et al., 2009; Alvaro et al., 2007; D'Angelo and Brecha, 2004)</td>
</tr>
<tr>
<td>NPY Y₅</td>
<td>Rat</td>
<td>mRNA in rat retina and cultured rat retinal cells;</td>
<td>(Alvaro et al., 2008; Alvaro et al., 2009; Alvaro et al., 2007; D'Angelo and Brecha, 2004)</td>
</tr>
<tr>
<td>Human</td>
<td>mRNA in human RPE.</td>
<td>(Ammar et al., 1998)</td>
<td></td>
</tr>
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</table>
**Table 3** – Roles of NPY receptors in mammalian retina

<table>
<thead>
<tr>
<th>NPY Receptors</th>
<th>Mammal Species</th>
<th>Function of NPY receptors in mammalian retina</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY Y₁</td>
<td>Rat</td>
<td>Receptor activation inhibits the increase in ([\text{Ca}^{2+}]) in rat retinal neurons;</td>
<td>(Alvaro et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proliferation of rat retinal cells in culture;</td>
<td>(Alvaro et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Receptor activation in neurons increases glutamate release that activates glutamate mGlu receptors of Müller cells and inhibits their osmotic swelling.</td>
<td>(Uckermann et al., 2006)</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td></td>
<td>NPY has a biphasic effect on Muller cells proliferation through NPY Y₁ receptor activation.</td>
<td>(Milenkovic et al., 2004)</td>
</tr>
<tr>
<td>NPY Y₂</td>
<td>Mouse</td>
<td>NPY Y₂ mRNA expression increase in post-natal oxygen-reared mice, suggesting that may have a dual role, vasoconstriction and angiogenesis, in the evolution of oxygen-induced retinopathy.</td>
<td>(Yoon et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Inhibition of voltage-dependent (\text{Ca}^{2+}) influx into rod bipolar cell terminals;</td>
<td>(D’Angelo and Brecha, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPY Y₂ receptor antisense oligonucleotide prevents hyperoxia-induced retinal neovascularization;</td>
<td>(Koulu et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proliferation of rat retinal cells in culture;</td>
<td>(Alvaro et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Receptor activation inhibits the necrotic cell death induced by glutamate in rat retinal cells in culture.</td>
<td>(Santos-Carvalho et al., 2013)</td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
<td>NPY signaling in cultured bovine RPE occurs mainly through NPY Y₂ receptor.</td>
<td>(Ammar et al., 1998)</td>
</tr>
<tr>
<td>NPY Y₄</td>
<td>Rat</td>
<td>Receptor activation inhibits the increase in ([\text{Ca}^{2+}]) in rat retinal neurons.</td>
<td>(Alvaro et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Receptor activation inhibits the necrotic cell death induced by glutamate in rat retinal cells in culture.</td>
<td>(Santos-Carvalho et al., 2013)</td>
</tr>
<tr>
<td>NPY Y₅</td>
<td>Rat</td>
<td>Receptor activation inhibits the increase in ([\text{Ca}^{2+}]) in rat retinal neurons;</td>
<td>(Alvaro et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proliferation of rat retinal cells in culture.</td>
<td>(Alvaro et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Receptor activation inhibits the necrotic and apoptotic cell death induced by glutamate in rat retinal cells in culture.</td>
<td>(Santos-Carvalho et al., 2013)</td>
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</table>
## Abbreviation List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BrdU</td>
<td>5-Bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular calcium</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>E18</td>
<td>Embrionic day 18</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<tr>
<td>GAD 65</td>
<td>Glutamic acid decarboxylase 65</td>
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<tr>
<td>GAT-1</td>
<td>GABA transporter 1</td>
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<td>GCL</td>
<td>Ganglion cell layer</td>
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<tr>
<td>hESC</td>
<td>Human embryonic stem cells</td>
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<td>iGluRs</td>
<td>Ionotropic glutamate receptors</td>
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<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
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<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
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<td>Leu</td>
<td>Leucine</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MDMA</td>
<td>3,4-Methylenedioxymethamphetamine</td>
</tr>
<tr>
<td>mGLuRs</td>
<td>Metabotropic glutamate receptors</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NOS–sGC</td>
<td>Nitric oxide synthase - soluble guanylyl cyclase</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>NPY–IR</td>
<td>Neuropeptide Y immunoreactivity</td>
</tr>
<tr>
<td>NPY Y₁</td>
<td>NPY receptor type 1</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>Outer plexiform layer</td>
</tr>
<tr>
<td>P7</td>
<td>Postnatal day 7</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<td>POS</td>
<td>Photoreceptor outer segment</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
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<tr>
<td>RPE</td>
<td>Retinal pigmented epithelium</td>
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