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# NPY in rat retina is present in neurons, in endothelial cells and also in microglial and Müller cells

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**Abstract**

NPY is present in the retina of different species but its role is not elucidated yet. In this work, using different rat retina in vitro models (whole retina, retinal cells in culture, microglial cell cultures, rat Müller cell line and retina endothelial cell line), we demonstrated that NPY staining is present in the retina in different cell types: neurons, macroglial, microglial and endothelial cells. Retinal cells in culture express NPY $Y_1$, $Y_2$, $Y_4$ and $Y_5$ receptors. Retina endothelial cells express all NPY receptors except NPY $Y_5$ receptor. Moreover, NPY is released from retinal cells in culture upon depolarization. In this study we showed for the first time that NPY is present in rat retina microglial cells and also in rat Müller cells. These in vitro models may open new perspectives to study the physiology and the potential pathophysiological role of NPY in the retina.


*Keywords:* Neuropeptide Y (NPY); Rat retina; Microglial cells; Müller cells; Endothelial cells; NPY receptors; NPY release

## 1. Introduction

The retina is a very organized structure of the nervous tissue that contains two main classes of cells: neurons and glial cells. Retinal neurons include photoreceptors, bipolar, ganglion, horizontal and amacrine cells that are strategically localized in various layers through. Glial cells comprise macroglial and microglial cells. Two main macroglial elements are present in the retina (Newman, 2004): Müller cells that span the entire thickness of the retina, give the support to the retina by regulating retinal metabolism and modulate the function of neurons and blood vessels; astrocytes are associated with optic nerve axons and retinal blood vessels. Microglia is normally quiescent but very sensitive to the retina homeostatic state. When the homeostasis is disturbed, microglia rapidly acts as phagocytes, or release inflammatory mediators (Chen et al., 2002).

Neuropeptide Y (NPY) is a highly conserved, 36 amino acid peptide, widely distributed in the central nervous system (Silva et al., 2005b) being also present in the retina of different species (Bruun et al., 1986; Hutsler and Chalupa, 1995; Jen et al., 1994; Oh et al., 2002). NPY-ir is mainly localized in amacrine cells but it is also present in ganglion cells (Ammar et al., 1998; Hutsler and Chalupa, 1994; Sinclair and Nirenberg, 2001). In rat retina, NPY-ir is present in two populations of amacrine cells, one located in the inner nuclear layer and the other in the ganglion cell layer. Little is known about the function of NPY system in the retina but some observations propose that NPY-ir cells participate in multiple circuits mediating visual information processing in the inner retina. It was suggested that these cells are involved in spatial tuning, specifically, in tuning ganglion cells to low spatial frequencies (Sinclair et al., 2004). In addition, exogenous application of NPY stimulates the release of glycine, dopamine, and 5-hydroxytryptamine from the rabbit retina, and gamma-aminobutyric acid (GABA) and

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choline from the chicken retina, suggesting that NPY modulates neurotransmitter release in the retina (Bruun and Ehinger, 1993). All these observations suggest that NPY is probably released in response to visual stimulus. However, the conditions required for NPY release must be investigated. The fact that NPY is released by high frequency light stimulation or depolarization was only demonstrated only in the frog retina (Bruun et al., 1991). The release of NPY in the retina of other vertebrates was not yet demonstrated.

NPY mediates its effects through the activation of six G-protein-coupled receptor subtypes named $Y_1$, $Y_2$, $Y_3$, $Y_4$, $Y_5$, and $y_6$. Five distinct NPY receptors have been cloned, $Y_1$, $Y_2$, $Y_4$, $Y_5$ and $y_6$ (Silva et al., 2002). The $Y_3$ receptor has not yet been cloned while $y_6$ receptor is not functional (Michel et al., 1998). The presence of mRNA for the $Y_1$, $Y_2$, $Y_4$ and $Y_5$ receptors was detected in intact rat retinas (D'Angelo and Brecha, 2004).

Endothelial cells are another cell type that forms an important structure of the retina: blood-retinal-barrier (BRB). This structure separates the retina from the blood circulation by a barrier located both at the retinal vessels, the inner BRB, and at the retinal pigment epithelium, the outer BRB. NPY has been reported to directly stimulate endothelial cell proliferation and migration (Ekstrand et al., 2003; Ghersi et al., 2001; Marion-Audibert et al., 2000; Zukowska-Grojec et al., 1998a,b). In the retina, some evidences have shown that NPY and $Y_2$ receptors are involved in development of diabetic retinopathy and retinal neovascularization (Koulu et al., 2004).

Several lines of evidence suggest that the expression of NPY and NPY receptors, in central nervous system, may not be restricted to neurons, but could also extend to glial cells, namely in astrocytes (Barnea et al., 1998; Gimpl et al., 1993; St-Pierre et al., 2000). The presence of NPY in retina glial cells, such as in microglial cells and in Müller cells, was not yet investigated.

The aim of the present study was to better characterize the presence of NPY on different in vitro rat retina models. Therefore, whole rat retinas, primary cell cultures of rat retinal cells, primary cell cultures of retina microglial cells, a cell line of rat Müller cells, and a cell line of rat retina endothelial cells were used. The mRNA for NPY and NPY receptors were detected in whole rat retinas, primary cell cultures of rat retinal cells and in a cell line of rat retina endothelial cells. Moreover, the NPY release was evaluated using the primary culture of rat retinal cells. The development and characterization of new in vitro models of the retina will allow defining the role of NPY in retinal physiology and pathophysiology.

## 2. Materials and methods

### 2.1. Materials

Minimum essential medium (MEM) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Dulbecco's modified Eagle medium (DMEM) and trypsin were purchased from GIBCO BRL, Life Technologies, Scotland, UK. Taq DNA polymerase was purchased from Amersham Biosciences Europe GmbH, Freiburg, Germany. First strand cDNA Synthesis Kit for RT-PCR (AMV) was purchased from Roche Applied Science, PO, Indianapolis, USA. Fetal bovine serum was obtained from Biochrom, Berlin, Germany.

### 2.2. Cell cultures

#### 2.2.1. Primary rat retina neural cell culture

Three to 5 days old Wistar rats were used to prepare primary rat retina cell culture as previously described (Santiago et al., 2006). All procedures involving animals were in agreement with the Association for Research in Vision and Ophthalmology (ARVO) statement in vision and ophthalmic research. Briefly, rat retinas were digested with 0.1% trypsin (w/v) for 15 min at 37 °C. Cells were plated on poly-D-lysine (0.1 mg/ml) coated wells with MEM, supplemented with 25 mM HEPES, 26 mM $NaHCO_3$, 10% FCS and penicillin (100 U/ml)–streptomycin (100 μg/ml) for 9 days (37 °C, 5% $CO_2$), at a density of $2 \times 10^6$ cells/cm$^2$. For immunocytochemistry experiments, cells were plated on glass coverslips and for RNA extraction cells were plated on 35 mm Petri dishes at the same density. For NPY assays retina neural cell cultures were plated on 60 mm Petri dishes at density of $3 \times 10^6$ cells/cm$^2$.

#### 2.2.2. Rat retina microglial cell culture

Primary rat microglial cell cultures were prepared from 3 to 5 days old Wistar rats retinas as described with some modifications (Wang et al., 2005). Briefly, retinas were dissected under sterile conditions using a light microscope in $Ca^{2+}$- and $Mg^{2+}$-free Hank's balanced salt solution (HBSS, in mM: 137 NaCl, 5.4 KCl, 0.45 $KH_2PO_4$, 0.34 $Na_2HPO_4$, 4 $NaHCO_3$, 5 glucose; pH 7.4). The retinas were digested in HBSS supplemented with 0.1% trypsin (w/v) and 0.001% DNase (w/v), for 15 min at 37 °C. After dissociation, trypsin was inactivated by 10% heat-inactivated fetal bovine serum (FBS) and the cells were pelleted by centrifugation. The cells were resuspended in DMEM/F-12 HAM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and plated at a density of $1.5 \times 10^6$ cells/cm$^2$ on 75 cm$^2$ growth surface area plastic flasks coated with poly-D-lysine (0.1 mg/ml). The cells were kept in culture at 37 °C under a 95% air/5% $CO_2$ atmosphere and the medium was changed every 3 days. After 2 weeks, microglia cells were harvested in culture medium containing serum by shaking the flasks at 150 rpm for 30 min. The detached cells were plated at a density of $25 \times 10^4$ cells/cm$^2$ on 16 mm glass coverslips coated with poly-D-lysine (0.1 mg/ml). The cells were used for immunocytochemistry experiments after 3 days in culture.

#### 2.2.3. Culture of rat retina endothelial cell line—TR-iBRB

Conditionally immortalized retinal capillary endothelial cell line (TR-iBRB—transgenic rat-inner blood-retinal barrier) was obtained from Dr. Ken-ichi Hosoya, and cultured as previously described (Hosoya et al., 2001). These cells were plated on glass coverslips and cultured in DMEM at 33 °C/5% $CO_2$ for 3–4 days until 80–90% of confluence.

#### 2.2.4. Culture of rat retina Müller cell line

Conditionally immortalized rat Müller cell line (TR-MUL—transgenic rat Müller cells) were cultured as previously described (Tomi et al., 2003). Müller cells were plated on glass coverslips and cultured in DMEM at 33 °C/5% $CO_2$ for 3–4 days until 80–90% of confluence.

### 2.3. Determination of NPY content and release in rat retina cell cultures

Retina cells in culture were washed three times with Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) containing Tween 20 (0.0001%) in order to prevent NPY sticking to plastic. After 20 min of pre-incubation, at room temperature in Krebs buffer, the cells were incubated with Krebs buffer for 10 min at room temperature with or without 50 mM KCl. NPY released without KCl (constitutive release) and with KCl was measured by an ELISA assay (described below). NPY intracellular content in whole retina or in cell cultures were determined in Krebs buffer with 50 mM EDTA and 0.0001% Tween 20 and after freeze–defreeze, and sonication for 12 s. The cell suspension was centrifuged (14,000 rpm/5 min/4 °C). NPY (released and content) was measured using a NPY sandwich-ELISA assay as previously described (Grouzmann et al., 1992a). Each well from 96 wells microplate (PolySorp, Nunc) was coated with 200 ng (100 μl) of the monoclonal antibody NPY02 diluted in Tris buffer (50 mM, pH 7.5) for 16 h at 4 °C.