Neuropeptídeo Y na retina: porquê? e para quê?

Neuropeptide Y in the retina: Why? and what for?

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Faculdade de Farmácia
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COVER: Painel of confocal images of R-iPS cells (in the corners, immunostained for NPY Y_1 receptor) and rat retinal cells (immunostained for NPY Y_1 and Y_2 receptors, TUJ1, GFAP, Vimentin, CD11b, Calretinin, Calbindin and Parvalbumin). Nuclei were visualized with Hoechst 33342 or DAPI.

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TABLE OF CONTENTS

List of abbreviations

$5HT_{2A}$	5-Hydroxytryptamine (serotonin) receptor 2A
AGEs	Advanced glycation end products
AIF	Apoptosis-inducing factor
AMD	Age-related macular degeneration
Am-P	Aminopeptidase P
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	Analysis of variance
Apaf-1	Apoptosis protease-activating factor 1
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BRB	Blood-retinal barrier
BrdU	5-Bromo-2'-deoxyuridine
$[Ca^{2+}]_i$	Intracellular calcium
cAMP	Cyclic adenosine monophosphate
CARD	Caspase activation and recruitment domain
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CMZ	Ciliary marginal zone
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CPB	Carboxypeptidase B
CPON	C-flanking peptide of NPY
CRF	Corticotrophin-releasing factor
C-terminal	Terminal carboxilic
DAG	Diacylglycerol
DAPT	N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
DCM	Mitochondrial transmembrane potential
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low pI;
DISC	Death-inducing signaling complex
Dkk-1	Dickkopf-related protein 1
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPP-IX	Dipeptidyl peptidase 9
DPP-VIII	Dipeptidyl peptidase 8
DPP-IV	Dipeptidyl peptidase IV
DR	Diabetic retinopathy
E18	Embrionic day 18
EAAT	Excitatory amino acid transporter
EdU	5-ethynyl-2′deoxyuridine
Endo G	Endonuclease G
EPSC	Excitatory postsynaptic current component
EPSP	Excitatory postsynaptic potential
ERG	Electroretinogram
ERK	Extracellular signal-regulated kinases
ES cells	Embryonic stem cells
FGF	Fibroblast growth factor
fRPE	Retinal pigmented epithelium obtain from fibroblats
GABA	γ-Aminobutyric acid
$GABA_A$	GABA receptor type A
GAD 65	Glutamic acid decarboxylase 65
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GAT-1	GABA transporter 1
GCL	Ganglion cell layer
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
G_i	Inhibitor G protein
GPCR	G-protein coupled receptor
GS	Glutamine synthetase
hES cells	Human embryonic stem cells
hiPS	Human iPS
HLEC	Human lens epithelial cells
HMGP1	High mobility group B1
HtrA2/OMI	High temperature requirement protein A2
IAP	Inhibitor of apoptosis protein
ICAM	Intercellular adhesion molecule
ICM	Inner cell mass
IGF- 1	Insulin-like growth factor
iGluRs	Ionotropic glutamate receptors
IL-1	Interleukin-1
Ile	Isoleucine
IMS	Mitochondrial intermembrane space
INL	Inner nuclear layer
IPL	Inner plexiform layer
iPS	Induced pluripotent stem cell
IUPHAR	International Union of Pharmacology
KA	Kainate
KCl	Potassium chloride
Klf-4	Kruppel-like factor 4
Leu	Leucine
LMP	Lysosomal membrane permeabilization
MAPK	Mitogen activated protein kinase
MDMA	3,4-Methylenedioxymethamphetamine
Mefs	Mouse embryonic fibroblats
mGluRs	Metabotropic glutamate receptors
MMP	Mitochondrial membrane permeabilization
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NCCD	Nomenclature Committee on Cell Death
NeuN	Neuronal nuclei
NFL	Nerve fiber layer
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS–sGC	Nitric oxide synthase - soluble guanylyl cyclase
NPDR	Nonproliferative DR
NPY	Neuropeptide Y
NPY Y_1	NPY receptor type 1
NPY–IR	Neuropeptide Y immunoreactivity
NPY–LI	Neuropeptide Y-like immunorecativity
NRL	Neural retina leucine zipper
Oct-4	Octamer-4
OLM	Outer limiting membrane
ONL	Outer nuclear layer
OPL	Outer plexiform layer
P7	Postnatal day 7

PACAP	Pituitary adenylate cyclase-activating polypeptide
PAM	Peptidylglycine alpha-amidating monooxygenase
PARP	Poly(ADP-ribose) polymerase
PAX6	Paired box protein 6
PCD	Programmed cell death
PDR	Proliferative diabetic retinopathy
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
POS	Photoreceptor outer segment
PP	Pancreatic polypeptide
Pro	Proline
PVR	Proliferative vitreoretinopathy
PYY	Peptide YY
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RGCs	Retinal ganglion cells
RIP	Receptor-interacting protein kinase
RNAi	RNA interference
ROS	Reactive oxygen species
RP	Retinitis pigmentosa
RPE	Retinal pigmented epithelium
RSC	Retinal stem cells
SAPK/JNK	Stress-activated protein kinase/c-Jun NH2-terminal kinase
SCNT	Somatic-cell nuclear transfer
SDIA	Stromal cell-derived inducing activity
S.E.M.	Standard error of mean
SFEB	Serum-free floating culture of embryoid body-like aggregates
SGZ	Subgranular zone
Sox-2	Sex determining region Y-box 2
ssDNA	Single stranded DNA
SVZ	Subventricular zone
Tir	Tyrosine
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TTFs	Tail-tip fibroblastos
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VGAT	Vesicular γ-aminobutyric acid transporter
VIP	Vasoactive intestinal polypeptide
Z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Resumo

A retina é atingida por várias doenças degenerativas, como o glaucoma, a retinopatia diabética e a degenerescência macular associada à idade. Os tratamentos disponíveis para estas doenças são escassos e pouco eficazes. Assim, os principais objetivos deste trabalho são investigar duas estratégias distintas para o tratamento de doenças degenerativas da retina. Uma das abordagens consiste na aplicação do neuropeptídeo Y (NPY) como uma molécula neuroprotetora enquanto a outra compreende a utilização de células pluripotentes induzidas de retina como potencial fonte de fotorrecetores para terapias de base celular.

A excitotoxicidade induzida pelo glutamato encontra-se associada à morte neuronal indutora do aparecimento de doenças degenerativas da retina (Kowluru et al., 2003; Santiago et al., 2008). O NPY e os seus recetores encontram-se distribuídos pelo sistema nervoso central, incluindo a retina. Sabe-se também que o NPY possui um papel neuroprotetor contra a excitotoxicidade do glutamato em hipocampo de rato através da ativação dos recetores Y_1, Y_2 e/ou Y_5 do NPY (Silva et al., 2003b; Xapelli et al., 2007). O nosso grupo mostrou também anteriormente que células neurais de retina de rato em cultura expressam o NPY e os seus recetores (Y_1, Y_2, Y_4 e Y_5) (Alvaro *et al*., 2007). Demonstrámos ainda que o NPY apresenta um papel protetor contra a toxicidade induzida pela MDMA em retina de rato (Alvaro et al., 2008), contudo os recetores do NPY envolvidos neste efeito ainda não foram investigados. Encontrava-se ainda por investigar a localização específica dos recetores do NPY nas células neurais de retina de rato em cultura.

Nesta tese, utilizando células neurais de retina de rato em cultura e células de Müller de rato em cultura mostrámos que os recetores Y_1 e Y_2 do NPY se encontram presentes em todos os tipos de células da retina. Neurónios, tais como fotorrecetores, células bipolares, horizontais, amácrinas e ganglionares expressam estes dois tipos de

recetores do NPY. Os recetores Y_1 e Y_2 encontram-se também expressos em células da macroglia (células de Müller e astrócitos) e da microglia.

Além do mais, o tratamento de células neurais de retina de rato em cultura com NPY (100nM) inibe a morte celular por necrose e apoptose induzida pelo glutamato. Observa-se uma redução do número de células positivas para iodeto de propídeo, tal como do número de células positivas para o TUNEL ou para a caspase 3. O NPY protege neurónios (positivos para a TUJ1), astrócitos e células de Müller (positivos para GFAP) e células da microglia (positivas para CD11b) da morte celular induzida pelo glutamato. Por outro lado, a utilização de ferramentas farmacológicas, tais como agonistas e antagonistas dos recetores do NPY, permitiram determinar que a ativação dos recetores Y_2, Y_4 e Y_5 do NPY inibem a morte por necrose, enquanto a ativação dos recetores Y_5 do NPY previne a morte celular por apoptose induzida pelo glutamato.

Outras doenças degenerativas da retina, como a retinite pigmentosa, são caraterizadas pela perda de fotorrecetores. Células pluripotentes induzidas de fibroblastos podem constituir uma fonte inesgotável de fotorrecetores para terapias de base celular. Contudo, uma das limitações desta estratégia é o baixo rendimento de fotorrecetores obtidos (Comyn et al., 2010; Huang et al., 2010; Lamba et al., 2010; Tucker et al., 2011). Recentemente, foi demonstrado que as células pluripotentes induzidas retêm uma memória epigenética transitória das suas células de origem que alteram o seu potencial em se diferenciar num tipo específico de células. Através do uso de retinas e fibroblastos da cauda de murganhos reprogramados com os fatores de pluripotência (Oct4, Klf4, Sox2 e c-Myc) foram geradas células pluripotentes de retina (R-iPS) e de fibroblastos (F-iPS). Após a caraterização da pluripotência e estudo da expressão de marcadores de fotorrecetores, estas células foram diferenciadas em fotorrecetores. As células pluripotentes obtidas da retina diferenciaram com maior eficiência que as provenientes de fibroblastos. Desta forma, confirma-se a importância de memória epigenética na reprogramação e diferenciação de células pluripotentes induzidas.

Com base em estudos prévios que mostraram as propriedades pró-neurogénicas do NPY em várias zonas do sistema nervoso central e o seu papel na manutenção da renovação e pluripotencia de células estaminais embrionárias humanas (Son et al., 2011), investigámos o sistema NPY nas células R-IPS. Estas células expressam NPY e os recetores Y_1 do NPY. Além disso, através da técnica de proliferação por citometria de fluxo com o 5-etinil-2-deoxiuridina (EdU) e imunocitoquímica para dois marcadores de proliferação (EdU e Ki-67), na presença e ausência de NPY, concluiu-se que o NPY não apresenta um efeito proliferativo em células R-iPS.

Em conclusão:

1) o NPY e os seus recetores poderão ser considerados potenciais alvos no tratamento de doenças, tais como o glaucoma e a retinopatia diabética.
2) as células pluripotentes induzidas provenientes de retina poderão ser uma nova fonte de fotorrecetores para técnicas de base celular.

Desta forma, quer o NPY, quer as R-iPS poderão, no futuro, abrir novas perspectivas no tratamento de doenças degenerativas da retina.

Summary

The retina is affected by several degenerative diseases like glaucoma, diabetic retinopathy and age-related macular degeneration (AMD). Moreover, the treatments available to treat these diseases are very scarce and not very effective. Thus the present study aims to investigate two different strategies to treat retinal degenerative diseases. First, neuropeptide Y (NPY) applied as a neuroprotective molecule. Second, R-IPS cells used as a new potential source of photoreceptors for retinal cell-based therapies.

It has been claimed that glutamate excitotoxicity is responsible for neuronal cell death giving rise to several retinal neurodegenerative diseases such as glaucoma and diabetic retinopathy (Kowluru et al., 2003; Santiago et al., 2008). It is also known that neuropeptide Y and NPY receptors are widely distributed in the central nervous system, including the retina. NPY has a neuroprotective role against excitotoxicity in rat hippocampus through the activation of Y_1, Y_2 and/or Y_5 receptors (Silva et al., 2003b; Xapelli et al., 2007). We previously showed that cultured rat retinal neural cells express NPY and NPY Y_1, Y_2, Y_4 and Y_5 receptors (Alvaro *et al*., 2007). Moreover, we have also demonstrated that NPY has a protective role against MDMA-induced toxicity in the retina (Alvaro et al., 2008), but the NPY receptor subtype(s) involved in the NPY neuroprotective effect were not investigated, neither their specific localization in rat retinal neural cell culture.

In the present work, using rat retinal neural cell cultures and pure rat Müller cell culture, we show that NPY Y_1 and Y_2 receptors are present on every cell type of rat retinal cells. Neurons, as photoreceptors, bipolar, horizontal, amacrine and ganglion cells express these two types of NPY receptors. NPY Y_1 and Y_2 receptors are also located in macroglial cells (Müller cells and astrocytes) and microglial cells.

Additionally, NPY (100 nM) pre-treatment of rat retinal neural cell culture inhibited glutamate-induced necrotic (PI-positive cells) and apoptotic (TUNEL-positive cells and caspase 3-positive cells) cell death compared to glutamate. NPY protected glutamate

induced cell death of neurons (TUJ1 positive-cells), astrocytes/Müller cells (GFAP positive-cells) and microglia cells (CD11b positive-cells).

Furthermore, using NPY receptor agonists and antagonists we observe that NPY Y_2, Y_4 and Y_5 receptors activation inhibited necrotic cell death, while only the activation of Y_5 receptor prevented apoptotic cell death triggered by glutamate,

Other retinal degenerative diseases are characterized by the loss of photoreceptors, like retinitis pigmentosa. Pluripotent stem cells (iPS cells) obtained from fibroblasts may provide an unlimited source of photoreceptors for cell based therapies. However one limitation of this approach is the low yield of photoreceptors obtained (Comyn et al., 2010; Huang et al., 2010; Lamba et al., 2010; Tucker et al., 2011). Recently, it has also been showed that iPS cells retain a transient transcriptional and epigenetic memory of their cell of origin which affects their potential to differentiate in specific cell types. Using collagen-OKSM (pluripotency factors: Oct4, Klf4, Sox2 and c-Myc) reprogrammable mice retinas and tail fibroblast, Retinal-iPS and Fibroblats-iPS cells were generated. After characterization of pluripotency and study of photoreceptor markers expression, these cells were differentiated in photoreceptors. R-iPS cells differentiated into photoreceptors with a much higher efficiency then F-iPS cells, which indicate the importance of epigenetic memory in cellular reprogramming and differentiation.

Based on previous studies showing the pro-neurogenic properties of NPY in several parts of the CNS and its role in the maintenance of human embryonic stem (hES) cells self-renewal and pluripotency (Son et al., 2011), we investigate the NPY system in these cells. We detected the expression of NPY and NPY Y_1 receptors on R-iPS cells. Moreover, we performed 5-ethynyl-2-deoxyuridine (EdU)-Flow cytometry proliferation assay and evaluated the expression of two different proliferation markers: EdU and Ki-67, in the presence and absence of NPY. NPY did not shown a proliferative effect in R-iPS cells.

In summary,

1) NPY and NPY receptors might be considered potential targets to treat diseases, such as glaucoma and diabetic retinopathy,

2) R-iPS cells are a new potential source of photoreceptors for cell-based therapies.

Therefore, we believe that NPY and R-iPS cells will open new perspectives to treat degenerative diseases in the retina.

Chapter I
General introduction

Chapter 1 – General introduction

1. The Retina

1.1. Retinal Structure

The retina is a neural tissue in the back of the eye between the retinal pigment epithelium (RPE) and the vitreous body. The vertebrate retina, like other regions of the central nervous system (CNS), is derived embryologically from the neural tube (Yang, 2004). Histologically, the retina is highly complex and organized in well structurally layers (Fig. 1.1). The retina is composed by four main groups of cells: five basic types of neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells), glial cells (astrocytes, Müller and microglial cells), epithelial cells (retinal pigment epithelium) and vascular cells (endothelial cells and pericytes). 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: outer plexiform layer, OPL; inner plexiform layer, IPL. The ONL contains cell bodies of photoreceptors (rods and cones), the INL contains cell bodies of the bipolar, horizontal and amacrine cells and the GCL contains cell bodies of ganglion cells and displaced amacrine cells. In the OPL occur connections between rod and cones, and vertically running bipolar cells and horizontally oriented horizontal cells. The second synaptic area is the IPL, and it functions as a relay station for the vertical-information-carrying nerve cells, the bipolar cells, to connect to ganglion cells. Thus, 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.

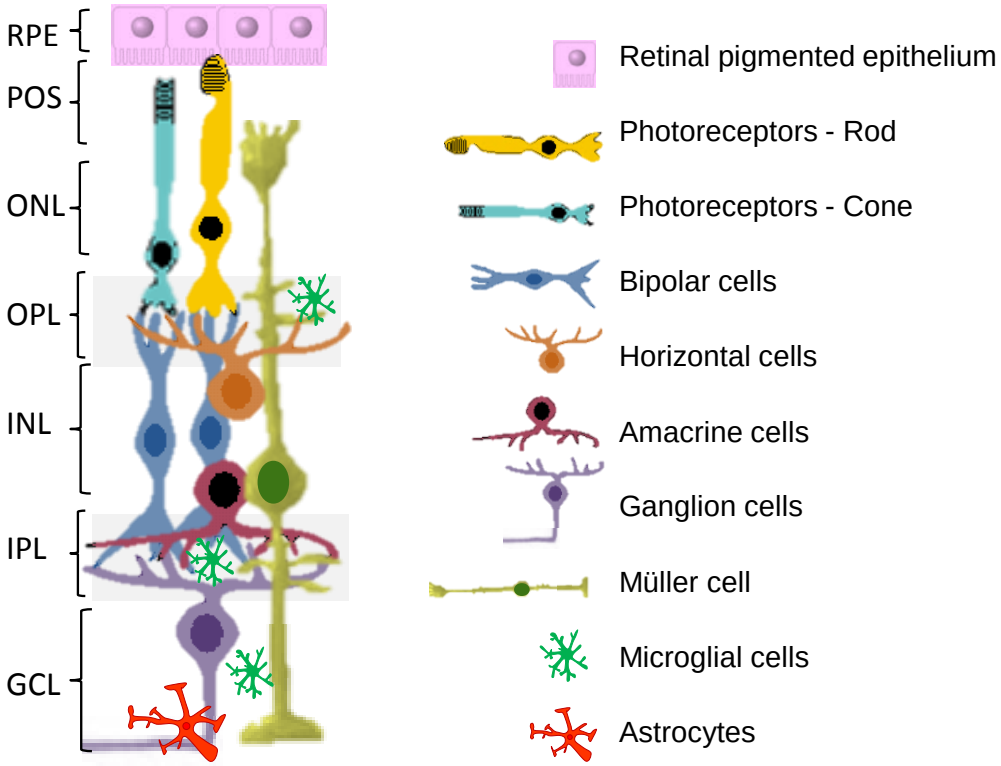


Fig. 1.1 - Diagram illustrating the organized layers of the retina with retinal neurons (photoreceptors, bipolar cells, ganglion cells, horizontal and amacrine cells), macroglia and microglial cells. In the more outer extremity of the retina, there are the retinal pigment epithelium (RPE), followed by the outer segment of photoreceptor (POS), cones and rods, which nucleus originate the outer nuclear layer (ONL). The photoreceptors contact with bipolar cells and horizontal cells at the outer plexiform layer (OPL). In the inner retina, especially in the inner nuclear layer (INL) could be found the nucleus of bipolar, amacrine and horizontal cells. The synapses between bipolar, amacrine and ganglion cells are established along the inner plexiform layer (IPL). Finally the ganglion cells in the ganglion cell layer transmit the information through the optic nerve until the cortex. The microglial cells are in the OPL, IPL and GCL. Macroglial cells present different distribution. The astrocytes are in the GCL, while the Müller cells extent through the entire thickness of the retina, extending processes from the outer until the inner limiting membrane.

Externally the RPE is in contact with the Brunch's Membrane adjacent to the choroid. In the central retina, these cells are cuboidal and rich in melanin, while in the periphery present an elongated shape. The tight junctions between the RPE cells form an important barrier between the retina and the vascular choroid system, the outer Blood Retinal Barrier (BRB). The RPE cells have as another main functions: transport and storing of nutrients (vitamin A), ions and water; absorption of scattered light (not absorbed by photoreceptors) and protection against photooxidation; photoreceptor outer segment renewal (phagocytosis of shed photoreceptor membranes); a role in visual cycle (reisomerization of all-trans-retinal); retina immune response and secretion of factors for retinal homeostasis and structural integrity (Simo et al., 2010; Sparrow et al., 2010).

1.1.1. RETINAL NEURONS

The retina is constituted by several types of neurons (photoreceptors, bipolar, amacrine, horizontal and ganglion cells) (Fig. 1.1).

The photoreceptors are the retinal sensory neurons responsible for light detection and the first actors of the visual perception pathway. The mammalian retinas present two types of photoreceptors: rods and cones. These two types of neurons have different morphological and functional characteristics. Usually rods are in major number in the retina than cones, except in fovea (Purves et al., 2004). Rods are lighter sensitive, present disc membranes in the outer segment and their photopigment is one of three types of opsins with different maximum wavelength absorption (blue - 419nm, green - 531nm and red - 559nm). Cones are faster integrators, their outer segments are invaginations of the plasma membrane and the photopigment is rhodopsin. Cones are responsible for color vision and rods for night vision (Nathans, 1989; Wang et al., 2011a).

The INL comprises the cell bodies of bipolar, horizontal and amacrine cells.

The bipolar cells are radial oriented neurons with one or more dendrites, which established synapses with photoreceptors or horizontal cells. Their terminals make synapses with ganglion or amacrine cells (Purves et al., 2004).

The horizontal cells are, in their majority, inhibitory neurons, which dendrites and axons distributed along the OPL. They connect with photoreceptors and bipolar cells. The amacrine cells, another type of lateral interneurons, establish connections between bipolar or ganglion cells. The high diversity of amacrine cells is categorized by the size of the dendritic tree, localization in IPL and neurotransmitters secreted. These cells detect movement and luminosity alterations (Purves et al., 2004).

Finally, deeper in the retina, the ganglion cells receive information from amacrine and bipolar cells. Their axons together constitute optic nerve that leads the visual information from retina to the visual cortex in the brain. It is described 15-20 types of ganglion cells (Wong et al., 2012). Size, extension of dendritic tree and axonal conduction speed variability let these cells to control contrast, color and movement (Guyton, 2006).

1.1.2. RETINAL GLIA

The retina presents three types of glial cells: microglial cells and macroglia cells, represented by astrocytes and Müller cells. In opposition to other parts of CNS, the retina does not have myelinated axons and oligodendrocytes are absent (Kolb, 1995).

Macroglial cells have neuroectodermal origin, although during development Müller cells arise from the same progenitor as retinal neurons while astrocytes migrate into the retina along the optic nerve (Watanabe et al., 1988; Ling et al., 1989). Müller cells span the entire thickness of the retina (Müller, 1851) extending processes through the outer until the inner limiting membrane. In close relation with neurons, Müller cells control the retina homeostasis through the glycogen storage and degradation, removal of cellular metabolites (carbon dioxide and ammonia), regulation of ionic concentration and neurotransmitters metabolism (specially glutamate). These cells also release neuronal and vascular trophic factors (as brain-derived neurotrophic factor, BDNF, and glial cell-

derived neurotrophic factor, GDNF) with important role in prevent neuronal cell death and endothelial dysfunction(Reis et al., 2007).

Astrocytes and their processes are restricted to the GCL and nerve fiber layer (NFL). Together with Müller cells end-feet they interact with inner retinal vasculature (Fig. 1.2). Astrocytes envelop the blood vessels, ensure the BRB, synthetizing factors to induce tight junctions between endothelial cells and give metabolic support to the neurons. Only present in vascularize retinas, astrocytes are a key piece in the development of vascular bed (Arthur et al., 1987; Stone et al., 1987a; Stone, 1997).

Microglial cells are the resident immune cells in the CNS. Coming from mesodermal/mesenchymal progenitors, microglial cells, migrate to the retina during embryonic and fetal periods of development (Chan et al., 2007). In physiological conditions, microglial cells present a ramified morphology and are mostly found in the OPL, IPL and GCL. In retina trauma or inflammation, these cells undergo a morphological retracting of processes reverting to a typical phagocyte appearance (Karlstetter et al., 2010). Microglia migrate to the injury site following chemotactic signals, where they phagocyte dead cells and clear tissue debris, playing scavenging functions. These cells play a dual role in the retina. They release pro-inflammatory cytokines and also neurotrophic factors (Hanisch, 2002; Butovsky et al., 2005). Microglia act as antigen presenting cells and are activated in retinal degeneration models, as ischemia, optic axotomy and kainic acid excitotoxicity (Zhang et al., 2005; Chang et al., 2006).

1.1.3. RETINAL VASCULATURE

The retina is a high oxygen consumption tissue irrigated by the choroid and retinal vasculature (Ames et al., 1992; Forrester et al., 2002). The choroid vasculature, more external, nourishes the photoreceptors, RPE and outer retina. It is characterized by a higher but variable blood flow rate (Yu et al., 2001). Complementary, the retinal vasculature provides lower and constant blood flow rate to the inner retina. The retinal

vessels are inner lining by endothelial cells, supported by the collagen fibers from the basal lamina and ensheathing by elongated and contractile cells, the pericytes (Chakravarthy et al., 1999) (Fig. 1.2). The tight junctions between endothelial cells form a Blood Retinal Barrier (BRB), a barrier that controls the exchanges between blood and retina (movement of ions and large molecules) and ensures the immunological privileged environment of the CNS, and the retina (Gardner et al., 1999). The capillaries are also enveloped by the end feet of astrocytes and Müller cells (Abbott et al., 2006). Conversely the choriocapillaris are fenestrae allowing the easy movement of water and solutes (Forrester et al., 2002).

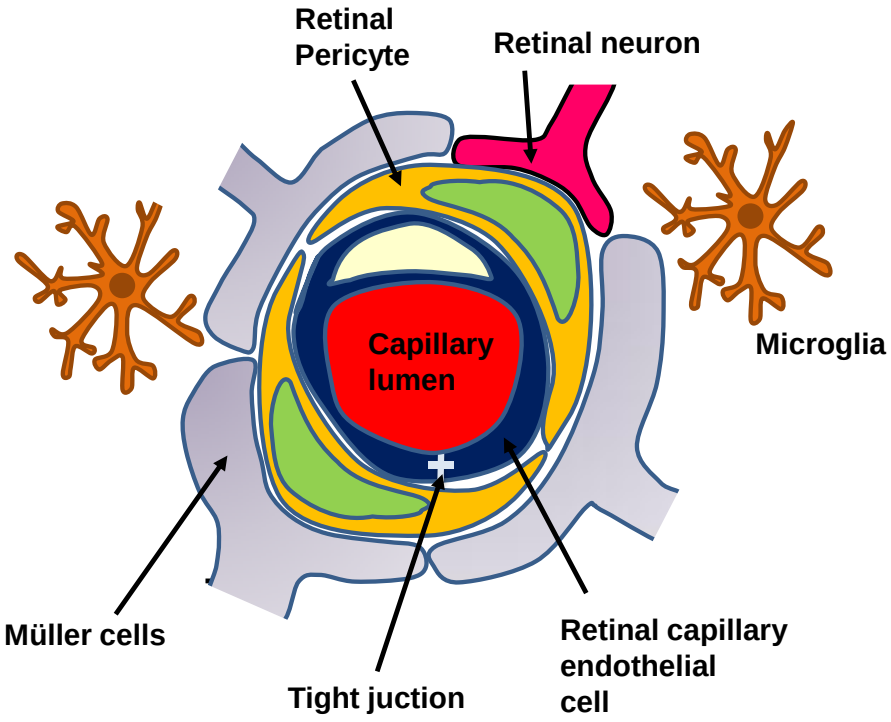


Fig. 1.2 – Scheme of inner Blood Retinal Barrier (BRB), a transversal section of a retinal capillary. The endothelial cells are ensheathed by retinal pericytes and these by the Müller cells and astrocytes end foots and neuronal terminals. Adapted from (Hosoya et al., 2005; Hosoya et al., 2009).

2. Function of the Retina

2.1. Visual pathways in the retina and neurotransmitters

The chemical transmission mediated by neurotransmitters predominates in the neuronal circuitry of the retina. The retina contains a variety of neurotransmitters, which glutamate and γ-aminobutyric acid (GABA) are excitatory and inhibitory neurotransmitters, respectively (Fig. 1.3). Glutamate is responsible for the radial flow of 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 cells 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. Inputs to bipolar cells are from both photoreceptors and horizontal cells. In the IPL, reciprocal synapses connect bipolar and amacrine cells and both type of cells send input to ganglion cells. Amacrine cells are involved in spatial and temporal integration of visual signals in the IPL.

Glutamate is the main excitatory neurotransmitter in the vertical/ radial flow visual signal in the retina. Glutamate binds to glutamate receptors, located in the extracellular side of the membrane.

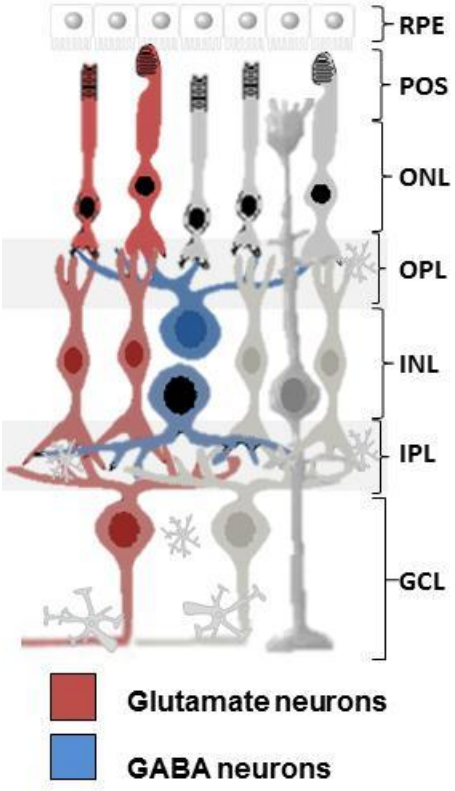


Fig. 1.3 - Diagram illustrating the distribution of the neurotransmitters, GABA and Glutamate, in the retinal neurons. Glutamate neurons (red) of the vertical information pathway (photoreceptors, bipolar cells and ganglion cells) and GABA neurons (blue) of the lateral information pathway (horizontal and amacrine cells). RPE, retinal pigment epithelium; POS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Adapted from (Yang, 2004).

Physiological, pharmacological and molecular properties divided the glutamate receptors in two main groups: ionotropic and metabotropic glutamate receptors. The ionotropic group included α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptors. These receptors form channels permeable to cations. AMPA and KA receptors are permeable to Na^+ and K^+ and with rare exceptions could be also permeable to Ca^{2+} and are characterized by fast kinetics. NMDA receptors are selectively permeable to Na^+, K^+, especially to Ca^{2+}, and are characterized by voltage-dependent block by Mg^{2+} and unusually slow activation/deactivation kinetics (Conn et al., 1997; Ozawa et al., 1998; Yang, 2004; Shen et al., 2006).

The metabotropic glutamate receptors are constituted by three different groups: group I (mGluR1 and mGluR5), coupled to phospholipase C (PLC) and intracellular calcium signaling; group II (mGLuR2 and mGluR3) and group III receptors (mGluR4 and mGluR6-8) conversely negatively coupled to adenylyl cyclase (Conn et al., 1997; Ozawa et al., 1998; Yang, 2004).

Non-NMDA ionotropic glutamate receptors are distributed all over the retina (bipolar, horizontal, amacrine and ganglion cells), while NMDA–glutamate receptors are more predominant in inner retina (amacrine and ganglion cells), however they were already detected in horizontal and bipolar cells (Yang, 2004; Shen et al., 2006; Connaughton, 2007).

Regarding metabotropic glutamate receptors, all subtypes were detected in the retina. However, they are distributed differently for the retinal layers (Yang, 2004; Connaughton, 2007).

GABA is the main inhibitory neurotransmitter in the retina. This neurotransmitter is synthetized from glutamate through the action of glutamic acid decarboxylase (GAD), present in horizontal and amacrine cells. These GABAergic neurons use GABA in the lateral visual pathway to modulate the synaptic transmission in OPL and IPL. Similarly to glutamate, the GABA receptors are divided in: ionotropic receptors, including $GABA_A$ and $GABA_C$ receptors and metabotropic receptors, $GABA_B$ receptors. The $GABA_A$ receptors activation leads to the influx of ions Cl^- through the receptor and consequently to membrane hyperpolarization, while $GABA_B$ inhibits the cyclic adenosine monophosphate (cAMP) synthesis and inhibition Ca^{2+} and K^+ channels. The $GABA_c$ receptors are predominantly detected in the vertebrate retina (Yang, 2004).

Although glutamate and GABA are the main neurotransmitters in the retina, other neurotransmitters like glycine, acetylcholine, dopamine, serotonin, adenosine triphosphate (ATP) and adenosine are also present in the retina.

3. Degeneration of the retina

The retina is triggered by several diseases. Some of them are characterized by degeneration processes.

Glutamate excitotoxicity in the retina

Neurotoxicity in the retina has as a principal actor - the glutamate. The excessive activation of ionotropic glutamate receptors, as NMDA and AMPA subtypes induces neurotoxicity. This process is named excitotoxicity and is indicated as a cause of several diseases in the CNS, including in the retina.

The term “excitotoxicity” was initially used to describe only the neuronal death induced by very high concentrations of exogenous glutamate or glutamate receptors agonists (Olney et al., 1972), later it was extended to the endogenous glutamate-mediated neurotoxicity (potential excitotoxicity of glutamate accumulates in the extracellular space) (Bradford, 1995).

The activation of NMDA or AMPA glutamate receptors causes changes in intracellular ions, especially Ca^{2+} and Na^{+}. The glutamate-induced elevated calcium levels proceed to overactivation of a number of enzymes, including protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II, phospholipases, proteases, phosphatases, nitric oxide synthase (NOS), endonucleases, and ornithine decarboxylase (reviewed in (Won et al., 2002)). Some of these enzymes may also produce positive feedback loops to accelerate the cascade of neuronal death. The generation of the second messenger NO could be another potential mechanism of glutamate neurotoxicity (Dawson et al., 1991; Stewart et al., 2002). More recently, the glutamate excitotoxicity was also associated with the upregulation of NMDA receptors caused by the lack of optic atrophy type 1 (OPA1), a mitochondrial pro-fusion protein (Nguyen et al., 2011) .

Neuronal cell loss in CNS is frequently associated with glutamate excitotoxicity (Sattler and Tymianski, 2000; Arundine and Tymianski, 2003). Several studies indicate that

glutamate excitotoxicity may be involved in neuronal cell loss in several neurodegenerative disease in the retina, like diabetic retinopathy during diabetes (Kowluru et al., 2003; Santiago et al., 2007) and glaucoma (Almasieh et al., 2012).

Necrosis and Apoptosis

Until forty year ago, cell death was synonymous of necrosis, but in 1972 Kerr and collaborators described a nonpathologic cell death in some tissues, later called apoptosis (Kerr et al., 1972). Since then, apoptotic cell death has been well characterized (genetic and functional/biochemical levels). More recently, some authors define necroptosis as regulated necrosis.

New guidelines from Nomenclature Committee on Cell Death (NCCD) (Galluzzi et al., 2012) indicate that, basis on the substantial progress in the biochemical and genetic exploration of cell death, the classification of cell death modalities should be define based on biochemical and functional rather than morphological criteria. Techniques as RNA interference (RNAi), knockout models and plasmid-driven overexpression systems, key signaling chemical inhibitors or activators are essential tools to distinguish cell death forms (Galluzzi et al., 2012).

Apoptosis and necrosis can often be initiated in response to different stimulus but also by the same type of insults with different dose, intensity and duration (Zeiss, 2003). At low doses, a variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anticancer drugs can induce apoptosis while in higher doses can result in necrosis. It is not clear yet if the two mechanisms occur independently, sequentially, or simultaneously (Hirsch et al., 1997; Zeiss, 2003), however NCCD refer that a 'specific' cell death-related phenomenon may occur along with the execution of another cell death model (Galluzzi et al., 2012).

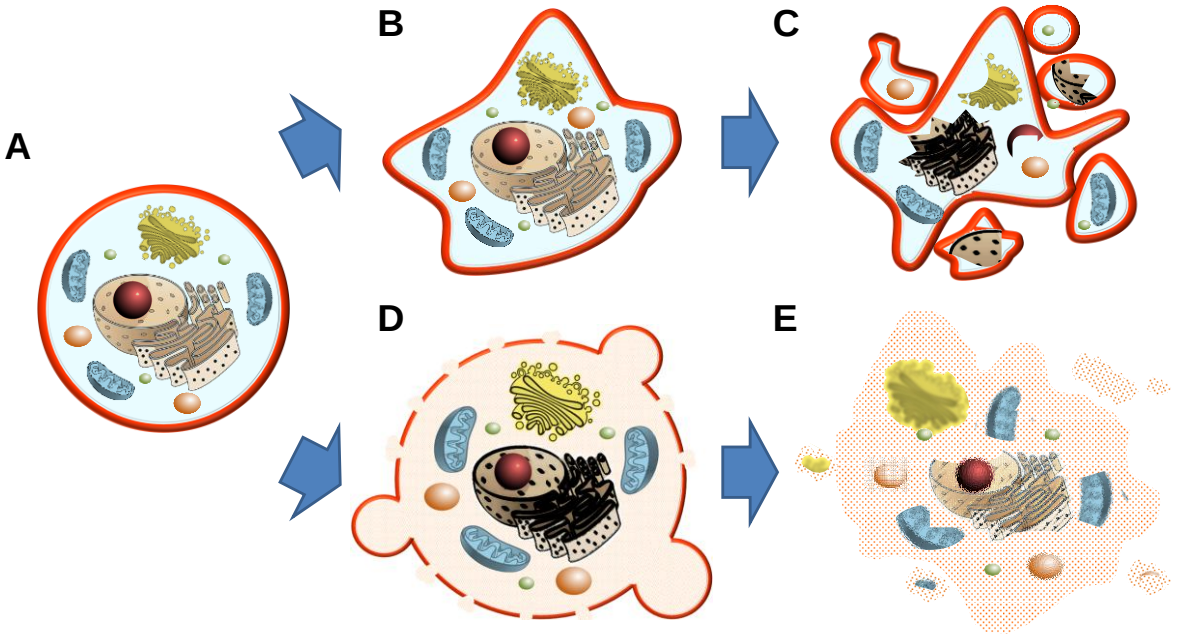


Fig.1.4 – Morphological differences between apoptosis and necrosis. (A) Normal cell. (B & C) Cell undergoes apoptosis. In the apoptotic cell death, the cell reduces its volume and plasma membrane blebbings appear. There are no significant differences in the cytoplasmic organelles, but nuclear fragmentation. (D & E) Cell undergoes necrosis. In necrotic cell death, the cell undergoes cytoplasmic and organelles swelling rupture of plasma membrane and moderate chromatin condensation.

Table 1.1 – Morphological and biochemical features of apoptosis and necrosis. Adapted from (Krysko et al., 2008; Kroemer et al., 2009; Galluzzi et al., 2012).

Differences	Apoptosis	Necrosis
Morphological features	· Rounding-up of the cell · Retraction of pseudopods · Reduction of cellular and nuclear volume (pyknosis) · Nuclear fragmentation (karyorrhexis) · Minor modification of cytoplasmic organelles · Plasma membrane blebbing · Engulfment by resident phagocytes, in vivo	· Cytoplasmic swelling (oncosis) · Rupture of plasma membrane · Swelling of cytoplasmic organelles · Moderate chromatin condensation
Biochemical features	· Activation of proapoptotic Bcl-2 family proteins (e.g., Bax, Bak, Bid) · Activation of caspases · $\Delta\Psi_m$ (mitochondrial transmembrane permeabilization) dissipation · MMP (mitochondrial membrane permeabilization) · Oligonucleosomal DNA fragmentation · Plasma membrane rupture · PS (phosphatidylserine) exposure · Reactive oxygen species (ROS) overgeneration · ss (single-stranded) DNA accumulation	· Activation of calpains · Activation of cathepsins · Drop of ATP levels · HMGB-1 release · Lysosomal membrane permeabilization (LMP) · Plasma membrane rupture · RIP1 phosphorylation · RIP1 ubiquitination · ROS overgeneration · Specific PARP1 cleavage pattern

Apoptosis is morphologically well characterized (Fig. 1.4 and Table 1.1). Cells undergoing apoptosis present plasma membrane blebbing, chromatin condensation with margination of chromatin to the nuclear membrane (pyknosis), karyorrhexis (nuclear fragmentation), and formation of apoptotic bodies (Kerr et al., 1972). Biochemically, apoptosis include alterations in phosphatidylserine kinetics in the plasma membrane (Fadok et al., 1992; Denecker et al., 2001), changes in mitochondrial membrane permeability and release of intermembrane space mitochondrial proteins (van Loo et al., 2002), caspase-dependent activation and nuclear translocation of a caspase-activated DNase causing internucleosomal DNA cleavage (Enari et al., 1998). In fact, apoptotic cells do not release their cellular constituents to the surround tissue, therefore they are quickly phagocytized and usually do not induce inflammatory reaction (Savill et al., 2000; Kurosaka et al., 2003).

Apoptosis is a complex and sophisticated mechanism involving an energy-dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The NCCD proposed an operational definition of extrinsic apoptosis. In summary, extrinsic apoptosis is a caspase-dependent cell death subroutine, and hence can be suppressed (at least theoretically) by pancaspase chemical inhibitors such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) or by the overexpression of viral inhibitors of caspases like cytokine response modifier A (CrmA). Extrinsic apoptosis is triggered by one of the following lethal signaling cascades: (i) death receptor signaling and activation of the caspase 8 (or 10)-caspase 3 cascade; (ii) death receptor signaling and activation of the caspase 8-tBID-mitochondrial outer membrane permeabilization(MOMP)-caspase 9-caspase 3 pathway; or (iii) ligand deprivation-induced dependence receptor signaling followed by (direct or MOMP-dependent) activation of the caspase 9-caspase 3 cascade (Galluzzi et al., 2012).

Similarly, the NCCD defined the 'intrinsic apoptosis' as a cell death process that is mediated by MOMP and hence is always associated with (i) generalized and irreversible mitochondrial transmembrane potential (DCM) dissipation, (ii) release of mitochondrial intermembrane space (IMS) proteins into the cytosol (and their possible relocalization to other subcellular compartments), as cytochrome C and (iii) respiratory chain inhibition. They also differentiate between caspase-dependent and caspase-independent intrinsic apoptosis based on the extent of cytoprotection conferred by inhibition of caspases (Galluzzi et al., 2012).

Both pathways converge on the same terminal, or execution pathway, initiated by the cleavage of caspase 3 (Galluzzi et al., 2012) and subsequently on other proteases and nucleases that drive the terminal events.

Recent studies have shown that **necrosis** is more than an accidental uncontrolled form of cell death (Fig. 1.4 and Table 1.1). Necrosis is a type of cell death finely regulated by transduction pathways and catabolic mechanisms (Kroemer et al., 2009; Galluzzi et al., 2012). Despite several mediators, organelles and cellular processes have been implicated in necrotic cell death, the cause elements and the effects still unclear and not correlated. Some of these phenomena are summarized in Table 1.1. However, no consensus was found on the biochemical changes that real identify necrosis; therefore necrotic cell death is still mainly identified by the absence of apoptotic or autophagic markers, especially when plasma membrane permeabilization is observed. Furthermore in the necrotic cell death the disruption of the plasma membrane leads to the release of the cytoplasmic contents which chemotactic signals recruit the inflammatory reaction.

Necrosis is also characterized by morphological changes, such as cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured

lysosomes and eventually disruption of the cell membrane (Kerr et al., 1972; Majno et al., 1995; Trump et al., 1997; Elmore, 2007; Kroemer et al., 2009).

Pyknosis and karyorrhexis are shared cytomorphological changes during apoptosis and necrosis (Krysko et al., 2008). Shared features as response to a death stimulus, indicate a "apoptosis-necrosis continuum" (Zeiss, 2003). In fact, dead cells in the late stages of apoptosis may present necrotic features due to the loss of cellular energy and plasma membrane integrity. This process is called "apoptotic necrosis" or "secondary necrosis" (Majno et al., 1995; Zong et al., 2006).

Programmed cell necrosis (as autolysis, oncosis (Majno et al., 1995), pyroptosis (Cookson et al., 2001), necrapoptosis (Lemasters, 1999), and necroptosis (Degterev et al., 2005), etc) is considered an early warning system to recognize and fight events that may comprise the integrity of the organism as a whole, essential for cell homeostasis. This mechanism showed to play a role in several diseases, like CNS disorders associated with excitotoxicity, as seizures, trauma, and possibly neurodegenerative diseases, such as Alzheimer's and Huntington's diseases (Zong et al., 2006). Apoptosis and necrosis as distinct cell death pathways has been recently defined by development of techniques, as RNA interference (RNAi) and chemical inhibitors, indicating that regulated forms of necrotic cell death exist nonetheless programmed cell necrosis may be just as an important cell fate as apoptosis (Zong et al., 2006).

In fact, apoptosis is not a synonym of programmed cell death or caspase activation while necrosis can also occur in a regulated fashion, involving a precise sequence of signals. Therefore, distinguish them demand caution.

4. Replacement therapy in the retina

4.1. Retinal degenerative diseases

The retina is affected by several types of diseases that may affect the outer retina (such as the age-related macular degeneration and retinitis pigmentosa) or the inner retina (such as the glaucoma and the retinopathies).

Age-related macular degeneration (AMD) is a very common outer retinal pathology. It presents genetic and environmental risk factors (Klein et al., 1992; Edwards et al., 2005). The dry-AMD is primary characterized by the degeneration of RPE leading to secondary photoreceptor loss (Klein et al., 2004) while wet-AMD shows choroidal neovascularization.

Retinitis pigmentosa (RP) is the leading cause of inherited retinal blindness in younger patients. Is a group of genetic disorders caused by mutations in over 150 genes discovered until now, causing the progressive photoreceptor or RPE degeneration, it typically progresses until involve the central retina and finally blindness (Hartong et al., 2006).

Glaucoma is one of the most common neurodegenerative diseases in the inner retina and the leading cause of irreversible blindness worldwide. In glaucoma occurs the death of RGC, causing optic nerve degeneration and lack in the connection retina-brain. Visual loss usually starts in the periphery and progresses to the central vision with understandable consequences to the quality of life of these patients. Glaucoma presents several risk factors to its progression. The most common risks are elevated intraocular pressure (AGIS, 2000; Leske, 2007) and age (Leske et al., 2001; Mukesh et al., 2002; Leske, 2007) however genetic background (Wolfs et al., 1998), thinner corneal thickness (Medeiros et al., 2003) and vascular deregulation were also proposed to contribute to this disease (Leske, 2009; Almasieh et al., 2012).

Another example of inner retina degeneration is **ischemic retinopathy**. This group of diseases is characterized by retinal vasculature damage, oxygen and nutrients deprivation and occasionally pathological neovascularization. As common causes, there are: diabetic retinopathy, retinopathy of prematurity and vascular occlusions. In particular, diabetic retinopathy is the leading cause of blindness in working-age adults in developed countries. There are several vascular alterations, like the loss of pericytes and endothelial cells, formation of microaneurysms, basement membrane thickening and blood-retinal barrier breakdown (Cai et al., 2002). Additionally, changes in the neural retina were also reported (Lieth et al., 2000; Barber, 2003; Abu El-Asrar et al., 2007; Barber et al., 2011) leading to loss in contrast sensitivity and color vision and alterations in the electroretinogram (Sokol et al., 1985; Roy et al., 1986).

4.2. Stem cells in the eye

Stem cells are tissue-specific ancestral cells characterized by the ability of both self-renew and differentiate in functional progeny cell types (nonrenewing progenitors and differentiated effector cells). This undifferentiated cells are classified by their development potential as totipotent (able to give rise/differentiate into all embryonic and extraembryonic lineages of the developing embryo, in mammals only the zygote and blastomeres), pluripotent (the unique capacity of a cell to differentiate into all somatic and germ line cells of the developing embryo, as example the embryonic stem cells), multipotent (cells with ability to form multiple cell types of one lineage, like adult stem cells), oligopotent (able to give rise to a more restricted subset of cell lineages than multipotent stem cells), unipotent (cells that generate only one mature cell type, like spermatogonial stem cells (can only give rise to sperm) (Ahmad, 2001; Wagers et al., 2004; Jaenisch et al., 2008; Dejosez et al., 2012). Stem cells present other properties such as asymmetrical division, mitotic quiescence, and regenerative capacity, however these are shared by some but not all stem cells Therefore, neural progenitor cells could be considered stem cells, and thus they self-renew and generate neurons, astrocytes

and oligodendrocytes (cells of the nervous tissue). Neurogenesis is by definition the process by which new nerve cells are generated. In neurogenesis, there is active production of new neurons, astrocytes, glia, and other neural lineages from undifferentiated neural progenitor or stem cells.

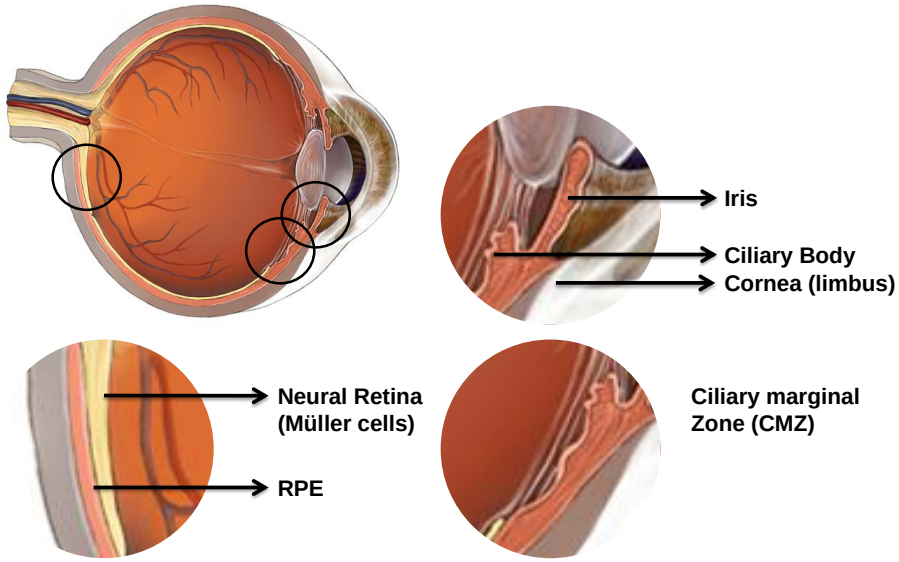


Fig. 1.5 – Localization of stem cell in the eye The eye presents several stem cells niches, such as neural retina, specifically in Müller cells; retinal pigment epithelium (RPE); iris, cornea (limbus) and ciliary marginal zone (CMZ, including the ciliary body). Adapted from http://ctcsherry.deviantart.com/art/Eye-Anatomy-160779859 and (MacNeil et al., 2007).

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 (Straznicky et al., 1971; Johns, 1977; Cepko et al., 1996; Tropepe et al., 2000; Ahmad, 2001; Reh et al., 2001; Coles et al., 2004; Xu et al., 2007; Martinez-Navarrete et al., 2008). 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 (RP), age-related macular degeneration (AMD), diabetic retinopathy and glaucoma (Tropepe et al., 2000; Ahmad, 2001; Ooto et al., 2004; Bernardos et al., 2007; Ohta et al., 2008).

Therefore, the transplantation of neural stem cells, in patients with retinal degenerative diseases, may help to repopulate the damaged retina and/or rescue retinal neurons from future degeneration (vision restoration or reduction of neuronal loss) (Ahmad, 2001).

The adult retina and structures nearby have stem/progenitor cells, which are mainly localized in ciliary body epithelium (Coles et al., 2004; Martinez-Navarrete et al., 2008; Cicero et al., 2009), iris pigment epithelium (MacNeil et al., 2007; Ohta et al., 2008; Locker et al., 2009) and peripheral retina (Fischer et al., 2003; Ooto et al., 2004; Das et al., 2006; Fausett et al., 2006; Raymond et al., 2006; Bernardos et al., 2007; Jadhav et al., 2009; Locker et al., 2009) (Fig. 1.5).

Single pigmented cells from the ciliary body of mammalian retina can clonally proliferate *in vitro* to form sphere colonies of cells that can differentiate into retinal-specific cell types, including rod photoreceptors, bipolar neurons, and Müller glia (Tropepe et al., 2000; Ahmad, 2001). Adult retinal stem cells are localized in the pigmented ciliary margin and not in the central and peripheral retinal pigmented epithelium, indicating that these cells may be homologous to those found in the eye germinal zone of other nonmammalian vertebrates (Straznicky et al., 1971; Johns, 1977; Tropepe et al., 2000; Ahmad, 2001; Reh et al., 2001). The ciliary epithelium in the adult mammalian eye harbors a population of neural stem cells, in a mitotically quiescent state, showing a long-term self-renewal since early postnatal until seventh-decade human eyes (Coles et al., 2004). Another study showed that human and mouse ciliary epithelium-derived spheres are made up of proliferating pigmented ciliary epithelial cells rather than retinal stem cells. It is an example of a differentiated cell that can form clonogenic spheres in culture, self-renew, express progenitor cell markers, and initiate neuronal differentiation (Cicero et al., 2009). Furthermore the iris pigment epithelium of chicken, rodent and porcine eyes display retinal stem/progenitor properties and neurogenic potential (MacNeil et al., 2007; Ohta et al., 2008; Locker et al., 2009).

Another source of progenitor cells in the retina is Müller cells. Some authors propose that Müller glial cells are latent neural stem cells, that after a retinal acute injury,

dedifferentiate to pluripotent retinal progenitor/stem cells with a high restricted capacity to express neuronal and photoreceptor proteins (Bringmann et al., 2012). Muller cells may act as retinal stem cells as described in several studies in different species: teleost fish (Fausett et al., 2006; Raymond et al., 2006), early postnatal chick (Fischer et al., 2003), adult rodents (Ooto et al., 2004; Das et al., 2006) and zebrafish (Bernardos et al., 2007). These cells could eventually be used in the future as cell-based therapeutic approaches in the retina (Jadhav et al., 2009).

Locker and collaborators summarize in a review the localization of stem/progenitor retinal cells, the different types and molecular fingerprints of retinal stem cells identified so far and also detail the current knowledge on molecular cues that influence their self-renewal and proliferation capacity (Locker et al., 2009).

One study described that retinal neurogenesis occurs in vivo in retina of adult monkeys and humans. The authors found cells expressing molecular markers of neural and retinal progenitors in the nonlaminated retinal margin and ciliary body *pars plana* of mature primates. These results extend to primates the idea of neurogenesis aimed at retinal cell turnover throughout life (Martinez-Navarrete et al., 2008).

4.2.1. Stem cell therapy to retinal neurodegeneration

The inefficient differentiation of the immature transplanted cells could originate a lack of functional integration in the retina, therefore the developmental age of the donor cells can be critical for the successful integration of the cells and better methods to improve differentiation should be developed (MacLaren et al., 2006).

The retina, and eye in general, congregate several advantages as a target for cell-based clinical therapies: the retina is an accessible tissue of the CNS; the retina is moderately small with a relatively immune privilege. Together these characteristics reduced the number of cells needed for cell-based therapies, the risk of systemic side effects and the rejection of transplanted cells. Additionally there are technological tools for measuring

ocular structure and function and to evaluate the therapy efficacy (Bull et al., 2011; Singh et al., 2011; Stern et al., 2011). All these characteristics make the retina a point of convergence for translating stem cell research into clinical therapy.

The differentiation of ES cells and RPC in photoreceptors, RPE and ganglion cells (Lamba et al., 2009) or even the use of retinal stem cells (ex: the ciliary body and the Müller glia) or iPS cells are possible strategies

Embryonic stem (ES) cells potential to treat retinal disorders

In the last decade, several groups showed that is easier obtain retinal progenitors cells and photoreceptors from ES cells than from retinal stem cells (iris (Sun et al., 2006) and ciliary tissue (Tropepe et al., 2000)). The potential of the ES cells for infinite proliferation facilitate the acquisition of enough number of cells for research and treatment of several diseases (Hoffman et al., 2005; Osakada et al., 2008).

In 2002, it was showed ES cell-derived neural progenitors express regulatory genes important for progenitors differentiation along the retinal lineage. In the presence of extracellular factors, progenitors give rise to retinal neurons, like photoreceptors expressing specific cell type genes but with no photoreceptors morfology. It was showed that ES cells may be used as an excellent model for understanding mechanisms that regulate specification of retinal neurons and as an unlimited source of progenitors for treating retinal degenerative diseases by cell replacement (Zhao et al., 2002). Later an eye-like structure (including lens, neural retina, and pigmented retina) was efficiently induced from embryonic stem cells (Hirano et al., 2003). Another method to obtain retinal cells from ES cells was stromal cell-derived inducing activity (SDIA) method. Through this technique ES cells were differentiated in RPE cells with normal morphologic and physiological properties but with low efficiency (Kawasaki et al., 2002; Haruta et al., 2004). To improve efficiency, a new approach was developed. Serum-free floating culture of embryoid body-like aggregates (SFEB) was associated with Dkk1, LeftyA,

serum, activin and coculture with embryoinic retinal tissue, in order to generate neural retinal precursors able to differentiate in photoreceptors. Lamba and colleagues confirmed that using embryoid bodies formation together with determined factors, like noggin, Dkk-1 and insulin-like growth factor-1 (IGF-1), 80% of the human ES cells (hES) can give rise to retinal progenitors cells, that integrate in degenerated mouse retina and differentiate in cells expressing photoreceptors markers (Lamba et al., 2006).

In a different approach, Ali and collaborators postulated that rod photoreceptors precursor cells expressing neural retina leucine zipper (Nrl) can incorporate healthy and degenerated mouse retinas, differentiate in rod photoreceptors, induce synaptic connections and contribute to visual function (MacLaren et al., 2006).

Takahashi group faces the absence of inductive signals of differentiation of hES cells in culture (absent of coculture with retinal tissues) using animal-derived substances. The low production and integration of photoreceptors was solve through the supplemented feeder- and serum-free culture with Wnt and Nodal inhibitors and application of retinoic acid and taurine to produce photoreceptors with a higher efficiency. These authors also defended that to induce a more efficient transplantation, RPE and photoreceptors should be applied together (Osakada et al., 2008). To avoid cross-species antigenic contamination in cell replacement therapy, recombinant Dkk1 and Lefty-A were replaced by small chemical molecules (CKI-7 and SB-4315429 respectively) that mimics the same effects. Rho associated kinase inhibitor, Y-27632 was also applied to increase survival of hES cells (Osakada et al., 2009). In parallel, Reh and collaborators showed that an extensive differentiation of hES cells prior to transplantation, do not induce teratomas “in vivo” and produce a sufficient numbers of cells which migrate, differentiate, and survive in the retina giving rise to a small functional restoration of vision to mice without a light response (Lamba et al., 2009).

The potential of Induced pluripotent Stem Cells (iPS cells) in retinal disease

Besides Nuclear Reprogramming illustrate funcional or molecular changes in cells fate, adult cells could be reprogrammable into pluripotent cells by four different methods: somatic cell nuclear transfer, fusion of somatic cells with pluripotent cells, explanting germline cells and expression of a defined set of transcription factors in somatic cells (Jaenisch et al., 2008; Hochedlinger et al., 2009) (Fig. 1.6).

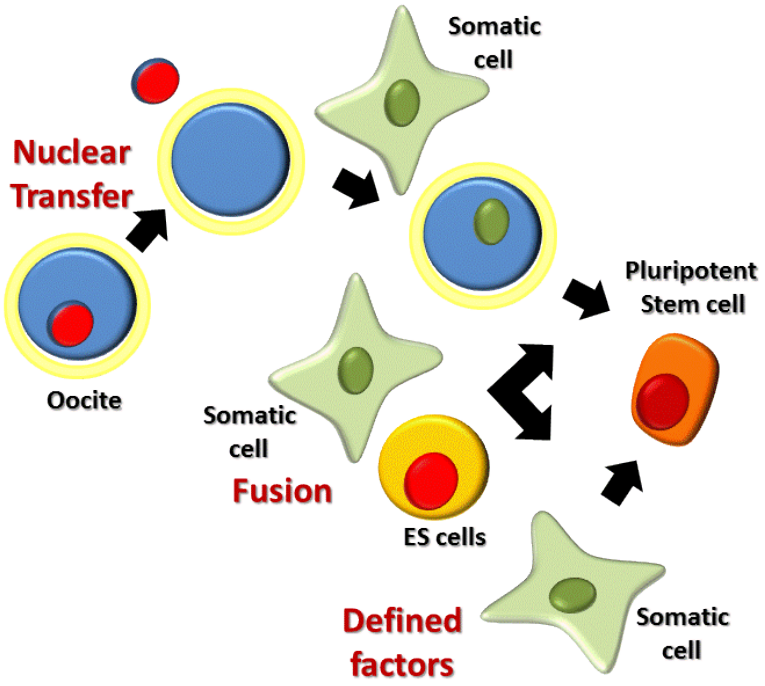


Fig. 1.6 – Scheme of different methods to obtain pluripotent cells from somatic cells. Adapted from (Yamanaka, 2008).

Induced pluripotent stem cells (iPS cells), obtained from somatic cells without nuclear transfer or fusion, were described for the first time in 2006 by Takahashi and Yamanaka. The adult mouse dermal fibroblasts were reprogrammed in iPS cells by using retroviral transduction of the four transcription factors: octamer-4 (Oct4), sex determining region Y-box 2 (Sox2), Kruppel-like factor 4 (Klf4) and c-Myc, combined with genetic selection for Fbx15 expression (Takahashi et al., 2006). Later, other authors using the same four factors, showed that fibroblasts that had reactivated the endogenous Oct4 (Oct4-neo) or Nanog (Nanog-neo) loci grew independently of feeder cells and are epigenetically identical to ES cell (Wernig et al., 2007). Maherali and collaborators (Maherali et al.,

2007) also demonstrated that the ectopic expression of four transcription factors is sufficient to globally reset the epigenetic state of fibroblasts into that of pluripotent cells similar to ES cells. These investigators suggest that retroviral transduction should be replaced by transient expression, of factors. In the same year, Yamanaka group originated iPS cells from adult human dermal fibroblasts with the same four factors. These hiPS cells were similar to hES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes and telomerase activity. They give rise to teratomas with the three germ layers (Takahashi et al., 2007). In order to circumvent the expression of the oncogene c-Myc factor, cause of death, and differentiation of hES cells, Yu and collaborators suggested a new combination of factors lacking this gene: Oct4, Sox2, Nanog, and Lin28. These factors were sufficient to reprogram the human somatic cells in hiPS cells similar to ES cells. However the viral integration reprogramming method and the immune rejection after transplantation still needed to be eliminated, an credible option is the formation of patient-specific iPS cell lines (Yu et al., 2007).

Since then, much other cell types, like blood cells, stomach and liver cells, keratinocytes, melanocytes, pancreatic β cells and neural progenitors have been reprogrammed into iPS cells (reviewed in (Hochedlinger et al., 2009; Stadtfeld et al., 2010a). Moreover, different and more developed protocols, delivery methods and reprogramming factors appear with the goal of decrease viral application and tumorigenic capacity and increase efficiency.

To avoid the several limitations of the viral vectors - secondary reprogramming system of somatic cells - some groups started to place directly doxycycline-inducible polycistronic cassette encoding the four factors c-Myc, Klf4, Oct4 and Sox-2 in the 3’ untranslated region of a specific gene. They were able to form chimeras and then reprogrammable mice which somatic cells are capable adding just doxycycline to the media originate iPS cells (Stadtfeld et al., 2010b). These iPS cells still show all the stringent criteria of pluripotency: form teratomas with the three embryonic germ layers, when injected into

murine blastocysts, contribute to all tissues and form tetraploid blastocyst (Kim et al., 2008; Zhao et al., 2009).

Each cell population has a characteristic epigenetic status correlated with its differentiation potential as Waddington showed in his epigenetic landscape model. More recently adapted by Hochedlinger and Plath (Hochedlinger et al., 2009), iPS cells, as in a pluripotent state, have only active X chromosomes, global expression of differentiation genes by Polycomb proteins and promoter hypomethylation. In a molecular level, during the epigenetic reprogramming of iPS cells, the promoter regions of pluripotency undergo demethylation (SCNT and induced by factors), in female cells, there are the reactivation of the somatically silent X chromosome. iPS cells also present global patterns of histone methylation (Okita et al., 2007; Maherali et al., 2008; Hochedlinger et al., 2009). At functional level completely reprogrammed iPS cells produce viable chimeric mice, contribute to the germline and support the development of embryos become from them (Hanna et al., 2008; Kim et al., 2008; Hirrlinger et al., 2010).

4.2.1.1. Studies with iPS cells in the retina

Since the appearance of iPS cells in 2006, several studies used iPS to generate retinal cells. In the Table 1.2 is summarized the studies and improvements obtain in this field.

Table 1.2 – Studies with retinal iPS cells

Year	Cell of origin	Method of generation of iPS cells	Main findings	References
2012	Human fibroblats	Lentiviral vectors carrying transcription factors Oct4, Sox2, Klf4 and MYC.	Human iPS cells were differentiated in RPE Functional recovery of Rpe65(rd12)/ Rpe65(rd12) mice, a clinically-relevant model of RP after iPS transplantation.	(Li et al., 2012)
	Human fibroblats	hiPS cells induced by transducing Oct3/4, Sox2, Klf4 and c-Myc were differentiated in RPE cells.	Human iPS cells were differentiated in RPE	(Kuroda et al., 2012)
	Human activated T-lymphocytes	Retroviral transduction with several combinations of pluripotency factors.	T-lymphocyte–derived iPScells optic vesicle like-structures (TiPS cells-OVs) self-assemble into rudimentary neuroretinal structures and express markers indicative of chemical and electrical synapses.	(Phillips et al., 2012)

	Human lens epithelial cells (HLECs) from age-related cataract patients.	Oct4, Sox2, and Klf4 lentiviral vectors transduction.	Generate lens progenitor cells from cataract patient HLEC-derived iPS cells.	(Qiu et al., 2012)
2011	Fibroblasts derived from cynomolgus monkey abdominal skin.	Retroviral transduction (retroviruses carrying Oct3/4, Sox2, Klf4, and c-Myc genes).	Monkey iPS cells differentiate into RPE cells.	(Okamoto et al., 2011)
	Adult dsRed mouse dermal fibroblasts.	Retroviral induction of the transcription factors Oct4, Sox2, Klf4 and c-Myc.	Generation of adult fibroblast-derived iPS cells.	(Tucker et al., 2011)
	Fibroblast from five RP patients with distinct mutations in the RP1, RP9, PRPH2 or RHO gene.	Ectopic expression of four key reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* genes).	Patient-derived iPS cells were differentiated into rod photoreceptor cells which recapitulated the disease phenotype in vitro and expressed markers of cellular stresses.	(Jin et al., 2011)
2010	Primary fetal RPE cells.	Lentiviral expression of Oct4, Sox2, LIN28, and Nanog.	RPE cells can be reprogrammed to pluripotency. The iPS cells obtained redifferentiate back into RPE, retaining ‘‘memory’’ of their origin.	(Hu et al., 2010b)
	Mouse tail-tip fibroblasts (TTFs).	Reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* genes).	iPS-derived RG-like cells were obtain from iPS by the addition of Dkk1, Noggin, DAPT and overexpression of *Math5*. Differentiated cells survived but were unable to be integrated into the normal retina after transplantation, however are valuable for regeneration research into retinal degeneration diseases.	(Chen et al., 2010)
	Human fibroblast culture.	Lentiviral vectors expressing Oct4, Nanog, LIN28 and Sox2.	FACS purified iPS cell-derived photoreceptors were able to integrate into a normal mouse retina and express photoreceptor markers.	(Lamba et al., 2010)
2009	Mesenchymal cells derived from human Oct4 knock-in ES.	Lentiviral vector of human Oct4, Sox2, Nanog and Lin28 genes.	iPS-RPE differentiated cells were morphologically similar to RPE, and expressed numerous markers of developing and mature RPE cells and were capable of phagocytosing photoreceptor material, in vitro and in vivo following transplantation.	(Carr et al., 2009)
	Population of pluripotent Xenopus laevis (blastula stage Embryos) cells.	Capped RNAs coding for Pax6, Tbx3, Rx1, Nr2e1, Six3, Six6, Otx2,and/or venus YFP.	The fate of pluripotent cells were altered to generate multipotent retinal progenitor cells, which differentiate into functional eyes (retinal cell classes) and form a neural circuitry sufficient for vision.	(Viczian et al., 2009)
	Human dermal fibroblasts.	Retroviral transduction of Oct3/4, Sox2, Klf4 with or without Myc.	These authors used low molecular- mass compounds to induce differentiation of iPS cells into retinal progenitors - RPE and photoreceptors.	(Osakada et al., 2009)
	Non indicated.	iPS cells were generated using Oct4, Sox2, Nanog, and Lin28.	The iPS cells spontaneously differentiate into RPE cells and could be cultured to form highly differentiated and functional RPE monolayers, which are quantitatively similar to fibroblast-RPE	(Buchholz et al., 2009)
	Human dermal fibroblasts and mouse embryonic fibroblasts.	Retroviral transfection of Oct3/4, Sox2, Klf4, w/or wo/ c-Myc	Mouse and human iPS cells can differentiate into retinal-like cells (retinal progenitor cells, RPE cells and photoreceptors) with defined factors (Wnt, Nodal antagonists, retinoic acid and taurine) in *vitro* .	(Hirami et al., 2009)

Epigenetic memory of iPS cells

In 2010, it was showed that genetically matched iPS cells retain a transient transcriptional and epigenetic memory of their cell of origin at early passage, which affect their potential to differentiate in specific cell type (Kim et al., 2010a; Polo et al., 2010). These differences are lost upon continuous passaging. So, there is a tendency of iPS cell lines to differentiate preferentially into the cell lineage of origin (epigenetic memory of iPS cells). An study suggested that reprogrammed RPE cells retain a memory of their previous state of differentiation (Hu et al., 2010b). That could be exploited in clinical practice to obtain specific cell types. This epigenetic memory of the tissue of origin is more marked in factor-based reprogramming than in nuclear transfer (Kim et al., 2010a). Low–passage factor based reprogramming iPS cells harbor DNA methylation signatures characteristic of their somatic cells of origin, which favor its differentiation along lineages related to the donor cell, restricting alternative fates. The authors also affirm that epigenetic memory could be reset by differentiation and serial reprogramming and also by chromatin-modifying drugs (Kim et al., 2010a).

These epigenetic differences are unlikely to be essential features of iPS cells but rather reflect stochastic variations associated with technical challenges of achieving complete reprogramming (Kim et al., 2010a).

5. Neuropeptide Y in the Retina

Neuropeptides are widely distributed both in the central and peripheral nervous system. 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 it is also present in peripherally innervated tissues of the eye (Duner et al., 1954; Stone et al., 1987b). 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 et al., 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).

5.1. NPY System

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) (Fig. 1.7). 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 et al., 2000). In mouse, the NPY gene is located in

chromosome 6, locus 6 B3; 6 26.0 cM while in rat is localized in chromosome 4, locus 4q24 (Pruitt et al., 2012).

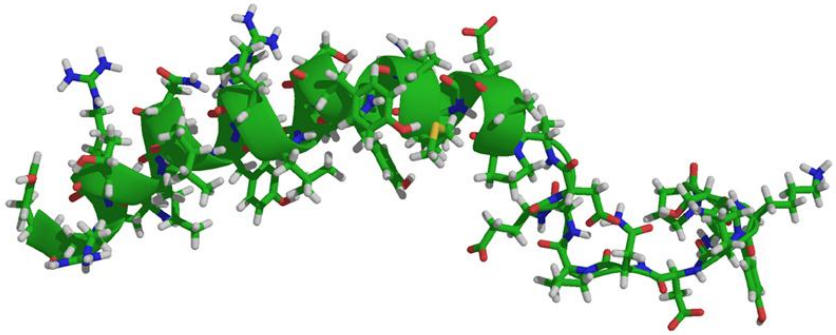


Fig. 1.7 – Scheme of NPY molecule (http://www.npy-pyy-pp.org/).

5.1.1. NPY synthesis, metabolization and localization

The prepro-NPY generated after translation is directed into the endoplasmic reticulum, where a 28 amino acid peptide is removed and Pro-NPY produced (Fig. 1.8). This NPY precursor, Pro-NPY, is a 69 amino acid peptide formed by NPY_{1-39} where the carboxylic group is flanked by a group of 33 amino acids called 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_{1-39} and CPON. Then, a truncation at the C-terminal end by a carboxypeptidase B (CPB) generates NPY_{1-37}, which is a substrate for the enzyme peptidylglycine alpha-amidating monooxygenase (PAM) and leads to the biologically active amidated NPY_{1-36} (NPY) (Medeiros Mdos et al., 1996). NPY can be further cleaved by two enzymes, dipeptidyl peptidase IV (DPP-IV) and aminopeptidase P (AmP) (Medeiros et al., 1994; Medeiros Mdos et al., 1996).

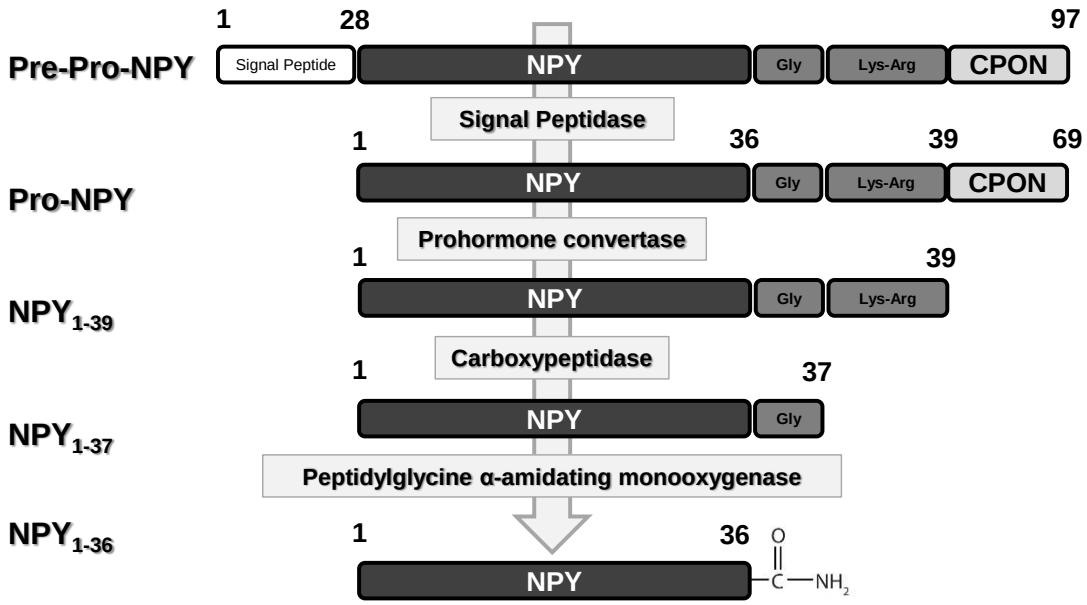


Fig. 1.8 – Scheme of the biosynthesis of NPY. The numbers are referred to base pairs; CPON: C-flanking peptide of NPY. Adapted from (Silva et al., 2002).

NPY is located in nervous system but also in several other systems. The Table 1.3 summarizes the NPY location in human body.

The dipeptidyl peptidase IV (DPPIV) is the peptidase with higher affinity to the active form of NPY. DPPIV is a membrane-bound protease which cleaves proline in the penultimate position (Hopsu-Havu et al., 1966). The connection Pro-Ser is cut and the Tyr-Pro dipeptide is removing from NPY N-terminal giving rise to $NPY_{3–36}$. This fragment lost the affinity for the NPY Y_1 receptor but retains its capacity to bind to the NPY Y_2 and/or Y_5 receptors (Medeiros et al., 1994; Medeiros Mdos et al., 1996). NPY is also metabolize by other aminopeptidases present in the cytoplasm, like dipeptidyl peptidase 8 (DPP-8; DPP-VIII) and dipeptidyl peptidase 9 (DPP-9; DPP-IX). They generate the same metabolite. Aminopeptidase P (AmP) can also hydrolyse NPY in the N-terminal side between the first and second amino acid residues (Yaron et al., 1968) removing the N-terminaltyrosine from NPY giving rise to NPY_{2-36}, a potentially semi-selective NPY Y_2 and Y_5 receptor agonist. The peptides NPY_{3-36} and NPY_{2-36} can be also degraded by endopeptidase neutral-24-11, in cleavage sites Tir^{20}-Tir^{21} and Leu^{30}-Ile^{31}, originating

biological inactive peptide as NPY_{1-20} e NPY_{31-36} (Yaron et al., 1968; Medeiros et al., 1994; Medeiros Mdos et al., 1996).

Table 1.3 – NPY distribution in human tissues and organs. Adapted from (Rosmaninho-Salgado et al., 2007b).

Distribution		References
Central Nervous System	Spinal cord, mesencephalon, metencephalon	Review in (Polak et al., 1984; Silva et al., 2005a)
	Cortex, hypothalamus, basal ganglia, thalamus, septum, striatum, amygdala	(Allen et al., 1983; de Quidt et al., 1986a; Silva et al., 2005a)
	Hippocampus (dentate gyrus)	(Caberlotto et al., 2000)
	Occipital lobe and temporal cortex	(Adrian et al., 1983; Beal et al., 1987; Delalle et al., 1997)
	Pituitary gland	(Grunditz et al., 1984; Jones et al., 1989; Silva et al., 2005a)
Immunologic system	Spleen	(Lundberg et al., 1989; Romano et al., 1991)
	Thymus	(Kranz et al., 1997)
Digestive system	Dental pulp	(Uddman et al. 1984)
	Colon, enteric neurons, pylorus	(Rettenbacher et al., 2001; Cox et al., 2002; Lindestrom et al., 2002; Anitha et al., 2006; Zalecki, 2012)
	Pancreas, Langerhans islets	(Jackerott et al., 1997; Ponery et al., 2000; Adeghate et al., 2001; Lambert et al., 2002)
	Liver	(Esteban et al., 2001)
Cardiovascular system	Heart and endothelial cells	(Ahmed et al., 1997; Jackerott et al., 1997; Zukowska-Grojec et al., 1998; Jacques et al., 2003; Silva et al., 2005a)
Endocrine system	Thyroid gland	(Grunditz et al., 1984)
	Adrenal glands	(de Quidt et al., 1986b; Pelto-Huikko, 1989; Fernandez-Vivero et al., 1993; Cavadas et al., 2001)
Reproductive system	Ovary and corpus luteum	(Keator et al., 2010)
Skin and sensory systems	Corti organ	(Gomide et al., 2009)
	Sebaceous and lacrimal glands	(Ebara et al., 1992; Kirch et al., 1996; Seifert et al., 1996)
	Nasal mucosa	(Zhao et al., 1998; Knipping et al., 2003)

5.2. NPY receptors

The 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 can be organized into three subfamilies (Larhammar et al., 2004): the Y_1 subfamily containing of subtypes Y_1, Y_4, Y_6 and Y_8; the Y_2 subfamily including the subtypes Y_2 and Y_7; and the Y_5 subtype, alone in it subfamily.

The seven receptors came from the same ancestral Y receptor and some subtypes were lost during the evolutionary lineages (Fig. 1.9). Therefore, there are 4–7 members of the NPY receptors family all over the diversity of jawed vertebrates (Sundstrom et al., 2012). All of these receptors are present in a cartilaginous fish the elephant shark (*Callorhincus millii*) (Larsson et al., 2009). Humans comprise the receptor subtypes NPY Y_1, Y_2, "*Y_3"*, Y_4, Y_5 and y_6 (Silva et al., 2005a; Xapelli et al., 2008).

Generally, NPY receptors use similar signal transduction pathways, acting via pertussis toxin-sensitive G-proteins, i.e., and 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 (Olasmaa et al., 1986; Michel, 1991). 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^{2+} or K^+ channels conductance (Gammon et al., 1990; Millar et al., 1991; Michel et al., 1995; Xiong et al., 1995). Moreover, there are also evidences suggesting that NPY may be associated to the activation of phospholipase A2 (Martin et al., 1989), MAPK (Keffel et al., 1999; Alvaro et al., 2008a; Rosmaninho-Salgado et al., 2009; Thiriet et al., 2011), PKC (Rosmaninho-Salgado et al., 2007a; Chen et al., 2008; Pons et al., 2008; Rosmaninho-Salgado et al., 2009), PI3K (Zhou et al., 2008), guanylyl cyclase (Rosmaninho-Salgado et al., 2007a), NO synthesis (Hodges et al., 2009; Rosmaninho-Salgado et al., 2009; Ferreira et al., 2010), or even with the inhibition of protein kinase A (PKA) (Pons et al., 2008; Rosmaninho-Salgado et al., 2009).

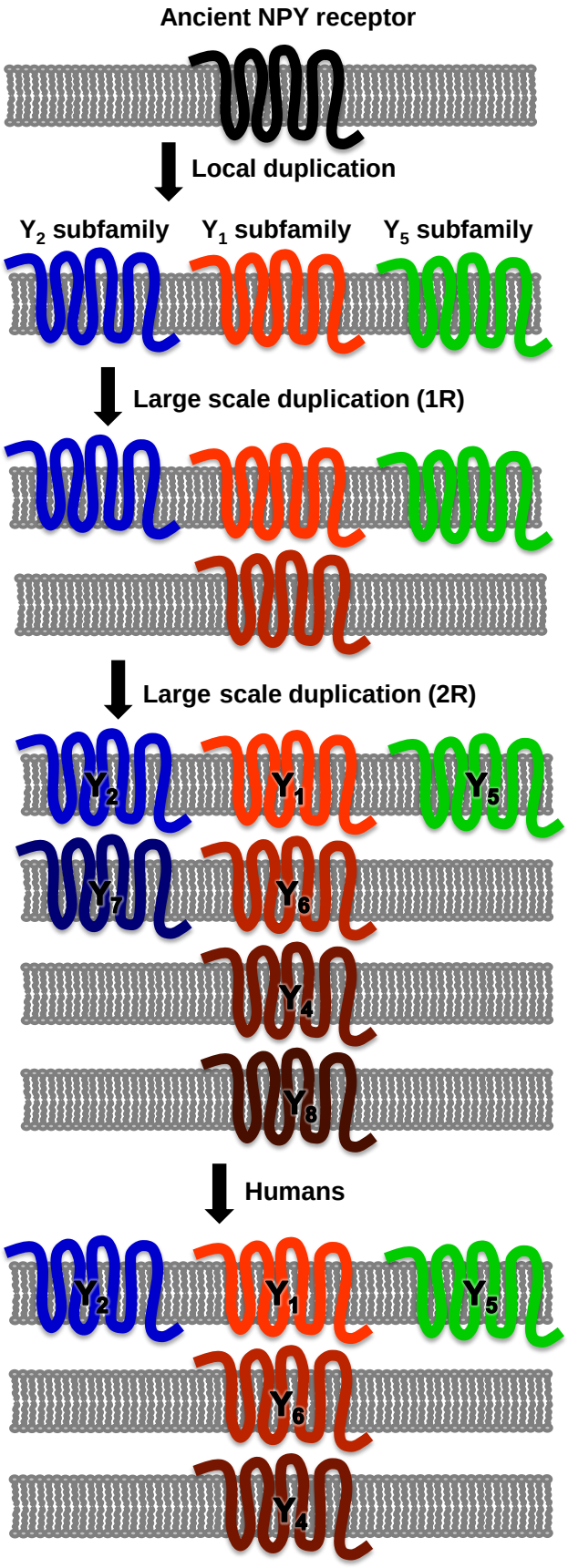


Fig. 1.9 - Proposed evolutionary history of the NPY receptor family by local and large-scale duplication events followed by loss of some receptors in humans. Adapted from (Larhammar et al., 2004; Larsson et al., 2008)

NPY Y_1 receptor was initially considered as post-synaptic. Only the NPY N-terminal is essential for the activation of Y_1 receptors since the NPY analogs, NPY_{2-36}, NPY_{3-36} and NPY_{13-36}, cleaved in this terminal, present a low affinity to this receptor. The carboxylic terminal had no effect on this receptor affinity (Silva et al., 2002). The NPY Y_1 receptor is richly expressed in the CNS and in blood vessels. It induces vasoconstriction and proliferation in several types of cells. Smooth muscle cells (Zukowska-Grojec et al., 1998), olfactory epithelium (Hansel et al., 2001), progenitor cell of the hippocampus (Howell et al., 2003; Howell et al., 2005; Howell et al., 2007), pancreatic cells (Cho et al., 2004), Müller cells (Milenkovic et al., 2004) and cancer cells (Korner et al., 2004) are some examples. The Y_1 receptor activation decreases anxiety and depression (Sajdyk et al., 1999; Redrobe et al., 2002), increases appetite (Corp et al., 2001; Lecklin et al., 2003) and alcoholic consumption (Kelley et al., 2001; Thiele et al., 2002). It also modulates pulpal inflammation (Rethnam et al., 2010) and the antagonism of this receptor increases bone mass (Baldock et al., 2007; Sousa et al., 2012).

NPY Y_2 receptor is considered a pre-synaptic receptor with high affinity to NPY and PYY as well as to NPY analogs cleaved in N-terminal, like NPY_{2-36}, NPY_{3-36} e NPY_{13-36} and in opposition of Y_1R (Michel et al., 1998). The activation of Y_2 inhibits the neurotransmitter release, regulates appetite (Naveilhan et al., 1999; Batterham et al., 2002; Sainsbury et al., 2002), is involved in neuronal excitability in epilepsy (El Bahh et al., 2002; Herzog, 2002; Vezzani et al., 2004), in angiogenesis (Zukowska-Grojec et al., 1998; A. Jonas Ekstrand, 2003; Lee et al., 2003) and presents a putative neuroprotective effect in Parkinson disease (Decressac et al., 2012).

Pharmacologically, the **NPY Y_4 receptor** offers higher affinity to PP than to PYY, and even smaller or inexistent to NPY (Lundell et al., 1995). Human Y_4R mRNA was found in prostate, colon, pancreas, small intestine, smooth muscle cells and brain, like hypothalamus. (Barrios et al., 1999; Berglund et al., 2003a; Misra et al., 2004). PP

through this receptor is able to inhibit exocrine release from pancreas, induce the relaxation of biliary vesicle and stimulate the release of luteinizing hormone (Horvath et al., 2001; Montet et al., 2005; Andersen, 2007).

The **NPY Y_5 receptor** is activated by NPY, PYY, [Leu^{31},Pro^{34}]-NPY, PYY analogs and fragmented peptides, as NPY_{3-36} e PYY_{3-36} (Gerald et al., 1996; Hu et al., 1996; Borowsky et al., 1998; Michel et al., 1998). This receptor is localized, centrally, in the hypothalamus, playing an essential role in appetite stimulation (Hwa et al., 1999; Cabrele et al., 2000; Lecklin et al., 2003; Beck, 2006) and peripherally, in human and murine adrenal glands (Cavadas et al., 2001; Cavadas et al., 2006).

The **NPY y_6 receptor** was already cloned, however its physiological functions still to unravel, therefore the International Union of Pharmacology (IUPHAR) receptor nomenclature committee has recommended that the mammalian receptor should be written with a small y, y_6 (Bromee et al., 2006). Some reports show the presence of this receptor in some mammals, like mouse, rabbit dog, cow and primates, including humans, but absent in rats (Burkhoff et al., 1998). The y_6 mRNA is located in hypothalamus, hippocampus, small intestine and adrenal glands of rabbits. It can also be found in heart, skeletal muscle and hypothalamus of humans (Gregor et al., 1996; Matsumoto et al., 1996; Weinberg et al., 1996). However the pharmacological properties of y6 are divergent, some authors defend that it is functional in mouse and rabbit and nonfunctional in human and other primates, as well as in guinea-pig and pig; result of frameshift mutations (Gregor et al., 1996; Matsumoto et al., 1996; Weinberg et al., 1996; Mullins et al., 2000; Starback et al., 2000) became a pseudogene in some mammals (Bromee et al., 2006). The NPY y_6 receptor is also present in chicken, amphibians and bony fishes and the y_6 gene appears to be functional in the shark, *Squalus acanthias* (Salaneck et al., 2003).

The **NPY Y_7 receptor** was more recently discovered in non-mammalian jawed vertebrates. Chicken, fishes, like zebrafish *Danio rerio*, rainbow trout *Oncorhynchus mykiss*, as well as amphibians, like two species of frogs (*Xenopus tropicalis* and the marsh frog *Rana ridibunda*) are examples of some species that no not have lost this receptor during evolution (Fredriksson et al., 2004; Bromee et al., 2006; Larsson et al., 2006; Larsson et al., 2009). In opposition mammalian do not present this receptor (Larhammar et al., 2004).

The **NPY Y_8 receptor** is present in fishes and frogs, like elephant shark, *Callorhinchus milii* and *Xenopus tropicalis*, respectively (Larsson et al., 2009). The Y_8 gene has been lost in the lineage leading to mammals. The Y_8 receptor is also missing in all amniote genomes; however is in duplicate (Y_8a and Y_8b) in the teleost fishes *Tetraodon nigroviridis* and *Takifugu rubripes*. In *T. rubripes*, Y_8 receptor is expressed in brain and in peripheral organs (Larsson et al., 2008).

5.3. NPY system in the retina

5.3.1. NPY in retinal development

Animals present different patterns of retina development and several studies indicate that NPY appears early during retinal development playing a role on it. Chicken amacrine NPY-IR cells appear at embryonic day 13, increase between day 13-15, but decrease after hatching (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 somas and just before metamorphosis. In the adult retina of this frog, NPY-IR is present in wide field of amacrine cells in the INL and GCL (Hiscock et al., 1990). In the retina of blue acara

(*Aequidens pulcher*), NPY-IR amacrine cells appear in IPL around hatching, at day 3-4 (Negishi et al., 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 et al., 1995).

Regarding the developing human retina, NPY-IR amacrine cells are found around 14 weeks of gestation (Jotwani et al., 1994). Other study indicates the presence of round and pear-shaped NPY-IR cells in INL at 15 weeks of gestation while NPY positive-ganglion cells were only observed at 17 weeks of gestation. These cells are located in INL and GCL at 28 weeks of gestation. Later, by 38-40 weeks of gestation, NPY-IR cells are present in INL, GCL and fibrous configuration in 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. Later at eye opening (P13) NPY-IR largely 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 developing retina circuitry (Ferriero et al., 1989).

5.3.2. Localization of NPY and NPY receptors in the retina

The presence of NPY in the retina was first described in guinea pig in 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 1.4.

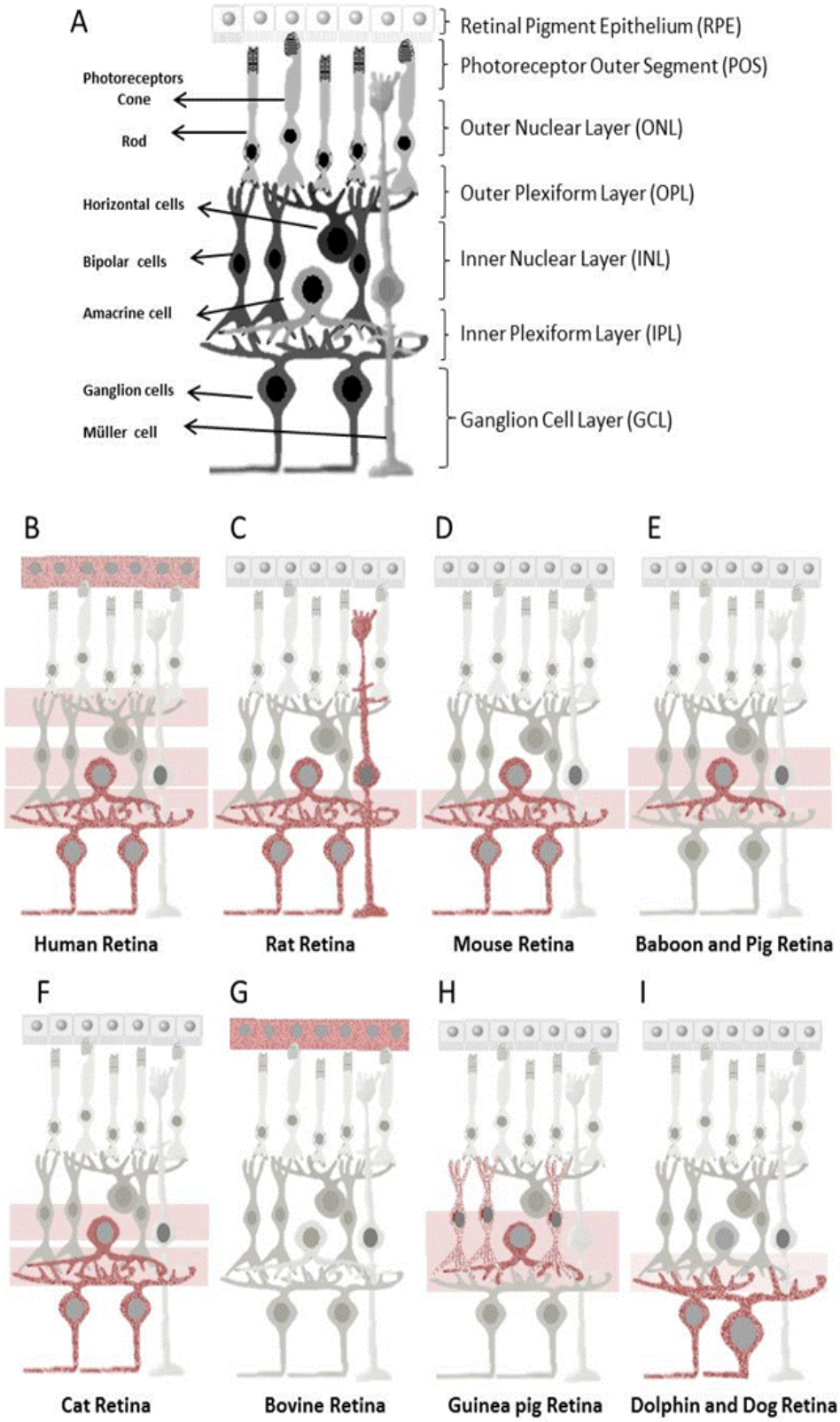


Fig. 1.10 – Localization of NPY-IR in different mammalian retinas. (A) Scheme of the retinal structure organized in layers; (B) In the human retina, NPY-IR is present in amacrine cells distributed by the innermost part of INL, in the middle of IPL and GCL. NPY-IR fibers are found between INL and OPL and also crossing the INL. NPY-IR is also found in RPE;(C) In rat retina, NPY-IR is present in amacrine cells in INL and GCL, as well as in macroglial and microglial cells in cultured rat retinal cells; (D) In mouse retina,

NPY-IR is found in amacrine cells in INL and GCL, and IPL; (E) In baboon and pig retina, NPY-IR is localized in amacrine cells in INL and IPL; (F) In cat retina, NPY-IR is found in amacrine cells in INL, in processes in IPL and in gamma-type retinal ganglion cells. (G) In bovine retina, NPY-IR is present in RPE cells; (H) In guinea-pig retina, NPY–IR fibers form a single layer at IPL, and NPY-IR cell bodies are in the innermost part of INL. NPY-IR is also present in some bipolar cells; (I) 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-IR.

Table 1.4 - NPY-IR localization in the retina of several non-mammalian species

		Species	NPY-IR localization in the retina	References
Non Mammals	Fishes	**Blue acara (*Aequidens pulcher*)**	amacrine cells and IPL	(Negishi et al., 1995)
		Carp	cell bodies of amacrine cells in INL and processes in the IPL	(Bruun et al., 1986)
		Gilthead seabream (*Sparus aurata L.)*	amacrine cells	(Pirone et al., 2008)
		Goldfish	cell bodies of amacrine in INL and cell processes in two layers in IPL	(Osborne et al., 1985; Bruun et al., 1986) (Muske et al., 1987)
		Killifish (*Fundulus heteroclitus*)	amacrine cell fibers in IPL	(Subhedar et al., 1996)
		Lamprey (*Lampreta fluviatilis*)	pyriform subclass of amacrine cells	(Negishi et al., 1986; Rawitch et al., 1992)
		Skates (*Raja clavata, Raja radiate and Raja oscellata*)	amacrine cells in the innermost part of INL and fibers in IPL	(Bruun et al., 1985)
		Squid	no NPY-IR	(Osborne et al., 1986)
		Trout	cell bodies of amacrine cells in INL and processes in IPL	(Bruun et al., 1986)
		Zebrafish	amacrine cells	(Mathieu et al., 2002)
	Amphibious	**Frog (*Bufo marinus and Xenopus laevis*)**	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.	(Osborne et al., 1985; Bruun et al., 1986; Hiscock et al., 1989; Hiscock et al., 1990; Zhu et al., 1995; Zhu et al., 1996)
	Reptiles	**Lizards (*Pogona vitticeps and Varanus gouldii*)**	amacrine cells: type A and type B in INL and displaced at GCL	(Straznicky et al., 1994)
		Turtle	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	(Isayama et al., 1988a; Isayama et al., 1988b; Wetzel et al., 1997)
	Birds	**Chicken**	cell bodies of amacrine cells in the middle and innermost INL and processes in the IPL	(Bruun et al., 1986)
		Pigeon	cell bodies of amacrine cells in INL and processes in the IPL	(Bruun et al., 1986; Verstappen et al., 1986)

In rat and mouse retinas, NPY-IR is present in the inner retina being localized in cell bodies in INL and GCL, and also in processes located in the IPL (Fig. 1.10) (Sinclair et

al., 2001; Oh et al., 2002). The NPY-IR in rodent retina is found mainly in amacrine cells and displaced amacrine cells with overlapping processes that ramify in the IPL as in baboon, pig, cat, chicken and pigeon among other species (Bruun et al., 1986; Sinclair et al., 2001; Oh et al., 2002). In cat and human retinas, the NPY-IR is localized in amacrine cells at INL and ganglion cells in GCL (Straznicky et al., 1989; Hutsler et al., 1994; Jen et al., 1994; Hutsler et al., 1995). NPY-IR is also present in bovine and human retinal pigment epithelium (RPE) (Ammar et al., 1998). Small amounts of NPY-IR are found in rabbit retina (Osborne et al., 1985). Furthermore, in rat, mouse and guinea pig retina all NPY-immunoreactive amacrine cells are also GABAergic, containing GABA or the GABA synthesizing enzyme glutamic acid descarboxylase 65 (GAD65) or even GAT-1, a vesicular GABA transporter (VGAT) (Kang et al., 2001; Sinclair et al., 2001; Oh et al., 2002). Other 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-IR (Chen et al, 1999)

Regarding non-mammalian retinas, in trout, carp and goldfish 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 (Osborne et al., 1985; Bruun et al., 1986). 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 killifish (*Fundulus heteroclitus*) retina NPY-IR is located in some amacrine cell fibers in the IPL showing a well-developed pattern (Subhedar et al., 1996). 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 (*Lampreta japonica*) NPY-IR is weakly positive in a subclass of pyriform amacrine cells which processes are located in sublamina a (Negishi et al., 1986). No NPY-IR is found in squid retina (Osborne et al., 1986).

Amphibious retina, like frogs (ex. *Bufo marinus* and *Xenopus laevis*), has the highest

concentration of NPY-IR levels among other species being characterized by seasonal variations (Hiscock et al., 1989). NPY-IR is located in a small population of amacrine cell bodies in INL co-localizing with GABA (Osborne et al., 1985; Bruun et al., 1986; Hiscock et al., 1990; Zhu et al., 1996), in bipolar-like cell bodies sparsely in the middle of INL, in GCL with ovoid shape, in Müller cells within the INL (Zhu et al., 1996) and in IPL processes (Bruun et al., 1986).

In reptiles, like lizards (*Pogona vitticeps*a nd *Varanus gouldii*), NPY-IR is present in the retina in two classes of amacrine cells: type A (large somata) and type B (small somata) located in INL and, occasionally, in cell somata displaced at GCL (Straznicky et al., 1994). Conversely, in turtles the retina presents NPY-IR in bipolar cells and three types of amacrine cells, evenly distributed in the retina: type A, with large somata located at INL, IPL and occasional processes at GCL; type B, with smaller somata at INL and IPL; type C, amacrine cells located at the periphery of retina (Isayama et al., 1988a). NPY-IR is also located in cytoplasm and within large vesicles of amacrine and bipolar cells in turtle retina (Isayama et al., 1988b; Wetzel et al., 1997).

We have also shown that NPY is present not only in retinal neurons, but also in retinal glial cells. The NPY-IR is present in rat macroglial (Müller cells) and microglial cells (Alvaro et al., 2007).

Regarding NPY receptors, there are only a few studies showing the presence and localization of these receptors in the retina (Table 1.5). In rat retina, we and others detected the presence of mRNAs for NPY Y_1, Y_2, Y_4 and Y_5 receptors (D'Angelo et al., 2004; Alvaro et al., 2007). The NPY Y_1 receptor-IR was found to be localized in horizontal and amacrine cell bodies and processes (D'Angelo et al., 2002). In mouse retina, NPY Y_1 and Y_2 mRNAs were also detected (Sinclair et al., 2001; Yoon et al., 2002). In primary cultures of Müller cells from guinea-pig retina, NPY has a biphasic effect on Müller cell proliferation through NPY Y_1 receptor activation (Milenkovic et al., 2004). In human RPE, NPY Y_1, Y_2 and Y_5 receptors mRNAs were detected, while in the bovine RPE only the NPY Y_1 and Y_2 receptors were detected (Ammar et al., 1998).

Table 1.5 – Localization of NPY receptors in mammalian retinas

NPY Receptors	Mammal Species	Localization and/or function of NPY receptors in mammalian retina	References
NPY Y_1	Mouse	NPY Y_1 mRNA expression increases in room air reared animals	(Yoon et al., 2002)
	Rat	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; Receptor activation inhibits the increase in $[Ca^{2+}]_i$ in rat retinal neurons; Stimulation of retinal cell proliferation.	(D'Angelo et al., 2002; Alvaro et al., 2007; Alvaro et al., 2008a; Alvaro et al., 2009)
	Guinea Pig	NPY has a biphasic effect on Muller cells proliferation through NPY Y_1 receptor activation.	(Milenkovic et al., 2004)
	Bovine	mRNA in cultured RPE cells.	(Ammar et al., 1998)
	Human	mRNA in RPE; Immunoreactivity in Müller cells in retina with proliferative vitreoretinopathy.	(Ammar et al., 1998; Canto Soler et al., 2002)
NPY Y_2	Mouse	NPY Y_2 mRNA expression increase in post-natal mice in oxygen-reared animals.	(Yoon et al., 2002)
	Rat	mRNA in intact retinas and cultured rat retinal cells; Inhibition of voltage-dependent Ca^{2+} influx into rod bipolar cell terminals; NPY Y_2 receptor antisense oligonucleotide prevents hyperoxia-induced retinal neovascularization; Stimulation of retinal cell proliferation.	(D'Angelo et al., 2002; D'Angelo et al., 2004; Koulu et al., 2004; Alvaro et al., 2007; Alvaro et al., 2008a)
	Bovine	mRNA in RPE and cultured RPE cells; NPY signaling in cultured bovine RPE occurs mainly through NPY Y_2 receptor.	(Ammar et al., 1998)
	Human	mRNA in RPE.	(Ammar et al., 1998)
NPY Y_4	Rat	mRNA in rat retina and cultured rat retinal cells; Receptor activation inhibits the increase in $[Ca^{2+}]_i$ in rat retinal neurons.	(D'Angelo et al., 2002; Alvaro et al., 2007; Alvaro et al., 2009)
NPY Y_5	Rat	mRNA in rat retina and cultured rat retinal cells; Receptor activation inhibits the increase in $[Ca^{2+}]_i$ in rat retinal neurons; Stimulates retinal cell proliferation.	(D'Angelo et al., 2002; Alvaro et al., 2007; Alvaro et al., 2008a; Alvaro et al., 2009)
	Human	mRNA in RPE.	(Ammar et al., 1998)

5.3.3. The role of NPY in cell proliferation and differentiation in the retina

In vitro and *in vivo* studies suggest that NPY has pro-neurogenic properties in CNS such as in the olfactory epithelium, subventricular zone (SVZ) and subgranular zone (SGZ) of dentate gyrus (Hansel et al., 2001; Howell et al., 2005; Howell et al., 2007; Agasse et al., 2008; Rodrigo et al., 2010; Decressac et al., 2011). Moreover, in CNS, NPY also 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 olfatory bulb and also increases the number of cells in rostral migratory stream, olfactory blub and striatum (Decressac et al., 2009). These NPY effects on neural cell proliferation and differentiation are mediated by the NPY Y_1-receptor activation (Hansel et al., 2001; Howell et al., 2003; Agasse et al., 2008; Stanic et al., 2008; Rodrigo et al., 2010; Decressac et al., 2011). The involvement of Y_2 receptor in these NPY effects is controversial (Stanic et al., 2008; Decressac et al., 2011). Moreover, the neurogenic effect of NPY requires ERK1/2 activation (Hansel, 2001; Howell et al., 2005; Agasse et al., 2008) 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 at an equal level or even greater than other trophic/growth factors such as ciliary neurotrophic factor, vascular endothelial growth factor, and transforming growth factor (Jin et al., 2002; Emsley et al., 2003; Decressac et al., 2011).

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 (Kishi et al., 1996). However, this proliferative effect on Müller cells is biphasic: at lower concentrations (0.1 ng/mL and 1 ng/mL) NPY decreases cell proliferation rate, while at higher concentration (100 ng/mL) increases Müller cell proliferation (Kishi et al., 1996).

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 (Straznicky et al., 1971; Johns, 1977; Cepko et al., 1996; Tropepe et al., 2000; Ahmad, 2001; Reh et al., 2001; Coles et al., 2004; Xu et al., 2007; Martinez-Navarrete et al., 2008). 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 (RP), age-related macular degeneration (AMD), diabetic retinopathy and glaucoma (Tropepe et al., 2000; Ahmad, 2001; Ooto et al., 2004; Bernardos et al., 2007; Ohta et al., 2008).

Therefore, the transplantation of neural stem cells, in patients with retinal degenerative diseases, may help to repopulate the damage retina and/or rescue retinal neurons from future degeneration (vision restoration or reduction of neuronal loss) (Ahmad, 2001). The adult retina and structures nearby have stem/progenitor cells, which are mainly localized in ciliary body epithelium (Coles et al., 2004; Martinez-Navarrete et al., 2008; Cicero et al., 2009), iris pigment epithelium (MacNeil et al., 2007; Ohta et al., 2008; Locker et al., 2009) and peripheral retina (Fischer et al., 2003; Ooto et al., 2004; Das et al., 2006; Fausett et al., 2006; Raymond et al., 2006; Bernardos et al., 2007; Jadhav et al., 2009; Locker et al., 2009).

Some studies suggest that NPY might have an important role on progenitor cells proliferation and/or differentiation in the nervous tissue (Thiriet et al., 2011; Baptista et al., 2012; Doyle et al., 2012). In cultured rat retinal cells, we showed that NPY stimulates the proliferation of neuronal progenitor cells (BrdU+/nestin+ cells), which means that NPY promotes the proliferation of committed neural immature cells, being this effect mediated by the activation of the NOS–sGC and ERK 1/2 signaling pathways (Alvaro et al., 2008a). Additionally, NPY, through Y_1 and Y_5 receptors activation, has the potential of maintaining human embryonic stem (hES) cells self-renewal and pluripotency (Son et al., 2011). NPY signaling can be useful to the development of defined and xeno-free culture conditions for the large-scale propagation of undifferentiated hES cells (Son et al., 2011). Thus, NPY system is a putative target to develop new strategies to increase retinal progenitor cells proliferation.

5.3.4. Modulatory effects of NPY in the retina

It is known that 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., 2005a). In different brain areas, NPY modulates the release of several neurotransmitters (Silva et al., 2005a), inhibiting the release of glutamate, aspartate, growth hormone, epinephrine and acetylcholine (Gu et al., 1983; Potter, 1987; Rettori et al., 1990b; Bleakman et al., 1992; Greber et al., 1994; Martire et al., 1995; Tsuda et al., 1995; Bitran et al., 1999; Silva et al., 2001; Rodi et al., 2003; Silva et al., 2003b; Hastings et al., 2004; Schwertfeger et al., 2004), and enhancing the release of somatostatin and dopamine and the production of nitric oxide (Rettori et al., 1990a; Ault et al., 1999; Bitran et al., 1999).

NPY also modulates the intracellular calcium concentration ($[Ca^{2+}]_i$) in the rat retina. NPY inhibits the depolarization-evoked Ca^{2+} influx into rod bipolar cells through the activation of NPY Y_2 receptors (D'Angelo et al., 2004). We also showed that NPY 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).

Moreover, others have shown that NPY may also modulate the spatial tuning. NPY-amacrine cells are involved in tuning ganglion cells to low spatial frequencies/large spatial patterns. The ablation of these cells causes alteration of size of the receptive field surround of retinal ganglion cells (Sinclair et al., 2004).

In the retina, the presence of NPY in amacrine cells suggests that this peptide may also have a role as a neuromodulator in the retina (D'Angelo et al., 2002; Oh et al., 2002). Actually, 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. This NPY neurotransmitter modulation requires energy (dependent on the activity of NA^+K^+-ATPase and calcium) (Bruun et al., 1993).

NPY also partially inhibited the KCl-evoked [^{3}H]-D-aspartate and [^{3}H]-GABA release in retinal cell cultures (Fig. 1.11). However, the NPY receptors mediating the effects of NPY on transmitter release are not yet known.

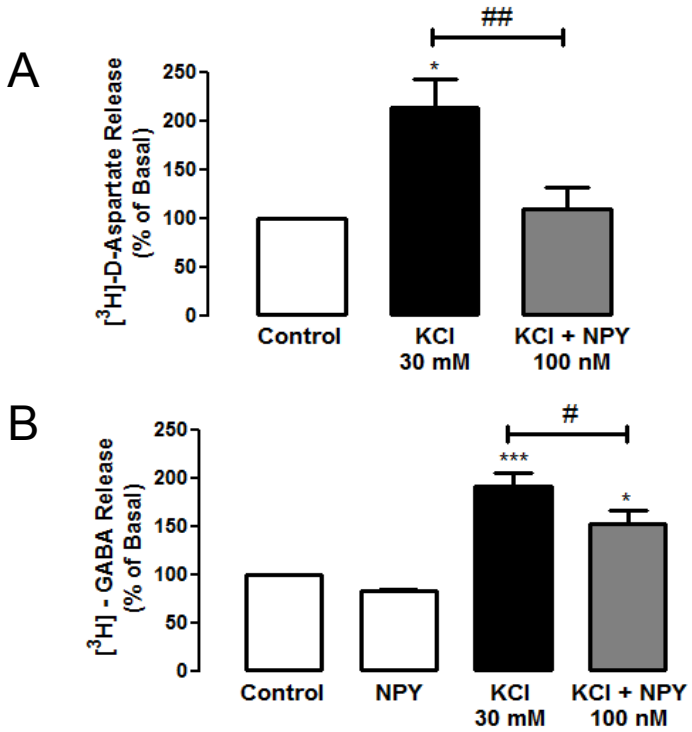


Fig. 1.11 – NPY decreases KCl-evoked [^{3}H]-D-aspartate and [^{3}H]-GABA release in retinal cell cultures. A – Cultured retinal neural cells were incubated with 40 nM D-aspartate and [^{3}H]-D-aspartate (1 mCi/ml) for 45 min, at 37°C. After this period, the cells were (or were not) pre-exposed to NPY (100 nM) for 10 min and then stimulated with 30 mM KCl for 1 min with or without NPY 100 nM present. The results represent the percentage of [^{3}H]-D-aspartate released relatively to the radioactivity of the control, and are expressed as mean±S.E.M., of at least three independent experiments. *p<0.05, significantly different of Control; $^{\#\#}$p<0.01, significantly different of KCl 30 mM; one-way ANOVA followed by Bonferroni's post-hoc test. B – Cultured retinal neural cells were incubated with 40 nM GABA and [^{3}H]-GABA (1 mCi/ml) for 45 min, at 37°C. After this period, cells were (or were not) pre-exposed to NPY (100 nM) for 10 min and then stimulated with 30 mM KCl for 1 min, with or without NPY 100 nM present. The results represent the percentage of [^{3}H]-GABA released relatively to the radioactivity of the control, and they are expressed as mean±S.E.M., of at least three independent experiments. ***p<0.001, *p<0.05, significantly different of Control; #p<0.05, significantly different of KCl 30 mM; one-way ANOVA followed by Bonferroni's post-hoc test.

In conclusion, retinal physiology is strictly dependent on adequate neurotransmission between different retinal neurons (photoreceptors, and bipolar, ganglion, horizontal and amacrine cells), which in turn depends on $[Ca^{2+}]_i$ regulation. In addition, visual processing depends on fine-tuning of neurotransmission that may result from the action

of neuromodulators, such as NPY. Thus, although additional studies are necessary to unravel the role(s) of NPY in retinal physiology the evidences mentioned above indicate that NPY can exert important neuromodulatory effects in retinal cells.

5.3.5. Neuroprotective effect of NPY in retinal cells

Neuroprotection is an important strategy to prevent cell death occurring in neurological disorders. The neuroprotective effects of NPY against excitotoxicity are well known in different brain regions, but also in the retina (Silva et al., 2003b; Silva et al., 2005a; Xapelli et al., 2006; Xapelli et al., 2007; Alvaro et al., 2008b; Alvaro et al., 2009; Smialowska et al., 2009). In rat and mouse organotypic hippocampal cultures, NPY is able to reduce cell death induced by glutamate receptor agonists, through NPY Y_1, Y_2 and/or Y_5 receptors activation (Silva et al., 2003b; Xapelli et al., 2007; Smialowska et al., 2009). Furthermore, NPY also exerts 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 is not yet clarified (Alvaro et al., 2008b). As mentioned above, we showed that NPY inhibits the increase in $[Ca^{2+}]_i$ in rat retinal neurons, through the activation of NPY Y_1, Y_4, and Y_5 receptors subtypes. Since sustained elevated cytosolic $[Ca^{2+}]_i$ levels have been linked to cell death, this inhibitory effect of NPY may also contribute to the neuroprotective effect of NPY in these cells (Alvaro et al., 2009). 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.

5.3.6. NPY involvement in retinal pathologies

A genetic study in a Finnish population has shown that a polymorphism in the NPY gene is associated with increased predisposition to develop diabetic retinopathy in Type 2

diabetic patients (Niskanen et al., 2000). This polymorphism refers to a leucine to proline substitution in codon 7 of the NPY gene (Leu7Pro). Despite the NPY receptor involved in this pathology is not completely established, Koulu and collaborators suggest that NPY Y_2 receptor may contribute for the progression of diabetic retinopathy, namely in neovascularization processes occurring in the late stages of the disease (Koulu et al., 2004). Previously another study implicated NPY and its Y_2 receptor in angiogenesis. Other results confirmed later that Y_2 receptor may be associated with angiogenesis and vasoconstriction in a mouse model of oxygen-induced retinopathy (Yoon et al., 2002).

6. Main Objectives

Nowadays the knowledge about the role of NPY in the retina is still scarce. Although there are some studies regarding the presence of NPY in the retina, just few studies adressed the function of this peptide and its receptors in the retina. Our group has shown that cultured rat retinal neural cells express NPY and NPY Y_1, Y_2, Y_4 and Y_5 receptors (Alvaro *et al*., 2007). However the specific localization of these receptors kept unknown. Additionally we also have shown that NPY has a protective role against MDMA-induced toxicity in the retina (Alvaro et al., 2008) and again which NPY receptor/s involved is still unknown. In fact, NPY is associated with several physiological and pathological functions in other parts of the CNS, including neuroprotection against degenerative diseases, but its role in the retina is not completely known. Therefore, NPY in the retina, Why? And what for?

NPY might be neuroprotective in some retinal diseases; however other strategies need to be developed in order to treat other retinal diseases, such as retinitis pigmentosa. The iPS cells are good strategy to be used in cell based therapies, but the low yield of photoreceptors obtained to transplant is still very low. Therefore new models need to be studied to supplant this problem.

Taking these evidences into account, we focused in three main objectives:

Aim 1 - to investigate the potential protective role of NPY against glutamate toxicity in cultured rat retinal neurons, unraveling the NPY receptors and intracellular mechanisms involved; In this first main objective we want to: a) Elucidate the distribution of NPY receptors in mixed rat retinal neural cell cultures, b) Investigate the type of cell death induced by glutamate and the neuroprotective role of NPY and NPY receptors against this insult, c) Identify the NPY receptors involved in the neuroprotective role of NPY against glutamate toxicity in cultured rat retinal neurons, using pharmacologic tools NPY receptor agonists and antagonists, d) Unravel the intracellular mechanisms that mediate the NPY protective role in this neuronal cell death using specific key proteins inhibitors;

Aim 2 - to characterize the pluripotency and capacity for photoreceptor cell differentiation of iPS cells lines obtained from murine retinas (R-iPS) as compared to those obtained from fibroblasts (F-iPS) harvested from the same donor. In this second main objective we want to: a) Characterize the pluripotency of iPS cells lines obtained from murine retinas; b) Evaluate the differentiation capacity of these cell into photoreceptors;

Aim 3 - To characterize the retinal-iPS cells in terms of NPY system and to evaluate the NPY proliferation effect in these cells.

Chapter II
Neuropeptide Y receptors Y_1 and Y_2 are present in neurons and glial cells in rat retinal cells in culture

Chapter 2 – Neuropeptide Y receptors Y_1 and Y_2 are present in neurons and glial cells in rat retinal cells in culture

1. ABSTRACT

PURPOSE. Neuropeptide Y (NPY) is one of the most abundant peptides in the central nervous system (CNS), including the retina. This peptide activates various different G coupled-receptors (NPY Y_1, Y_2, Y_4, and Y_5) that are also present in the retina. However, the localization of NPY receptors in the several types of retinal cells is not completely known. In this study, we have looked at the distribution of NPY Y_1 and Y_2, receptors in rat retinal cells to reveal new perspectives on the role of NPY receptors in retina physiology.

METHODS. Rat retinal neural cell cultures were prepared from newborn Wistar rats (P3-P5) and pure rat Müller cell culture was obtained after treatment of these cells with ascorbic acid. The presence of NPY Y_1 and Y_2 in retinal cell types was studied by immunocytochemistry.

RESULTS. We show that NPY Y_1 and Y_2 receptors are present on every cell type of rat retinal cell cultures. Neurons, as photoreceptors, bipolar, horizontal, amacrine and ganglion cells express these two types of NPY receptors. NPY Y_1 and Y_2 receptors are also located in macroglial cells (Müller cells and astrocytes) and microglial cells.

CONCLUSIONS. We have clarified the presence of the NPY Y_1 and Y_2 receptors in all different cell types that constitute the retina, which we believe will help open new perspectives for studying the physiology and the potential pathophysiological function of NPY and its receptors in the retina.

2. INTRODUCTION

Neuropeptide Y (NPY) is a member of the NPY or “PP-fold” family (Michel et al., 1998). This 36-amino acid peptide was first isolated from the pig brain in 1982 by Tatemoto.(Tatemoto et al., 1982) NPY is one of the most abundant peptides in the central nervous system (CNS), including the retina, and is involved in numerous physiological functions, such as feeding, memory processing, and cognition (Wettstein et al., 1995; Silva et al., 2005a). NPY actions are mediated by G protein-coupled receptors, which have been named NPY Y_1, Y_2, Y_4, Y_5 and y_6 (Silva et al., 2005a; Xapelli et al., 2008).

NPY is expressed in the retina of mammals and non-mammals (Osborne et al., 1985; Bruun et al., 1986; Isayama et al., 1988a; Hiscock et al., 1989; Jen et al., 1994; Mathieu et al., 2002; Oh et al., 2002). In rodent retinas, NPY-immunoreactivity (NPY-IR) is present in the inner retina, it is localized in cell bodies at both inner nuclear layer (INL) and ganglion cell layer (GCL), and is also present in processes located in the inner plexiform layer (IPL) (Sinclair et al., 2001; Oh et al., 2002). NPY shows one of the highest degrees of phylogenetic preservation, compared with other neuropeptides, and does not show marked differences between species (Larhammar et al., 1992; Larhammar et al., 2004; Larsson et al., 2008). From a consideration of the phylogenetic preservation, it is expected that the NPY and NPY receptor distribution in the retina does not vary significantly between species. NPY-immunoreactivity is present in amacrine cells in the majority of species studied (fish, frogs, lizards, rodents, baboon, pig, cat, chicken and pigeon). Cats, dogs, dolphins and humans also show NPY in their ganglion cells (Straznicky et al., 1989; Hutsler et al., 1994; Jen et al., 1994; Hutsler et al., 1995; Chen et al., 1999) while some turtles, lizards and frogs present NPY-immunoreactivity in bipolar cells (Isayama et al., 1988a; Zhu et al., 1996; Wetzel et al., 1997). Few studies have analyzed the distribution of NPY receptors either in the retina or in specific retinal cell types. The presence of both Y_1 and Y_2 receptors in the mouse retina has been

confirmed by mRNA analysis (Sinclair et al., 2001; Yoon et al., 2002); however, it is unclear which cell types express these receptors. Moreover, in primary cultures of Müller cells isolated from the retina of guinea-pig, a functional assay of cell proliferation suggested the presence of a NPY Y_1 receptor in these glial cells (Milenkovic et al., 2004). In human retinal pigment epithelium (RPE), mRNA encoding for NPY Y_1, Y_2 and Y_5 receptors has been detected, while in bovine RPE only mRNAs encoding for NPY Y_1 and Y_2 receptors have been found (Ammar et al., 1998). Although the presence of NPY receptors in the rat retina has been described, in particular NPY Y_1, Y_2, Y_4 and Y_5 mRNA expression (D'Angelo et al., 2004; Alvaro et al., 2007), little is known about their localization. The NPY Y_1 receptor-ir is localized in horizontal and amacrine cell bodies and involved in their processes (D'Angelo et al., 2002), while NPY Y_2 receptor can be found in terminals of rod bipolar cells (D'Angelo et al., 2004).

NPY and its receptors play important roles in other parts of CNS. However their role in the retina is little known. Therefore, a deeper investigation into how NPY is distributed in the retinal cells that have specific roles in retina physiology will contribute to a better understanding of the human retina and will open new avenues for application of that knowledge in human retinal diseases. Our group has previously shown that NPY activates different NPY receptors to perform the same effect. More specifically, NPY induces retinal cell proliferation through the activation of NPY Y_1, Y_2 and Y_5 receptors (Alvaro et al., 2008a) and also inhibits the $[Ca^{2+}]_i$ increase by the activation of NPY Y_1, Y_4 and Y_5 receptors in rat retinal cells (Alvaro et al., 2009).

The involvement of several NPY receptors in the same NPY effect (Alvaro et al., 2007; Alvaro et al., 2008a; Alvaro et al., 2008b; Alvaro et al., 2009) and the shortage of information on the distribution of NPY receptors in the retina have led us to characterize the localization of NPY receptors in the different cell types present in retina neural cell cultures, namely retinal neurons, macroglial and microglial cells.

3. MATERIAL AND METHODS

3.1 Primary rat retina neural cell cultures

Three- to five-day-old Wistar rats were used to prepare primary rat retina neural cell cultures, 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 for the Use of Animals in Ophthalmic and Vision Research. Briefly, rat retinas were dissected under sterile conditions, using a light microscope (Zeiss, Jena, Germany), in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (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) and digested with 0.1% trypsin (w/v) for 15 min at 37ºC. Cells were resuspended in Eagle's Minimum Essential Medium (MEM - Sigma Chemical, MO, USA), supplemented with 25 mM HEPES (Sigma Chemical), 26 mM $NaHCO_3$, 10% Fetal bovine serum (FBS - Gibco BRL, Life Technologies, Scotland, UK), and penicillin (100 U/mL) / streptomycin (100 mg/mL) (Gibco BRL), plated on glass coverslips coated with poly-D-lysine (0.1 mg/mL - Sigma Chemical) at a density of 2 x 10^6 cells/cm^2, and cultured for 8 days (37ºC, 5% CO_2).

3.2 Primary rat Müller cell culture

The preparation of primary rat Müller glial cell cultures was based on a protocol previously described by Reis and colleagues (Reis et al., 2002), with the following modifications: 8- to 9–day -old primary rat retina neural cell cultures in poly-D-lysine-coated coverslips were treated with ascorbic acid (4 mM - Sigma Chemical) for 3 h and then washed abundantly in order to eliminate all neurons. The Müller glial cell culture was maintained for up to two weeks with MEM medium [supplement with 25 mM HEPES, 26 mM $NaHCO_3$, 10% FBS, and penicillin (100 U/mL) / streptomycin (100 mg/mL)] which was changed every 3 days. Immunocytochemical experiments were performed after that period.

3.3 Immunocytochemistry

Cells cultured on glass coverslips were washed twice with PBS. They were fixed with 4% paraformaldehyde (20 min; room temperature), permeabilized with 1% Triton/PBS for 5 min, and, to prevent nonspecific binding, were blocked with 3% (w/v) fatty acid-free BSA in 0.2% Tween 20/PBS, for 1 h at room temperature. Cells were then incubated with the selected primary antibodies overnight at 4ºC (Table 2.1). To identify photoreceptors, rhodopsin antibody was used. Rhodopsin is a photopigment found in rods - the most common type of photoreceptor in the retina (Gunhan et al., 2003). Bipolar cells were identified by Protein Kinase C (PKC) alfa (Osborne et al., 1992; Gunhan et al., 2003; Lameirao et al., 2009). Different sub-populations of amacrine cells were identified by calcium binding proteins: calbindin, calretinin and parvalbumin (Haverkamp et al., 2000; Hwang et al., 2005; Kim et al., 2010b; Hirano et al., 2011). The same proteins may also be present in ganglion cells. However a more specific marker of ganglion cells was used - Brn3a (Xiang et al., 1995; Nadal-Nicolas et al., 2009). Additionally, calbindin can also identify horizontal cells. GFAP and vimentin were used as macroglial cell markers (astrocytes and Müller cells), while CD11b identifies resting and activated microglial cells (Schnitzer, 1988; Seth et al., 2008). After washing, the cells were incubated for 1 h at room temperature with the respective secondary antibodies: Alexa^{TM} 488 anti-sheep IgG, Alexa^{TM} 488 anti-mouse IgG, Alexa^{TM} 594 anti-mouse IgG, Alexa^{TM} 594 anti-rat IgG, Alexa^{TM} 488 anti-rabbit IgG or Alexa^{TM} 594 anti-rabbit IgG (1:200 - Invitrogen, Eugene, OR, USA). Finally, after 5 min washing, cell nuclei were stained with Hoechst 33342 (1 mg/mL in PBS - Invitrogen) for 5 min, and upon rinsing twice with PBS, the coverslips were mounted on glass slides using Dako Fluorescent mounting medium (Dako Cytomation, Glostrup, Denmark).

3.4 Sequential Immunocytochemistry

In the case of double labeling with primary antibodies made with the same species, a sequential immunolabeling was performed. For that, the first primary antibody (Table 2.1) was incubated overnight at 4ºC, followed by the respective secondary antibody (1h at room temperature). The cells were then blocked to prevent all nonspecific binding (1h at room temperature). In the second part of the immunolabeling, cells were incubated with the second primary antibody (overnight at 4ºC) followed by the respective secondary antibody (1h at room temperature). Between the various incubations, cells were washed three times with PBS 1x. Cell nuclei were detected by Hoechst 33342 (as previously referred) and coverslips were mounted.

3.5 Tissue preparation

Adult Wistar rats eyes were removed and fixed in 4% paraformaldehyde solution (overnight at 4ºC), transferred into 30% sucrose in PBS, embedded in Tissue Tek OCT Compound (Sakura Finetek Europe B.V., AV Alphen aan den Rijn, The Netherlands) and frozen. Eyes were then cryosected in a cryostat (Leica, 3050-S, Wetzlar, Germany) in 7 micrometer sections.

Table 2.1 – Primary antibodies

Primary antibody	Immunolabelled retinal cell / receptors Immunogen	Supplier	Host/ Dilution	Specificity/Control
Anti-Rhodopsin	Rhodopsin is a photoreceptor marker (Gunhan et al., 2003). Antibody raised against amino acids 1-100 mapping at the N-terminus of rhodopsin of human origin.	Santa Cruz Biotechnology Inc, CA, USA	Mouse 1:50	Staining absent when primary antibody was omitted. Western blot analysis of rhodopsin expression in mouse eye tissue extract revealed a single band at 40 kDa (manufacturer statement).The use of this antibody has been previously reported – Western Blotting (WB) and Immunocytochemistry (ICC) (Gandorfer et al., 2004; Kolesnikov et al., 2011; Costa et al., 2012).
Anti-PKC alfa	PKC alfa is a retinal bipolar cells marker (Osborne et al., 1992; Gunhan et al., 2003; Lameirao et al., 2009).	Cell Signalling Technology, Danvers, MA.	Rabbit 1:200	Staining absent when primary antibody was omitted. Use of this antibody has been previously reported – WB and ICC (Cohen et al., 2009; Zhang et al., 2010; Costa et al., 2012).
Anti-Calbindin	Calbindin is a marker of horizontal cells and subpopulations of amacrine and ganglion cells (Haverkamp et al., 2000; Hirano et al., 2011). Antibody obtained from recombinant calbindin.	Merck Millipore, Billerica, MA (AB 1778)	Rabbit 1:100	Staining absent when primary antibody was omitted. This antibody was characterized by Western Blotting and Immunofluorescence. It shows no cross-reactivity to calretinin by Western blot. The antibody produces specific staining of cerebellum Purkinje cells, molecular layer dendrites and axonal fibers, stains cell bodies and fibers in neuronal subpopulations (manufacturer statement).Use of this antibody has been previously reported - immunohistochemistry (IHC) and WB (Berger-Sweeney et al., 2001; Matilla et al., 2001; de Melo et al., 2003; Joly et al., 2008; Kim et al., 2009; Cammas et al., 2010; Gallagher et al., 2010; Lee et al., 2010; Brzezinski et al., 2011; Caprara et al., 2011; Cho et al., 2011). A primary antibody negative control and preabsorbation control were previously performed (Lee et al., 2010).
Anti-Calretinin	Calretinin is an amacrine and ganglion cells marker (Hwang et al., 2005). Antibody obtained from recombinant rat calretinin.	Merck Millipore (MAB1568)	Mouse 1:200	Staining absent when primary antibody was omitted. Use of this antibody has been previously reported – ICC and IHC (Bender et al., 2001; Perrotti et al., 2004; Baizer et al., 2005; Liu et al., 2009; Pinzon-Duarte et al., 2010).
Anti-Parvalbumin	Parvalbumin is a marker of amacrine and ganglion cells (Haverkamp et al., 2000; Kim et al., 2010b). Antibody obtained from parvalbumin purified from frog muscle.	Merck Millipore (MAB 1572)	Mouse 1:200	Staining absent when primary antibody was omitted. By immunoblot it recognizes a protein of 12 kDa. The antibody is directed against an epitope at the first Ca^{2+}-binding site and specifically stains the Ca^{2+}-bound form of parvalbumin. Use of this antibody has been previously reported – ICC and IHC (Perrotti et al., 2004; Baizer et al., 2005; Liu et al., 2009; Runyan et al., 2010; Kroehne et al., 2011).
Anti-Brn3a	Brn3a is a ganglion cells marker (Xiang et al., 1995; Nadal-Nicolas et al., 2009). Antibody obtaioned from amino acids 186-224 of Brn3a fused to the T7 gene 10 protein	Merck Millipore (MAB 1585)	Mouse 1:25	Staining absent when primary antibody was omitted. This antibody shows no reactivity to Brn3b or Brn3c by Western blot and no reactivity to Brn3a knock-out mice. Use of this antibody has been reported previously – ICC, IHC and WB (47kDa single band) (Xiang et al., 1995; Joly et al., 2008; Cammas et al., 2010; Caprara et al., 2011; D'Autreaux et al., 2011).
Anti-GFAP	GFAP is a macroglial cells (astrocytes and Müller cells) marker (Schnitzer, 1988). Immunogen is GFAP, Clone G-A-5.	Sigma Chemical	Mouse 1:400	Staining absent when primary antibody was omitted. Use of this antibody has been previously reported – IHC and WB (Ruiz-Ederra et al., 2003; Alunni et al., 2005; Joly et al., 2008; Burgi et al., 2009; Caprara et al., 2011).
Anti-CD11b	CD11b is a microglial cells marker (Seth et al., 2008).	AbDSerotec, Kidlington,	Mouse 1:200	Staining absent when primary antibody was omitted. This antibody recognizes the rat

	This antibody was obtained from rat peritoneal macrophages.	UK		equivalent of human CD11b, the receptor for the iC3b component of complement. The antigen is expressed on most macrophages, including resident and activated peritoneal macrophages and Kupffer cells and around 35% of alveolar macrophages. The antibody also labels dendritic cells, granulocytes and microglial cells in the brain (manufacturer statement).Use of this antibody has been previously reported – IHC and immunoprecipitation (Robinson et al., 1986; Chew et al., 2011; Morales-Garcia et al., 2011; Spencer-Segal et al., 2011).
Anti-TUJ 1	This antibody identifies neurons (Snow et al., 1995) expressing neuronal class III ß-Tubulin - Clone TUJ 1.	Covance Research Products Inc, CA	Mouse 1:500	Staining absent when primary antibody was omitted. Antibody highly reactive to neuron specific Class III β-tubulin (βIII). TUJ1 does not identify β-tubulin found in glial cells. Use of this antibody has been previously reported – ICC, FACS and flow cytometry (Kunath et al., 2007; Heanue et al., 2011; Jeon et al., 2011; Du et al., 2012; Mak et al., 2012; Vazquez et al., 2012).
Anti-Vimentin	Vimentin identifies macroglial cells (astrocytes and Müller cells) (Lemmon et al., 1983). This antibody was obtained from vimentin clone V9.	Thermo Fisher Scientific, Waltham, MA	Mouse 1:400	Staining absent when primary antibody was omitted. Use of this antibody has been previously reported – ICC, IHC and WB (48kDa) (Helboe et al., 1999; Chen et al., 2009).
Anti NPY Y_1 receptor	Peptide sequence: KQASPVAFKKINNN, a synthetic peptide corresponding to amino acids 365 378 of human NPY_1R, conjugated to KLH.	AbDSerotec	Sheep 1:500	Staining absent when primary antibody was omitted. Cross reactivity with rat. Use of this antibody has been previously reported (Ferreira et al., 2010; Baptista et al., 2012). This antibody detects a band of approximately 55kDa in human brain, 40 kDa in dentate gyrus neurosphere-derived cultures (Baptista et al., 2012) and murine N9 microglial cell line (Ferreira et al., 2010).
Anti-NPY Y_2 receptor	Peptide sequence CEQRLDAIHSEVSMTFKAK, corresponding to amino acid residues 346-364 of mouse NPY_2R.	Alomone Labs, Jerusalem, Israel	Rabbit 1:100	Staining absent when primary antibody was omitted. Use of this antibody has been previously reported (Baptista et al., 2012). Western blot analysis of rat hippocampus, rat whole brain and dentate gyrus neurosphere-derived cultures reveals a 50kDa single band (Baptista et al., 2012). The preabsortion control has shown no immunostaining.

3.6 Immunohistochemistry

Eye sections were fixed with acetone at -20ºC for 20 min, blocked in PBS containing 10% newborn goat serum (NGS; Gibco) and 0.3% Triton X-100 (Sigma) and incubated in primary antibody overnight at 4ºC. Primary antibody (rabbit anti-NPY Y1R, Alomone) or rabbit anti- NPY Y1R, 1:500, Alomone) were diluted in 2% NGS and 0.3% Triton X-100 in PBS. After washing, slices were incubated with AlexaTM 488 anti-sheep IgG and AlexaTM 488 anti-rabbit IgG (1:200, Invitrogen) for one hour at room temperature. Finally, after 5 min washing, cell nuclei were stained with Hoechst 33342 (1 mg/mL in

PBS; Invitrogen) for 10 min, and upon rinsing twice with PBS, the slides were mounted using Dako Fluorescent mounting medium (Dako Cytomation).

Cells and retinal slices were visualized using a fluorescence microscope (Zeiss Axioshop 2 Plus) coupled to a digital camera (AxiocamHRc) and a laser scanning confocal microscope LSM 510 META (Zeiss, Jena, Germany). Images were analyzed using Adobe Photoshop (Adobe, San Jose, CA) or Image J (National Institutes of Health, Bethesda, MD). Negative controls were performed for each individual experiment by staining the cells without the primary antibodies. Each antibody was tested separately to determine its correct dilution..

4. RESULTS

Our group has previously shown that mRNAs coding for NPY Y_1, Y_2, Y_4 and Y_5 receptors are expressed in primary rat retina neural cell cultures (Alvaro et al., 2007). In the present study, we have used immunocytochemistry to analyze the distribution and localization of NPY Y_1 and Y_2 receptors in the different cell types present in the rat retina neural cell culture.

Cultured retinal cells were immunoreactive for NPY Y_1 and Y_2 receptors (Fig 2.1). NPY Y_1 receptor immunoreactivity (-IR) and NPY Y_2 receptors-ir were localized in cells with distinct morphologies, specifically neurons (small cell bodies and long processes), astroglial and microglial cells (Fig. 2.1A, B). However, some cells were not double labeled for both NPY Y_1 and Y_2 receptors-IR (Fig. 2.1C).

We carried out immunohistochemistry studies in adult rat retina slices (Fig. 2) to confirm the NPY Y_1 and Y_2 immunoreactivity localization in rat retina cells. NPY Y_1 and Y_2 receptor immunoreactivities were detected in RPE, photoreceptor outer and inner segments (P), in cell processes of OPL, INL, IPL, and in GCL. To study the distribution of the NPY receptors in retinal neurons, and, specifically, those in distinct subtypes of neurons, different markers were used for double immunostaining (Fig. 2.3-2.5). NPY Y_1- and Y_2 receptor-ir were detected in cells which were immunoreactive for TUJ1, a neuronal marker (Fig. 2.3A, B). The immunoreactivity of NPY receptors in retinal neurons was found to be localized mainly in the neuronal cell bodies, but was also seen to be distributed along the cell processes (Fig. 2.3). In this retinal neural cell culture, we detected: rhodopsin-positive cells (photoreceptors), anti-PKC α-positive cells (bipolar cells), calbindin-positive cells (horizontal cells), Brn3a-positive cells (ganglion cells), parvalbumin-, calretinin-, and calbindin-positive cells (subsets of amacrine cells and ganglion cells). As can be seen from Figures 2.4 and 2.5, some of the cells were immunoreactive for rhodopsin, suggesting that some photoreceptors are present in our model of rat retinal neural cells. Furthermore, in some cases, the immunostaining results

resembled the shape of photoreceptor outer segments. PKC-α immunoreactivity (bipolar cells) was also present in several neurons. Calbindin and calretinin immunoreactivity was detected in a number of cells, and the staining was particularly evident in cell bodies, although some processes were also positive for both calcium binding proteins. In contrast, the other calcium binding protein, parvalbumin, was much less expressed in this culture, and was only identified in a small number of neurons. Brn3a was also present in a few neuronal cells. NPY Y_1 and Y_2 receptors-IR were detected in all types of neurons present in this cell culture, specifically in rhodopsin-, PKC α-, calbindin-, parvalbumin-, calretinin- and Brn3a-positive cells (Fig. 2.4, 2.5).

The presence of NPY Y_1 (Fig. 2.6) and Y_2 (Fig. 2.7) receptors was also assessed by double-immunolabeling both in macroglial (Müller cells and astrocytes) and microglial cells. The presence of NPY Y_1 and Y_2 receptors was also observed in purified rat Müller cell cultures (Fig. 2.6, 2.7).

In retinal primary cell cultures, microglial cells were identified using an antibody against CD11b, and macroglial cells (astrocytes and Müller cells) were characterized using anti-GFAP and anti-vimentin antibodies. CD11b-positive cells presented a positive labeling for each NPY receptor subtype analyzed (Figs. 2.6, 2.7), indicating that both NPY Y_1 and Y_2 were distributed in microglial cells. GFAP- and vimentin-positive cells were immunoreactive for NPY Y_1 and Y_2 receptor subtypes (Fig. 2.6, 2.7). The presence of NPY receptor subtypes in Müller cells was confirmed by double staining with antibodies for each NPY subtype receptor and vimentin in purified Müller cell cultures.

In summary, our results show that two types of NPY receptors (NPY Y_1 and Y_2) are present in neurons, macroglial and microglial cells.

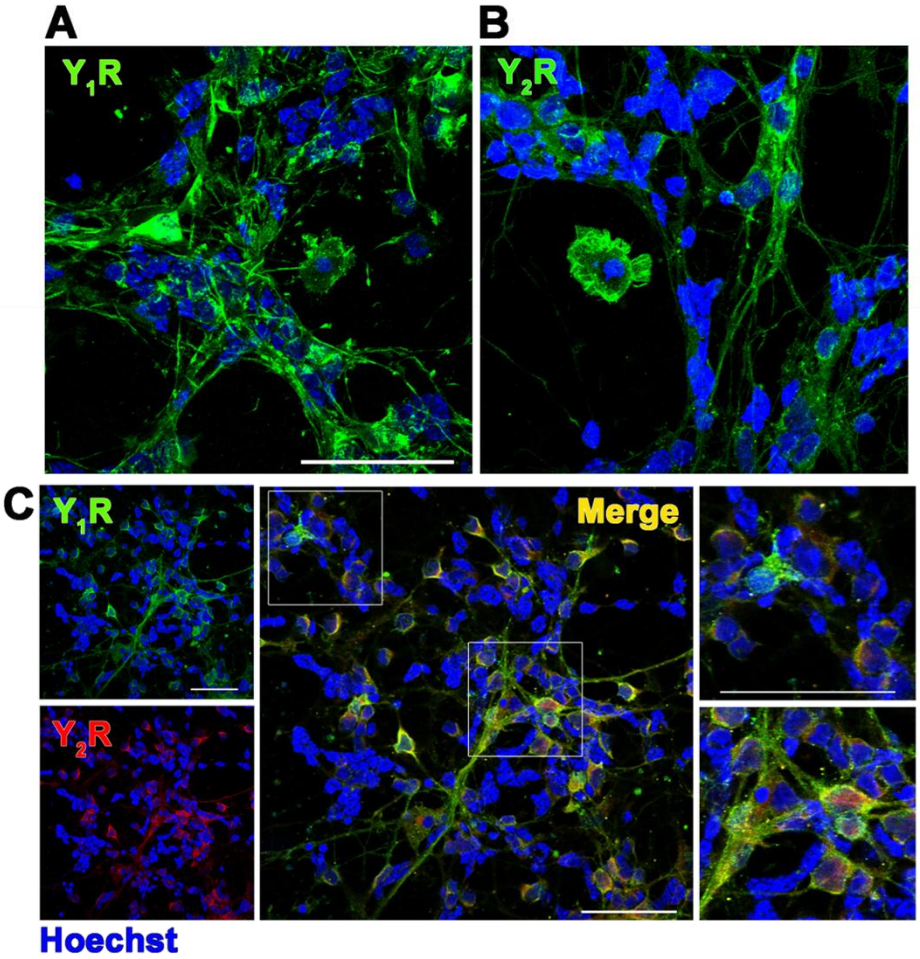


Fig. 2.1 – The immunoreactivity (green fluorescence) for NPY Y_1 and Y_2 receptors in primary rat retinal neural cell cultures: (A) NPY Y_1 receptor was detected (green) with an anti-NPY Y_1 antibody; (B) NPY Y_2 receptor was identified (green) with an anti-NPY Y_2. Filled arrow represents cells with neuron-like shape. Head arrow shows cells with microglia-like shape. (C) Double immunolabeling of rat retinal cells with NPY Y_1 (green) and NPY Y_2 receptor (red). No double-labeled cell (top image) and double-labeled cells (bottom image) were magnified on right column. Cell nuclei were stained with Hoechst 33342 (blue). Negative controls were stained without primary antibodies. The images are representative of three independent cell cultures. Scale bar: 50µm.

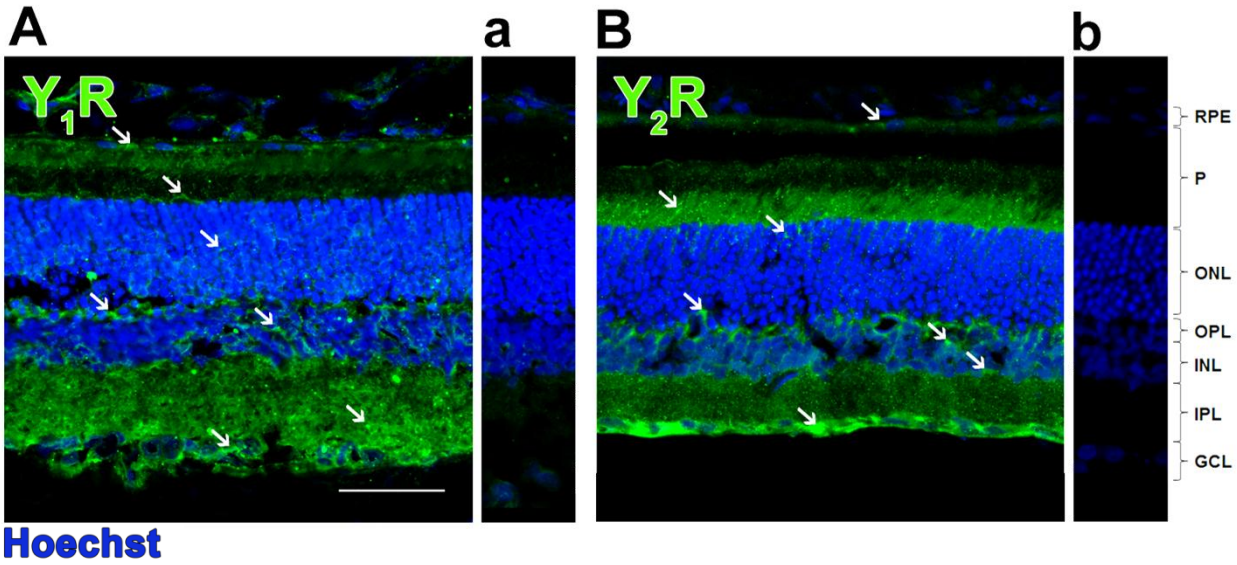


Fig. 2.2 - NPY Y_1 and Y_2 receptors immunoreactivity in rat retinal slices: Rat retinal slices were immunostained against NPY Y_1 (A) and Y_2 (B) receptors antibodies. (A) NPY Y_1 receptor immunoreactivity is particularly evident in the inner retinal layers, mainly in inner nuclear and plexiform layers as well as in retinal ganglion cell layer (GCL). NPY Y_1R immunoreactivity is also observed in retinal pigment epithelium (RPE), photoreceptors (P) and in outer layers: outer nuclear layer (ONL) and outer plexiform layer (OPL). (B) NPY Y_2R immunoreactivity is mainly detected in inner and outer plexiform layers and GCL. NPY Y_2 receptors immunoreactivity is also identified in RPE, photoreceptors, and nuclear layers (INL and ONL).White arrows highlight NPY Y_1 and Y_2 receptors immunostaining. Cell nuclei were stained by Hoechst 33342 (blue). Negative controls were performed without respective primary antibody (a, b). Scale bar: 50 µm.

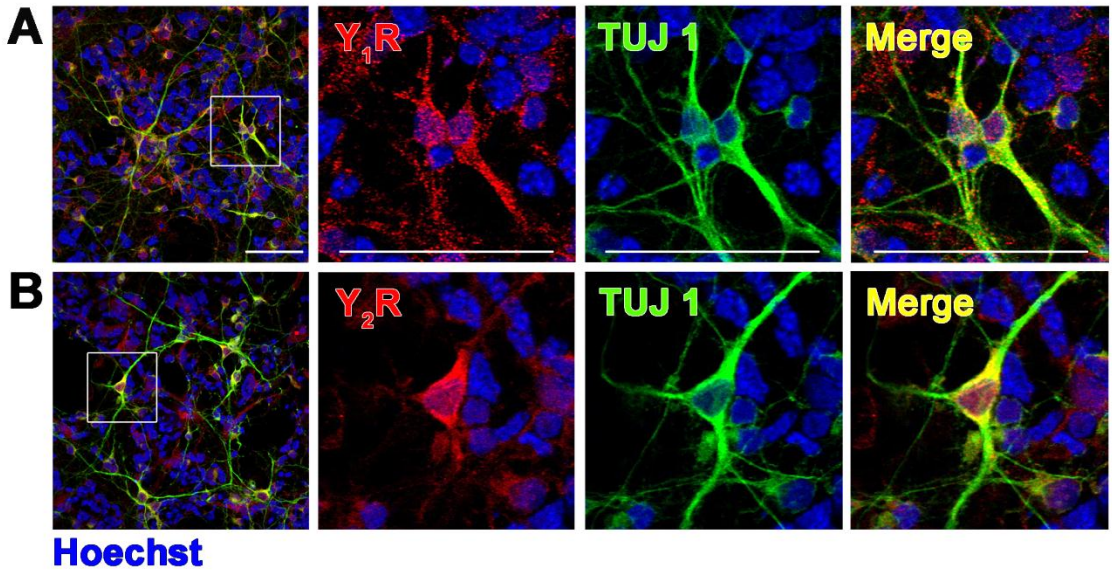


Fig. 2.3 - NPY Y_1 and Y_2 receptors are present in rat retinal neurons. Retinal neurons were double-labeled with the TUJ1 antibody (green) and the different antibodies against NPY receptors (NPY Y_1 and Y_2- red). A representative double-stained neuron is highlighted in a small square and presented on the right - Magnification of double-labeled neurons. (A) TUJ 1-positive neuron expressing NPY Y_1 receptors; (B) TUJ 1-positive neuron expressing NPY Y_2 receptors.. Cell nuclei were identified by Hoechst 33342 staining (blue). The images were representative of three independent cell cultures. Scale bar: 50 µm

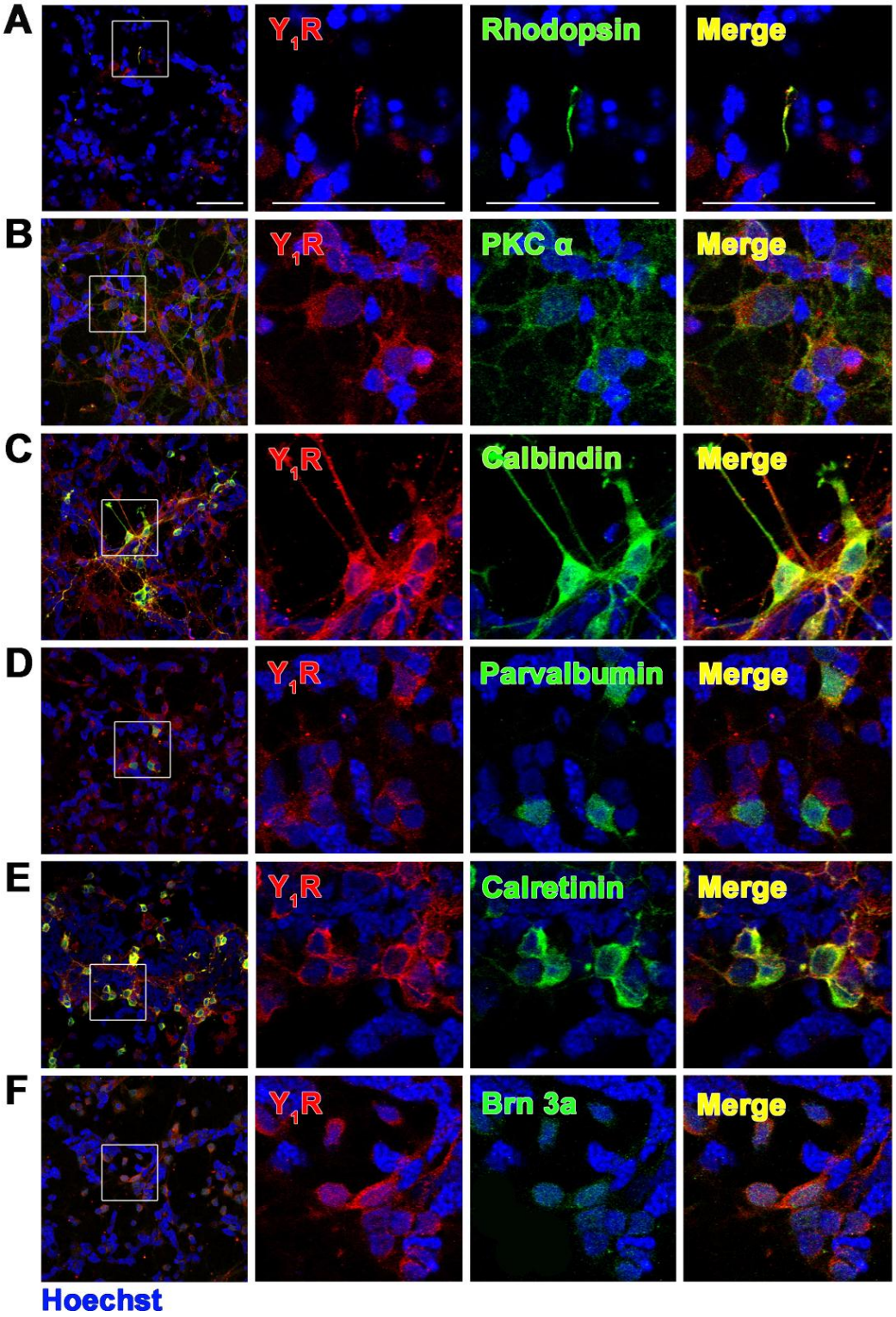


Fig. 2.4 - NPY Y_1 receptors are present in several types of cultured retinal neurons. Retinal cells were immunolabeled using an antibody against NPY Y_1 receptor (A-F). Photoreceptors were identified with anti-rhodopsin (A); bipolar cells (B) were recognized using anti-PKC α; horizontal cells were marked using anti-calbindin (C); amacrine cells sub-populations were identified using anti-parvalbumin (D) and anti-calretinin (E); ganglion cells were identified using anti-Brn3a (F). Double immunolabeling of NPY Y_1 receptor and each cell type marker was highlighted in a square. and presented on the right – Magnification of double labeled neurons. Cell nuclei were identified by Hoechst 33342 (blue). The images are representative of three independent cell cultures. Scale bar: 50 µm.

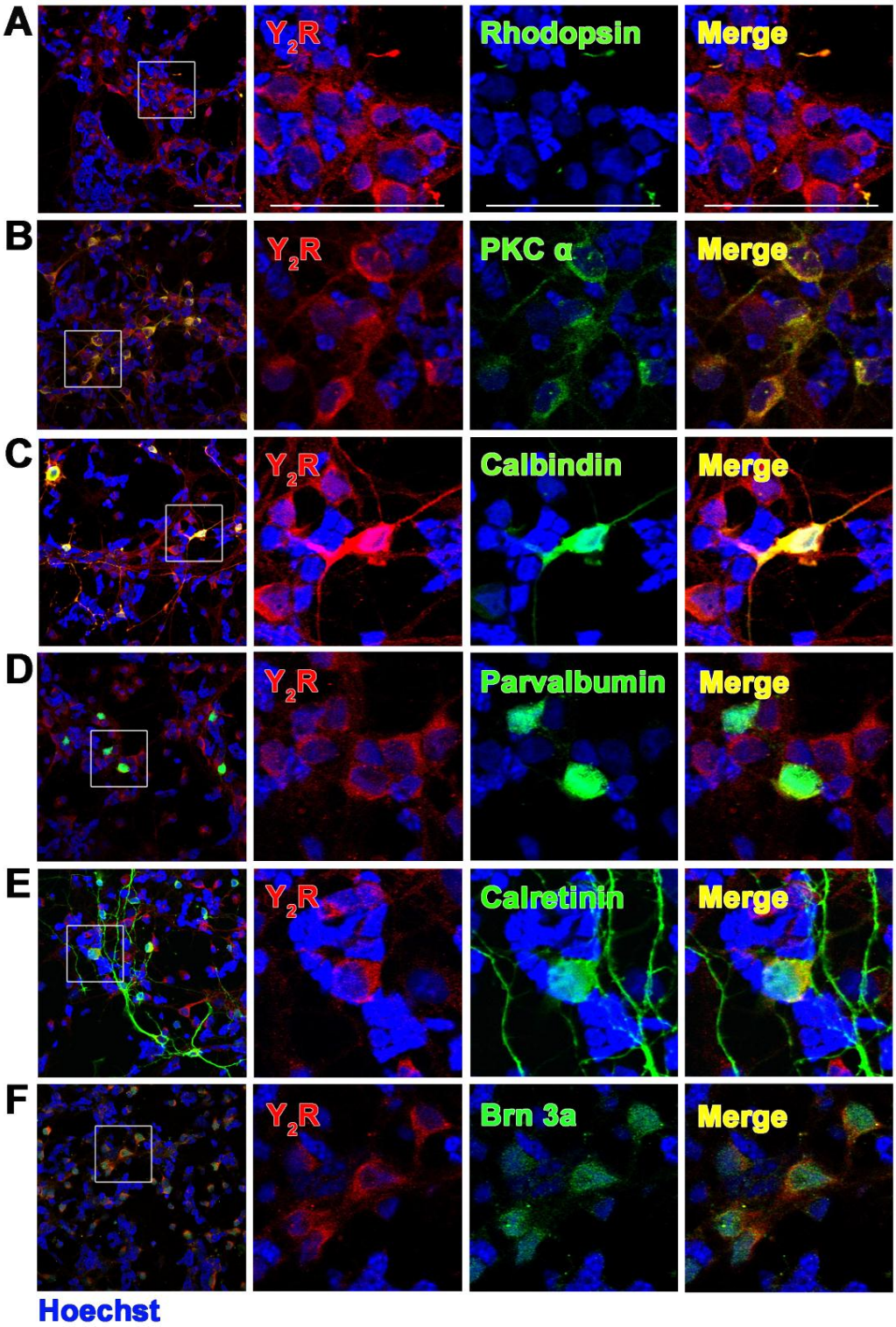


Fig. 2.5 - NPY Y_2 receptors are present in several types of cultured retinal neurons. Retinal cells were immunolabeled using an antibody against NPY Y_2 receptor (A-F). Photoreceptors were identified with anti-rhodopsin (A); bipolar cells (B) were identified using anti-PKC α; horizontal cells were identified using anti-calbindin (C); amacrine cells sub-populations were labeled using anti-parvalbumin (D) and anti-calretinin (E); ganglion cells were identified using anti-Brn3a (F). Double immunolabeling of NPY Y_2 receptor and each cell type marker was highlighted in a square. and presented on the right– Magnification of double labeled neurons. Cell nuclei were identified by Hoechst 33342 (blue). The images are representative of three independent cell cultures. Scale bar: 50 µm.

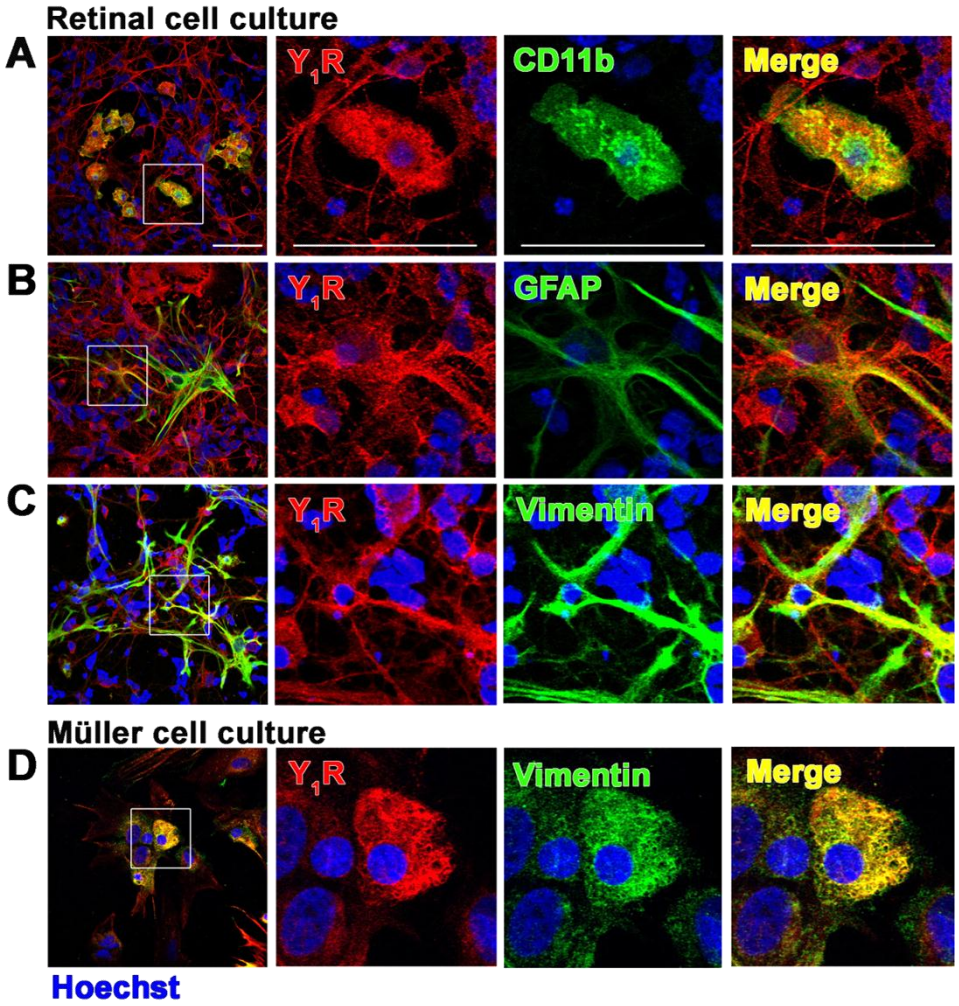


Fig. 2.6 - NPY Y_1 receptors are present in rat retina microglial and macroglial cells. An antibody against NPY Y_1 receptor was used to identify the expression of this receptor in microglial and/or macroglial cells present either in retinal neural cell cultures (A-C - red) or purified Müller cell cultures (D - red). Retinal microglial cells were immunolabeled with an antibody against CD11b (A - green), and macroglial cells (astrocytes and Müller cells) were identified by anti-GFAP (B – green) and anti-vimentin (C – green) specific antibodies. Cells/cell co-expressing NPY Y_2 receptor and each specific cell type marker were highlighted in the right column. Cell nuclei were identified by Hoechst 33342 (blue). The images are representative of three independent cell cultures. Scale bar: 50 µm

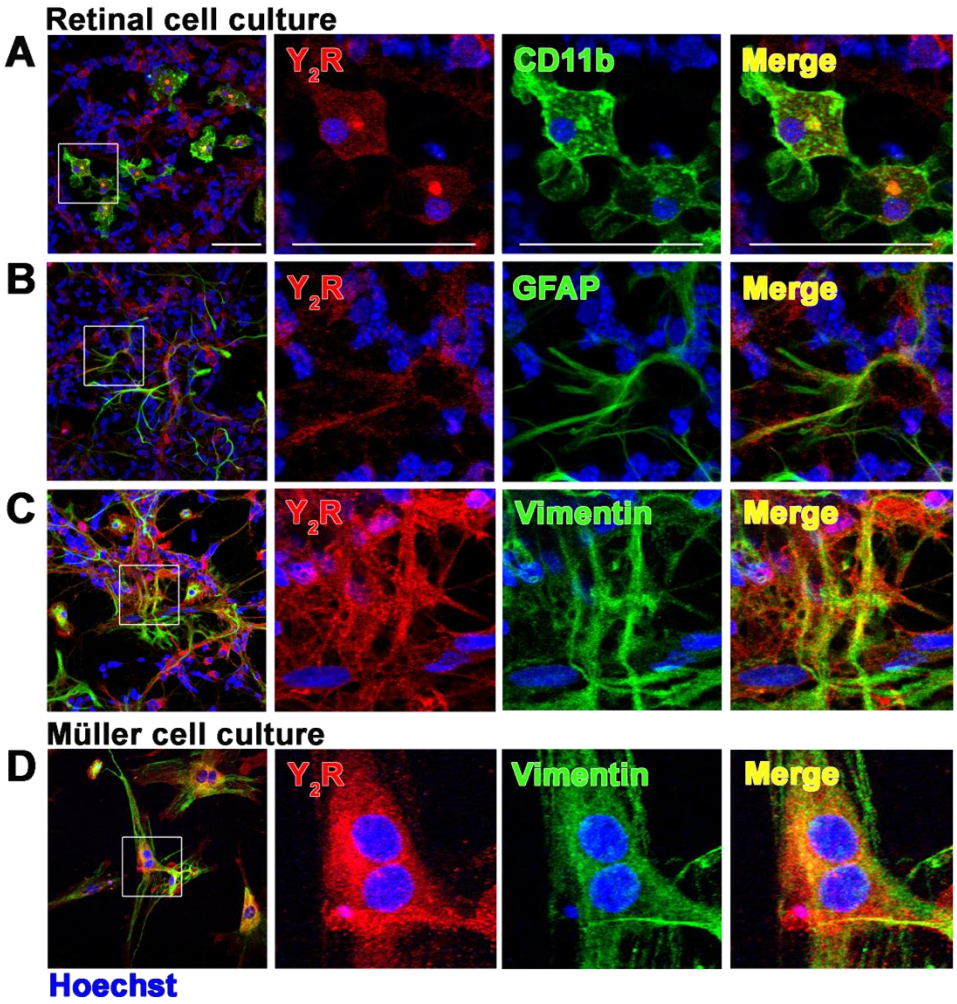


Fig. 2.7 - NPY Y_2 receptors are present in rat retina microglial and macroglial cells. An antibody against NPY Y_2 receptor was used to identify the expression of this receptor in microglial and/or macroglial cells present either in retinal neural cell cultures (A-C - red) or purified Müller cell cultures (D - red). Retinal microglial cells were immunolabeled with an antibody against CD11b (A - green), and macroglial cells (astrocytes and Müller cells) were identified by anti-GFAP (B – green) and anti-vimentin (C – green) specific antibodies. Cells/cell co-expressing NPY Y_2 receptor and each specific cell type marker were highlighted in the right column. Cell nuclei were identified by Hoechst 33342 (blue). The images are representative of three independent cell cultures. Scale bar: 50 µm.

5. DISCUSSION

It is well known that NPY and NPY receptors are present in the retina of a variety of species (Osborne et al., 1985; Bruun et al., 1986; Isayama et al., 1988a; Hiscock et al., 1989; Jen et al., 1994; Oh et al., 2002). However, to the best of our knowledge, no previous studies have provided a full characterization of the localization and distribution of NPY and NPY receptors in the retina. The high degree of phylogenetic preservation of NPY and its receptors (Larhammar et al., 1992; Larhammar et al., 2004; Larsson et al., 2008) make their distribution similar between species. In fact, among the species for which data is available, the retina of fishes, frogs, rodents, baboon, pig, cat, chicken and pigeon show NPY-immunoreactivity in amacrine cells and displaced amacrine cells (INL and cell processes in IPL) (Bruun et al., 1986; Tornqvist et al., 1988; Bruun et al., 1991; Hutsler et al., 1994; Sinclair et al., 2001; Oh et al., 2002). In addition, NPY-IR in cat, dog, dolphin and human retinas is also localized in ganglion cells at GCL (Straznicky et al., 1989; Hutsler et al., 1994; Jen et al., 1994; Hutsler et al., 1995; Chen et al., 1999). Furthermore, the bovine and human retinal pigment epithelium (RPE) shows NPY-IR (Ammar et al., 1998). Our group has previously shown that NPY-ir is present in macroglial cells (Müller cells) and microglial cells in rat retinal neural cell cultures (Alvaro et al., 2007). In relation to fish retinas, NPY-immunoreactivity is detected in amacrine cells, which processes originate distinct sub-layers in the IPL (Osborne et al., 1985; Bruun et al., 1986). By contrast, the NPY-immunoreactivity of amacrine cells of river lamprey is weakly positive (Negishi et al., 1986) and even absent in squid retina (Osborne et al., 1986). Lizard retina show NPY-immunoreactivity in amacrine cells in INL, and are sporadically displaced at GCL (Straznicky et al., 1994). Turtle retina shows NPY-immunoreactivity in bipolar cells and amacrine cells, evenly distributed in the retina (in INL, IPL and GCL and located in peripheral retina) (Isayama et al., 1988b; Wetzel et al., 1997). Furthermore, frogs have the highest concentration of NPY-immunoreactivity levels, which are characterized by seasonal variations (Hiscock et al., 1989); NPY-

immunoreactivity is localized in a small population of amacrine cell bodies in INL (Osborne et al., 1985; Bruun et al., 1986; Hiscock et al., 1990; Zhu et al., 1996), in bipolar-like cell bodies of INL, in GCL, in Müller cells within in INL (Zhu et al., 1996) and in various processes in IPL (Bruun et al., 1986). Our group has previously shown the presence of mRNA encoding for different NPY receptor subtypes in the retina and in the same retinal cell culture model used in this study (Alvaro et al., 2007). In the present study, through immunocytochemistry techniques we have shown that NPY Y_1 and Y_2 receptors are present in different subtypes of rat retinal neurons. In addition, this study is the first to report that cultured cells expressing rhodopsin also express the NPY Y_1 and Y_2 receptors studied. Furthermore, bipolar, horizontal, amacrine and ganglion cells express NPY Y_1 and Y_2 receptors. PKC α-positive cells (bipolar cells) were frequently distributed in our cell culture model and also expressed the Y_1 and Y_2 receptors. This finding is consistent with a previous study, which has localized the NPY Y_2 receptor in rod bipolar cell terminals in a rat retinal culture (D'Angelo et al., 2004).

In rat retinal slices, NPY Y_1 and Y_2 receptor immunoreactivities were detected in: photoreceptor outer segment; processes of OPL, layer composed by synapses between photoreceptors, bipolar cells and horizontal cells; INL, layer composed by horizontal cells, bipolar cells and amacrine cells; cell processes localized in IPL, layer composed by synapses between bipolar cells, amacrine cells and ganglion cells; and also in ganglion cell layer. The localization of the immunoreactivity in retinal slices is comparable to the immunocytochemistry in the rat retinal cell culture model. In retinal cell cultures, cells are not arranged in organized layers as in the retina, and many cells may not achieve full maturity (Haverkamp et al., 2000). It is difficult to identify in culture all the neuronal cell types that are normally present in the retina compared with retina slices. Calbindin, one of the calcium binding proteins present in the retina is a common marker used to detect horizontal cells (Kim et al., 2010b). However, subsets of amacrine and ganglion cells may also express calbindin (Haverkamp et al., 2000). From the same group of calcium-binding proteins, parvalbumin and calretinin are more common in specific subpopulations

of amacrine cells and some ganglion cells (Haverkamp et al., 2000; Kim et al., 2010b). We observed that rat retinal cells are immunoreactive for all these calcium binding proteins and also for NPY Y_1 and Y_2 receptors. Additionally, we confirmed that retinal ganglion cells are present in the studied culture, through positive immunostaining for Brn3a, a transcription factor expressed only by ganglion cells in the retina (Xiang et al., 1995; Nadal-Nicolas et al., 2009). Overall, our observations suggest the presence of horizontal cells, subsets of amacrine cells and ganglion cells expressing NPY Y_1 and Y_2 receptors in this rat retinal neural cell culture model. Our results, in retina slices and retinal cell culture, are in agreement with a previous study showing that NPY Y_1 receptor-ir is mainly localized in horizontal cell bodies in the INL and in cell processes in the OPL, in cholinergic amacrine cell processes in the IPL and in all calbindin horizontal cells in rat retina (D'Angelo et al., 2002). Other groups have reported that NPY Y_1, Y_2 and Y_5 receptors are present in bovine and/or human RPE (Ammar et al., 1998). In this study, NPY Y_1 and Y_2 receptors immunoreactivity was also detected in adult rat retina slices. However, RPE cells are not present in our cell culture.

NPY and its receptors play a number of different and important roles in CNS that could also occur in the retina, such as neuroprotection, neurogenesis and neuromodulation regulation. In fact, previous studies have shown that NPY has a neuroprotective role in the hippocampus and striatum (Silva et al., 2003b; Xapelli et al., 2008; Decressac et al., 2012). In rat and mouse organotypic hippocampal cultures, the involvement of NPY Y_1, Y_2 and/or Y_5 receptors has been shown in the protection against cell death induced by glutamate (Silva et al., 2003b; Xapelli et al., 2007; Smialowska et al., 2009). In contrast, we have shown that NPY has a protective role against 3,4-methylenedioxy-N-methylamphetamine (MDMA)-induced toxicity in rat retinal cells (Alvaro et al., 2008b). However, the NPY receptor subtypes involved in this neuroprotective effect have not yet been identified. In this study we show that NPY Y_1 and Y_2 receptors are present in different types of neurons in rat retinal neural cell cultures, suggesting that they may putatively mediate the neuroprotective effect of NPY. We, therefore, speculate that NPY

Y_1 and/or Y_2 receptor agonists might be viewed as putative therapeutic drugs against neural cell degeneration in retinal degenerative diseases. Other studies suggest that NPY might have an important role in progenitor cell proliferation and/or differentiation in nervous tissue (Thiriet et al., 2011; Baptista et al., 2012; Doyle et al., 2012). Our group has demonstrated, using the same model of cultured rat retinal cells, that NPY stimulates the proliferation of neuronal progenitor cells (BrdU+/nestin+ cells) (Alvaro et al., 2008a). Additionally, NPY, through NPY Y_1 and Y_5 receptor activation, has the potential to maintain human embryonic stem (hES) cell self-renewal and pluripotency.(Son et al., 2011) Consequently, NPY system is a putative target to develop new strategies to increase retinal progenitor cell proliferation.

Both we and other groups have shown that NPY modulates the intracellular calcium concentration ($[Ca^{2+}]_i$) in the rat retinal neurons (D'Angelo et al., 2004). In fact, NPY inhibits the depolarization-evoked Ca^{2+} influx into rod bipolar cells through the activation of NPY Y_2 receptors (D'Angelo et al., 2004). We have also demonstrated that NPY 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). The localization of NPY Y_1 and Y_2 receptors in retinal neurons described in the present study are in agreement, at least in part, with those studies. Furthermore, we have shown that amacrine cells expressing NPY are involved in tuning ganglion cells to low spatial frequencies/large spatial patterns (Sinclair et al., 2004). This provides another possibility for NPY Y_1 and Y_2 receptor function in retinal cells. The visual processing is severely dependent on fine-tuning neurotransmission between different retinal neurons (photoreceptors, and bipolar, ganglion, horizontal and amacrine cells), which depend on $[Ca^{2+}]_i$ regulation and may result from the action of neuromodulators as the NPY. NPY modulates the release of neurotransmitters in some areas of CNS (Silva et al., 2005a). In the retina, at least in chickens and rabbits, this peptide plays a similar role when applied exogenously(Bruun et al., 1993). Therefore, as has previously been suggested (D'Angelo et al., 2002; Oh et al., 2002), the localization of NPY in several types of retinal cells may indicate a

neuromodulatory role of this peptide in the retina.

Our results have shown the presence of NPY Y_1 and Y_2 receptors in the different types of retinal cells. Therefore, since both NPY and its receptors are present in the retinal cells, this suggests an NPY autocrine effect that might play a variety of roles in the retina. These include homeostasis, modulation, and even protection of retinal cells against toxic effects. However, additional studies are necessary to unravel the role(s) of NPY Y_1 and Y_2 receptors in retinal physiology and pathophysiology. Following the evidence of previous studies suggesting that NPY and its receptors are not restricted to neurons in the retina (Zhu et al., 1996; Canto Soler et al., 2002; Milenkovic et al., 2004; Alvaro et al., 2007), we have also found that NPY Y_1 and Y_2 receptors, can be expressed in glial cells, specifically astrocytes and Müller cells. In fact, GFAP- or vimentin-positive cells were immunolabeled for the two NPY receptors analyzed. These findings are supported by the presence of NPY Y_1 and Y_2 receptors in the purified primary culture of Müller cells. Previous studies have reported the presence of Y_1 receptors in Müller cells of guinea pigs and diseased human retina (Canto Soler et al., 2002; Milenkovic et al., 2004), and the authors suggested that the overexpression of NPY Y_1 receptors in glial cells could be involved in retinal glial (Müller) cells proliferation and the development of proliferative vitreoretinopathy (PVR) (Milenkovic et al., 2004). Other groups have previously shown that NPY effects on neural cell proliferation and differentiation in different parts of CNS (such as, olfactory epithelium, subventricular zone (SVZ) and subgranular zone (SGZ) of dentate gyrus, rostral migratory stream, striatum and the olfatory bulb) were mediated by the NPY Y_1-receptor activation (Hansel et al., 2001; Howell et al., 2003; Agasse et al., 2008; Stanic et al., 2008; Rodrigo et al., 2010; Decressac et al., 2011). The involvement of NPY Y_2 receptor is controversial (Stanic et al., 2008; Decressac et al., 2011). However, to the best of our knowledge, there are no studies that have demonstrated the participation of the NPY Y_2 receptor in the physiology of Müller cells. Further studies are needed to dissect the role of NPY receptors in Müller cells.

We have also demonstrated the presence of NPY Y_1 and Y_2 receptors in retinal microglial cells (CD11b-positive cells). Microglial cells are involved in neuronal homeostasis and innate immune defense in the retina and are considered to mediate several pathogenic mechanisms in a variety of retinal degenerative diseases (Langmann, 2007; Wang et al., 2011b). In contrast, some studies have suggested that NPY is a key modulator of the crosstalk between the brain and the immune system in both health and disease (Bedoui et al., 2003; Ferreira et al., 2012). Further, in a microglial cell line, it was shown that NPY modulates microglial cell responses, regulating phagocytosis under inflammatory conditions through the Y_1 receptor (Ferreira et al., 2010; Ferreira et al., 2011b; Ferreira et al., 2012). In addition, NPY, through NPY Y_1 receptor activation, inhibits the release of nitric oxide (NO) and interleukin-1β by microglial cells exposed to lipopolysaccharide (LPS). Hence, the presence of NPY Y_1 and Y_2 receptors in microglial cells suggests the involvement of these receptors in the immune responses occurring in the retina under pro-inflammatory conditions found in retinal degenerative diseases. In our study, the NPY Y_1 receptor immunoreactivity is distributed in the microglia cell while the NPY Y_2 receptor is also highly expressed close to the nucleus of these cells. Some studies have shown nuclear and perinuclear localization of G protein-coupled receptors (Jacques et al., 2006; Valdehita et al., 2010). One specific group reported the localization of a NPY receptor in the nucleus membrane. However, in their studies the NPY Y_1 receptor was localized in the nuclear envelope of 20-week human fetal endocardial endothelial cells and cells of adult rat hearts (Jacques et al., 2003; Jacques et al., 2006).

We have previously shown the presence of mRNA encoding for NPY Y_1, Y_2, Y_4 and Y_5 receptor subtypes in the retina (Alvaro et al., 2007). However, in spite of our findings regarding Y_1 and Y_2 NPY receptors, further studies are needed to characterize the localization of the remaining NPY receptors in rat retinal cells.

Other studies developed by us have shown that different NPY receptors are activated to perform the same effect, such as retinal progenitor cell proliferation (Alvaro et al., 2008a)

and protection against retina degeneration (Alvaro et al., 2009). In this study, we show that rat retinal cell cultures present cells that do not express simultaneously the NPY Y_1 and Y_2 receptors. Therefore, the fact that not all retinal cells present all NPY receptors simultaneously indicates that different receptors may have similar roles. In fact, we have previously shown that various NPY receptors are able to prevent the increase of intracellular calcium concentration induced by the depolarizing agent KCl (Alvaro et al., 2009).

The main technique used in our study was immunocytochemistry, as an identification and characterization method. The main limitations of this technique involve the possibility of some non-selective binding of the antibodies used and that some antibodies identify more than one retinal cell type. The use of positive and negative controls may not completely abolish these limitations. However, in immunocytochemistry the cell morphology is preserved. Therefore this technique is a powerful method to visualize and analyze specific biochemical structures (proteins, such as receptors) and cellular compartments (cell body, axons and dendrites) of the cell. In conclusion, we have demonstrated that NPY Y_1 and Y_2 receptors are expressed in the different retinal cell types, although not necessary simultaneously. These observations suggest that NPY and NPY receptors have important functions in the retinal physiology, and that alterations in their expression and/or function may also be implicated in the pathogenesis of retinal degenerative diseases.

Chapter III
Neuropeptide Y receptors activation protects rat retinal cells against necrotic and apoptotic cell death induced by glutamate

Chapter 3 – Neuropeptide Y receptors activation protects rat retinal cells against necrotic and apoptotic cell death induced by glutamate

1. ABSTRACT

It has been claimed that glutamate excitotoxicity might play a role in the pathogenesis of several retinal degenerative diseases, including glaucoma and diabetic retinopathy. Neuropeptide Y (NPY) has neuroprotective properties against excitotoxicity in the hippocampus, through the activation of Y_1, Y_2 and/or Y_5 receptors. The principal objective of this study is to investigate the potential protective role of NPY against glutamate-induced toxicity in cultured rat retinal neural cells, unraveling the NPY receptors and the intracellular mechanisms involved. Rat retinal neural cell cultures were prepared from newborn Wistar rats (P3-P5) and exposed to glutamate (500 μM) for 24h. Necrotic cell death was evaluated by propidium iodide (PI) assay and apoptotic cell death using TUNEL and caspase 3 assays. The cell types present in culture were identified by immunocytochemistry. The involvement of NPY receptors was assessed using selective agonists and antagonists. Pre-treatment of cells with NPY (100 nM) inhibited both necrotic cell death (PI-positive cells) and apoptotic cell death (TUNEL-positive cells and caspase 3-positive cells) triggered by glutamate, with the neurons being the cells most strongly affected. The activation of NPY Y_2, Y_4 and Y_5 receptors inhibited necrotic cell death, while apoptotic cell death was only prevented by the activation of NPY Y_5 receptor. Moreover, NPY neuroprotective effect was mediated by the activation of PKA and p38K. In conclusion, NPY and NPY receptors can be considered potential targets to treat retinal degenerative diseases, such as glaucoma and diabetic retinopathy.

2. INTRODUCTION

Neuropeptide Y (NPY) is one of the most abundant peptides in the mammalian Central Nervous System (CNS) (Tatemoto et al., 1982; Dumont et al., 1992; Silva et al., 2005a). NPY is a highly conserved peptide containing 36 amino acids. Its biological effects are mediated by six G protein-coupled receptors Y_1, Y_2, Y_4, Y_5 and y_6 (Tatemoto et al., 1982; Michel, 1991; Silva et al., 2002). The retina is a specialized nervous tissue where NPY and its receptors are expressed in retina of different species (Bruun et al., 1986; Hutsler et al., 1995). The presence of mRNA for Y_1, Y_2, Y_4 and Y_5 NPY receptors has been detected in rat retinas (D'Angelo et al., 2004; Alvaro et al., 2007) and in cultured rat retinal neural cells (Alvaro et al., 2007), but their distribution in different cell types location and their function in the retina is poorly understood.

Glutamate is the main excitatory neurotransmitter in the CNS, including in retina (Kishida et al., 1967). Excitotoxicity, which is considered as an overactivation of glutamate receptors triggering neuronal cell death, has been associated with several acute and chronic neurodegenerative disorders (Lucas et al., 1957; Ozawa et al., 1998) and in retinal degenerative disorders, such as glaucoma (Dkhissi et al., 1999; Martin et al., 2002; Gupta et al., 2007) and diabetic retinopathy (Lieth et al., 1998; Kowluru et al., 2003; Santiago et al., 2008).

NPY has been linked to several physiological and pathological functions, such as feeding behaviour, memory processing, pain, anxiety, cell proliferation and many other processes in the central and peripheral nervous systems (Wettstein et al., 1995; Hokfelt et al., 1998). Some studies have demonstrated putative neuroprotective effects of NPY in various regions of the CNS. In particular, NPY inhibits the glutamate release in rat hippocampus, and is neuroprotective in rat hippocampus and striatum (Silva et al., 2003b; Silva et al., 2005a; Silva et al., 2005b; Xapelli et al., 2007; Xapelli et al., 2008; Smialowska et al., 2009). Moreover, the activation of NPY Y_1, Y_2 and Y_5 receptors mediates the neuroprotective effect of NPY against AMPA- and kainate-induced

excitotoxicity in organotypic rat hippocampal slice cultures (Silva et al., 2003b). It has also been suggested that selective activation of Y_1 and Y_2 receptors protects mouse hippocampal cells from excitotoxic lesions (Xapelli et al., 2008). Similarly, NPY Y_2 and Y_5 are implicated in the neuroprotective role against kainate-induced excitotoxicity in hippocampus even after delayed application of the respective agonists. Specific activation of NPY Y_2 receptor is also effective in a transient middle cerebral artery occlusion (MCAO) model of ischemia (Smialowska et al., 2009). Recently, it was shown that NPY, also through NPY Y_2 receptor activation, mediates the survival of dopaminergic neurons in Parkinson's disease models (Decressac et al., 2012). In addition, NPY was suggested as a potential neuroprotective agent in Alzheimer's disease through counteracting the toxic effect of β-amyloid in an *in vitro* model (Rose et al., 2009; Croce et al., 2011).

We have also shown that NPY in the retina presents neuroprotective properties. Specifically, NPY protected rat retinal cells in culture against 3,4-methylenedioxy-N-methylamphetamine (MDMA)-induced toxicity (Alvaro et al., 2008b), although the NPY receptor subtype(s) involved in this neuroprotective effect is unknown.

Since the retina is affected by various degenerative diseases, where glutamate excitotoxicity might eventually play a role (Gupta et al., 2007; Santiago et al., 2008), our major goal in the present work is to evaluate the putative neuroprotective role of NPY and NPY receptors against glutamate excitotoxicity in retinal cells. We have evaluated the involvement of the different NPY receptors, as well as the possible intracellular signaling pathways involved in the neuroprotective effects of NPY in retinal cells, using primary rat retinal neural cell cultures.

3. MATERIAL AND METHODS

3.1 Primary rat retinal neural cell cultures

Three- to five-day old Wistar rat pups were used to prepare primary rat retinal cell cultures, as previously described (Santiago et al., 2006; Alvaro et al., 2007). All procedures involving animals were in agreement with the Association for Research in Vision and Ophthalmology (ARVO) statement on vision and ophthalmic research for experimental models. Briefly, rat retinas were dissected under sterile conditions, using a light microscope, in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (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) and digested with 0.1% trypsin (w/v, Gibco, Life Technologies Corporation, Paisley, UK) for 15 min at 37ºC. Cells were plated on glass coverslips coated with poly-D-lysine (0.1mg/mL, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) using Minimum Essential Medium Eagle (MEM, Sigma-Aldrich) , supplemented with 25mM HEPES (Sigma-Aldrich), 26mM $NaHCO_3$, 10% Fetal bovine serum (FBS, Gibco) and penicillin (100U/mL)–streptomycin (100mg/mL, Gibco) for 8/9 days (37ºC, 5% CO_2), at a density of $2x10^6$ cells/cm^2.

3.2 Immunocytochemistry

After treatment, cells cultured on glass coverslips were washed twice with PBS and fixed in 4% paraformaldehyde (20min; room temperature - RT). The cells were then permeabilized with 1% Triton for 5min, and blocked with 3% (w/v) fatty acid-free bovine serum albumin (BSA, Sigma-Aldrich), supplemented with 0.2% Tween 20, to prevent nonspecific binding, for 1h at room temperature. Cells were incubated with primary antibodies for 90min at RT: rabbit anti-glial-fibrilliary acid protein (GFAP; 1:400; Dako, Glostrup, Denmark); mouse anti-GFAP protein (1:500, Sigma-Aldrich); rat anti-CD11b and mouse anti-CD11b (1:200; AbD Serotec, Kidlington.UK); mouse anti-TUJ1 (1:500, Covance Research Products Inc, Berkeley, CA, USA); anti-vimentin (1:400, Thermo

Fisher Scientific, Waltham, MA, USA); rabbit anti-cleaved caspase 3 (1:1600, Cell Signaling Technology, Danvers, MA; USA) (1:1600, Cell Signaling); mouse anti-CD68/ED1 (1:200, AbD Serotec). All antibody solutions were prepared in 3% fatty acid–free BSA solution.

After washing, the cells were incubated for 1h at RT with secondary antibodies: Alexa^{TM} 488 anti-mouse IgG, Alexa^{TM} 594 anti-rat IgG or Alexa^{TM} 594 anti-rabbit IgG (1:200, Invitrogen, Life Technologies Corporation). Finally, after 5min washing, cell nuclei were stained with Hoechst 33342 (1 mg/mL in PBS, Molecular Probes, Eugene, OR, USA) for 5min, and, following rinsing twice with PBS, the coverslips were mounted on glass slides using Dako Fluorescent mounting medium (Dako). Cells were visualized using a fluorescence microscope (Zeiss Axioshop 2 Plus) coupled to a digital camera (Axiocam HRc) and a scanning laser confocal microscope LSM 510 META (Zeiss, Jena, Germany). Images were analyzed using Adobe Photoshop or ImageJ, as indicated in figure legends.

The number of cleaved caspase-3 positive cells was counted in 10-12 random fields on each coverslip, while CD11b and CD68/ED 1-positive cells were counted in 6 random fields. The average number of cleaved caspase-3; CD11b- or CD68/ED 1-positive cells per random field was determined for each condition tested (control – no drug; NPY; glutamate; and glutamate + NPY).

Immunoreactivity was quantified on micrographs taken after immunocytochemical experiments. Images were acquired using identical settings. The fluorescence levels (arbitrary units) were quantified using image analysis software (image J), considering the mean grey value in 6 random fields per coverslip of at least 3 independent experiments. Negative controls were stained without primary antibodies per each immunocytochemistry performed.

3.3 Cell viability studies

3.3.1 Hoechst staining

The Hoechst 33342 marker was used to label cell nuclei in rat retinal cells in culture. The Hoechst 33342 fluorescence intensity was evaluated in images captured in a confocal microscope (LSM 510 Meta; Zeiss) using identical settings and image analysis software (Image J). The fluorescence intensity was obtained by the mean grey value in 6 random fields per coverslip of at least 3 independent experiments. The average of mean grey value was determined in arbitrary units in each experimental condition and the results were expressed as a percentage of control.

3.3.2 Propidium iodide (PI) staining

PI [3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide, Sigma-Aldrich] is a polar substance that only stains the nucleus of dead or dying cells with disrupted cell membranes. In cells undergoing necrosis or late apoptosis, PI binds to DNA, emitting a bright red fluorescence (630nm) when excited by blue–green light (493nm). Cells plated on coverslips were exposed to 100, 250 or 500µM glutamate (Sigma-Aldrich) for 24h, at 37°C. In order to test for a potential protective role of NPY, cells were incubated with 100nM NPY (Novabiochem, Laufelfingen, Switzerland) at three different times: 1h before exposure to glutamate (500µM), simultaneously with the addition of glutamate and 30min after exposure to glutamate. The agonists for NPY receptors (Y_1 ([Leu^{31}Pro^{34}]NPY), Y_2 (NPY_{13-36}), Y_4 (r-PP) and Y_5 ((Gly^{1}, Ser^{3,22}, Gln^{4,34}, Thr^{6}, Arg^{19}, Tyr^{21}, Ala^{23,31}, Aib^{32})PP); 100nM, Bachem, Bubendorf, Switzerland) were also tested 1h before the exposure to glutamate. Cells were incubated with antagonists of NPY receptors (Y_1 - BIBP3226; Y_2- BIIE0246 and Y_5 - L-152,804; 1µM, Tocris Bioscience, Bristol, UK) 30min before incubation with the agonists of these receptors.

Inhibitors of key proteins in important signaling pathways were used to elucidate the signaling pathways mediating the neuroprotective effect of NPY against glutamate, The

inhibitors were introduced 1h before glutamate addition. H89 (1µM, Merck Millipore, MA, USA) was used as a Protein Kinase A (PKA) inhibitor (Tocris Bioscience). SB203580 (1µM), L-NAME (500µM), Calphostin C (10nM), LY294002 (1µM) and U0126 (1µM) were used as inhibitors of p38K inhibitor, NOS (Nitrate Oxide Synthase), PKC (Protein Kinase C), PI3K (Phosphoinositide 3-Kinase) and MEK1/2 proteins (Merck Millipore), respectively. After 24h exposure to glutamate, cells were washed twice and incubated with PI (7.5µM) for 10min, washed again twice and fixed with 4% PFA for 20min. Cells were then observed with a fluorescence microscope (Zeiss Axioshop 2 Plus) coupled to an Axiocam HRc camera. The number of PI-positive cells was counted in six random fields on each coverslip (2 per condition), and the average number of PI-positive cells per random field was determined for each condition tested.

3.3.3 Terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) assay

Cells cultured on coverslips were exposed to 500µM glutamate for 24h, with the drugs described above. After incubation, cells were washed twice and then incubated for 1h at 37ºC with the TUNEL mix (*in situ* cell death kit; Roche Applied Science, Mannheim, Germany), washed again, and, finally, nuclei were stained with Hoechst33342 for 5min. Coverslips were mounted in DAKO mounting media and images were acquired on a Zeiss PALM Microscope. The number of TUNEL-positive cells was counted in six random fields on each coverslip (2 per condition) and the average number of TUNEL-positive cells per random field was determined for each condition tested.

3.4 Statistical analysis

All data are presented as mean±S.E.M. Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's post-tests, as indicated in the figure legends.

4. RESULTS

4.1 NPY protects neurons against necrotic and apoptotic cell death induced by glutamate

Necrotic and late apoptotic cell death of cultured rat retinal neural cells was evaluated by propidium iodide (PI) uptake assay. Retinal cells were exposed to 100, 250 or 500µM glutamate for 24h (Fig. 3.1A, B). The number of PI-positive cells in coverslips exposed to 100, 250 or 500 µM of glutamate was 175.7± 27.1%, 364.7±64.4% and 617.3±71.7% of the control, respectively. These results indicate that the cell viability decreases significantly with increased glutamate concentrations. To investigate the potential neuroprotective role of NPY against glutamate induced-toxicity, retinal cells were incubated with NPY (100nM) at three different times: 1h before the incubation with glutamate (100, 250 and 500µM), simultaneously with the addition of glutamate (500µM), and 30min after the exposure to glutamate (500µM). NPY did not affect the increase in the number of PI-positive cells induced by exposure to 100µM glutamate, since the number of PI-positive cells in this case in the presence NPY (179.9± 25.0% of control - NPY applied 1h before glutamate) was similar to that when they were exposed to glutamate alone. When cells were exposed to 250µM glutamate, there was a tendency, although not significant, for a protective effect of NPY when applied before glutamate. A neuroprotective effect of NPY was observed when 500µM glutamate stimulus was applied. When cells were exposed to 500µM glutamate, and NPY (100nM) was added 1 h before glutamate, there was a significant neuroprotective effect of NPY, as shown by a decrease on the number of PI-positive cells to 409.4±41.8% of the control (Fig. 3.1A, Bd), which can be compared with the glutamate condition (617.3±71.7% of control), indicating a decrease in the number of PI-positive cells of 34%. However, when cells were exposed to NPY, either simultaneously or 30min after adding 500µM glutamate, the neuroprotective effect was lost. Under the two conditions, the number of PI-positive cells was 614.7±80.5% and 756.9±78.0% of control, respectively, similar to percentage found

when cells were exposed to 500µM glutamate (617.3±71.7% of control). The exposure of cells to NPY alone did not change cell viability. Based on these results, 100nM NPY was applied 1h before glutamate (500µM) for the subsequent experiments.

The effects of glutamate and/or NPY treatments on the total number of cells were also assessed (Fig. 3.1C). For this, cells were stained with Hoechst33342, and then the fluorescence intensity (arbitrary units) was measured. Glutamate (500µM, 24h) was found to decrease the Hoechst 33342-fluorescence intensity to 50.8±7.0% of the control (untreated cells). NPY partially prevented this effect triggered by glutamate, since the decrease in fluorescence intensity was attenuated by NPY (75.4±9.8% of control). NPY *per se* did not affect Hoechst-fluorescence intensity (Fig. 3.1C).

Apoptotic cell death was assessed using the TUNEL assay to obtain a better characterization of the protective role of NPY against retinal cell death caused by exposure to glutamate (Fig. 3.1D, E). Glutamate (500µM) increased the number of apoptotic cells to 294.1±41.7% of the control. When NPY (100nM) was applied 1h before glutamate, the increase in the number of TUNEL-positive cells triggered by glutamate was reduced to 206.2±32.6% of the control, representing a 30% reduction. Again, NPY *per se* had no effect on cell death since it did not affect the number of TUNEL-positive cells compared with the control.

In addition, while glutamate (500µM, 24h) increased the number of active caspase 3-positive cells to 201.9±12.8% of the control (Fig. 3.2A, Bc), NPY pre-treatment reduced the increase in the number of caspase 3-positive cells triggered by glutamate to 120.7±16.7% of the control (Fig. 3.2Bd). NPY did not affect caspase 3 activation (Fig. 3.2Bb).

To further elucidate the protective effect of NPY against glutamate-induced cell death, while taking into account the fact that these cell cultures are mixed ones, composed of neurons, macroglial and microglial cells, we evaluated, by immunocytochemistry, which cell types could be most strongly affected by glutamate and, eventually, protected by NPY (Fig. 3.3, 3.4). To quantify the effects of glutamate and NPY on different cell types,

the immunoreactivity (fluorescence intensity) of different cell markers was measured. Under control conditions (untreated cells), a normal distribution of TUJ1-positive neurons was observed (Fig. 3.3Ca). When cells were exposed to 500µM glutamate for 24h, the number of neurons decreased and their neurite integrity was dramatically affected (Fig. 3.3Cc). Determination of TUJ-1-immunoreactivity (Fig. 3.3A) showed that glutamate induced significant decrease in the content of this neuronal marker to 26.0±4.9% of the control (Fig. 3.3A). In cells pre-incubated with NPY before glutamate application, the decrease in TUJ-1 immunoreactivity was attenuated (49.2±8.5% of control), compared with cells just exposed to glutamate. NPY *per se* did not affect TUJ-1 immunoreactivity (Fig. 3.3Ca, Cb).

To evaluate the effects of glutamate and NPY on macroglial cells, we analyzed the immunoreactivity of GFAP, a macroglial cell (astrocytes and Müller cells) marker. The morphology of GFAP-positive cells, compared with control cells, changed markedly when they were exposed to glutamate (Fig. 3.3B, D). A decrease in the number of cells, as well as in the number of cell processes, was clearly observed, in addition to an increase of their thickness (Fig. 3.3Dc, Da). However, quantification of GFAP immunoreactivity revealed no significant differences between cells exposed to glutamate and controls. NPY was unable to significantly prevent the alterations in the morphology of GFAP-positive cells triggered by glutamate (Fig. 3.3Dc). NPY alone did not affect either the morphology of GFAP-positive cells or the intensity of GFAP immunoreactivity (Fig. 3.3Dd).

The effects of glutamate and NPY on microglial cells were assessed by analyzing the immunoreactivity of two microglial cell markers: CD11b and CD68/ED1 (Fig. 3.4). CD11b labels resting and activated microglial cells, while ED1 is a marker of activated microglia (Streit, 2005). Two different parameters were evaluated for these markers: both the number of CD11b- and CD68/ED1-positive cells per field, and the CD11b- or CD68/ED1-immunoreactivity (Fig. 3.4). NPY increased the number of microglial cells (resting and activated; Fig.4Cb and Fb). Similarly, glutamate or NPY plus glutamate also increased

the number of CD11b- and CD68/ED1-positive cells. As with the results obtained for the number of CD11b-positive cells, the fluorescence intensity measurements showed that NPY, glutamate and NPY *plus* glutamate increased the immunoreactivity of CD11b- and CD68/ED1-positive cells (Fig. 3.4B, E).

4.2 Activation of NPY Y_2, Y_4 or Y_5 receptors inhibits the increase in necrotic cell death induced by glutamate

We have evaluated the effects of NPY receptor agonists and antagonists to determine which NPY receptors could be mediating the protective role of NPY against necrotic cell death induced by glutamate (Fig. 3.5A, B). In this analysis, we compared the number of PI-positive cells for each experimental condition with the number of PI-positive cells in cultures exposed to glutamate, taken as 100%. NPY decreased the number of PI-positive cells to 72.4±3.7% relative to glutamate. The NPY Y_1 receptor agonist ([Leu^{31},Pro^{34}]NPY) did not inhibit glutamate-induced necrotic cell death (Fig. 3.5A, B). However, the NPY Y_2 receptor agonist (NPY_{13-36}) inhibited the increase in PI-positive cells (68.8±6.4%, compared with glutamate; Fig. 3.5A). This protective effect was partially prevented by the NPY Y_2 receptor antagonist BIIE0246 (83.4±7.2% compared with glutamate condition). Furthermore, the NPY Y_4 agonist (r-PP, 100nM) also partially protected retinal cells exposed to glutamate, as shown by the number of PI-positive cells decreasing to 60.2±15.5% relative to glutamate condition. In addition, the NPY Y_5 receptor agonist (Gly^1, $Ser^{3,22}$, $Gln^{4,34}$, Thr^6, Arg^{19}, Tyr^{21}, $Ala^{23,31}$, Aib^{32})PP) also exerted a protective effect, as seen by the increase in the number of PI-positive cells induced by glutamate, which was attenuated to 73.0±4.4%, compared with glutamate alone (Fig. 3.5A, B). This effect was completely blocked by the NPY Y_5 receptor antagonist. The NPY receptor agonists or antagonists *per se* did not increase the number of PI-positive cells, compared to control (data not shown).

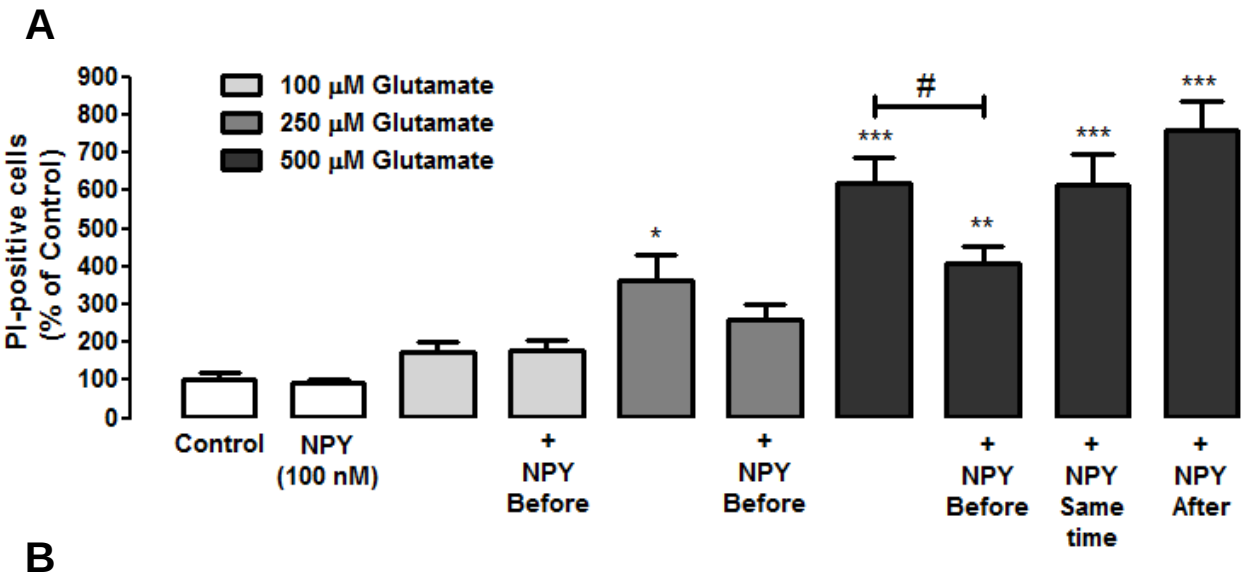
4.3 NPY Y_5 receptor activation inhibits apoptotic retinal cell death induced by glutamate

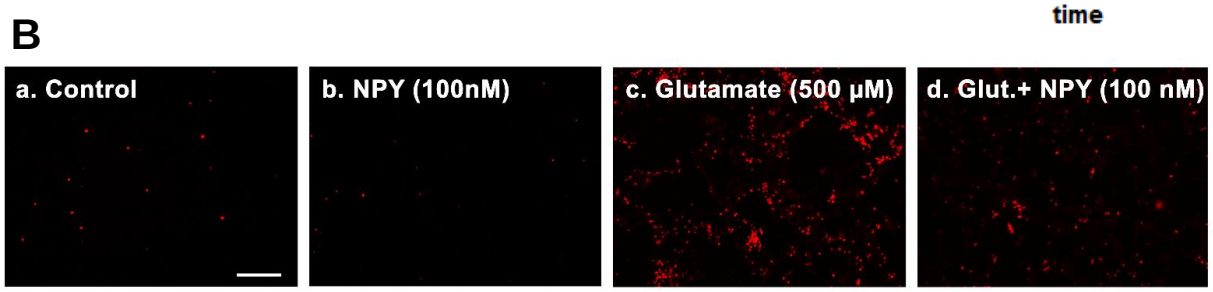
We have evaluated the potential neuroprotective effect of NPY receptor agonists against the increase in apoptotic cells (TUNEL-positive cells) number by exposure to glutamate. NPY was able to reduce 30% the number of apoptotic cells to 69.7%±3.8%, compared with glutamate conditions. NPY receptor agonists and antagonist were used to investigate those involved in this neuroprotective effect (Fig. 3.6A, B). The NPY Y_5 receptor agonist mimicked the effect of NPY, inhibiting the increase in the number of TUNEL-positive cells triggered by glutamate; the percentage of apoptotic cells decreased to 68.2±6.0%, compared to the glutamate condition (Fig. 3.6A, B). This effect was completely blocked by the NPY Y_5 receptor antagonist (L-152,804). The activation of NPY Y_1, Y_2 or Y_4 receptors by their selective agonists did not decrease the number of TUNEL-positive cells in cultures exposed to glutamate. NPY receptors agonists and antagonist alone did not increase the number of TUNEL-positive cells, compared to control (data not shown).

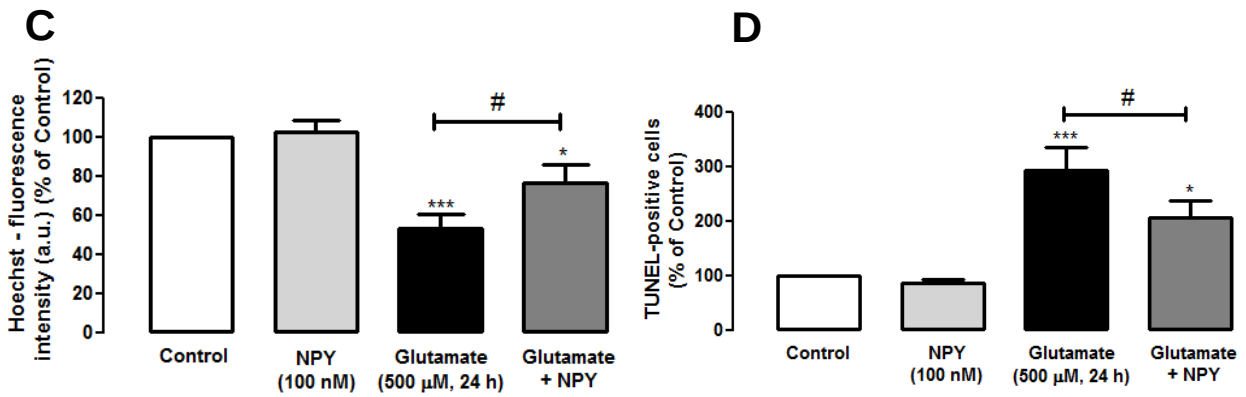
4.4 PKA and p38K proteins mediate the neuroprotective effect of NPY against glutamate-induced necrotic retinal neural cell death

Inhibitors of key proteins in different intracellular pathways were used to elucidate the intracellular pathways that mediate the neuroprotective effect of NPY when cells are exposed to glutamate or/and NPY (Fig. 3.7). The PKA inhibitor, H89 (1µM), prevented the neuroprotective effect of NPY (63.2±5.5%, compared with glutamate condition). The number of PI-positive cells exposed to glutamate, or to glutamate *plus* NPY and H89, was similar. In order to confirm the involvement of PKA in the neuroprotective effect of NPY against glutamate-induced excitotoxicity, we have evaluated the effect of the PKA activator, forskolin (10µM), with cells exposed to glutamate. Forskolin decreased the number of PI-positive cells (69.6±7.1%, compared with glutamate) to a similar extent as

NPY (63.2±5.5%, compared with glutamate; Fig 3.7). The protective effect of NPY against the increase of PI-positive cells triggered upon exposure to glutamate, was also partially prevented (85.6 ±2.7%, compared with glutamate) by the presence of the p38K inhibitor (SB203580; Fig. 3.7). The inhibitors of nitric oxide synthase (NOS), PKC, PI3K and MEK1/2, namely L-NAME, calphostin C, LY294002 and U0126, respectively, did not affect the neuroprotective effect of NPY against glutamate-induced toxicity (Fig. 3.7). The inhibitors used in these experiments did not cause any toxic effect on retinal cells, as seen by the number of PI-positive cells with cultures exposed to them being similar to that found in control conditions (data not shown).


A
PI-positive cells (% of Control)
100 µM Glutamate
250 µM Glutamate
500 µM Glutamate
Control
NPY (100 nM)
+ NPY Before
+ NPY Before
+ NPY Before
+ NPY Same time
+ NPY After


B
a. Control
b. NPY (100nM)
c. Glutamate (500 µM)
d. Glut.+ NPY (100 nM)


C
Hoechst - fluorescence intensity (a.u.) (% of Control)
Control
NPY (100 nM)
Glutamate (500 µM, 24 h)
Glutamate + NPY
D
TUNEL-positive cells (% of Control)
Control
NPY (100 nM)
Glutamate (500 µM, 24 h)
Glutamate + NPY

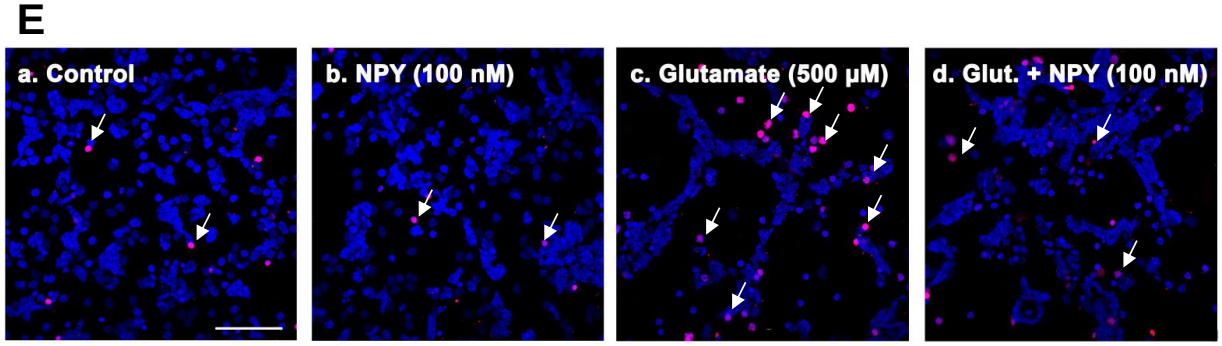

E
a. Control
b. NPY (100 nM)
c. Glutamate (500 µM)
d. Glut. + NPY (100 nM)

Fig. 3.1 – NPY protects against necrotic and apoptotic retinal cell death induced by glutamate. Necrotic (A, B) cells were assessed by Propidium Iodide (PI) incorporation assay. Cell nuclei were stained by Hoechst 33342 (C). Apoptotic (D, E) cells were assessed by TUNEL assay, respectively. (A) Quantification of PI-positive cells (% of control). Retinal cells were exposed to different concentrations of glutamate (100, 250 and 500µM) for 24h and treated with NPY (100nM) at three different time points: 1h before, simultaneously, and 30min after glutamate, as indicated below bars. The results represent the mean±S.E.M of n=4-11 independent experiments; ***p<0.001, **p<0.01, *p<0.05, compared with control; #p<0.05, compared with glutamate (500 µM); One way ANOVA followed by Bonferroni's post-hoc test. (B) Representative images of control, and cultures treated with NPY, glutamate or glutamate + NPY (1h before), showing PI positive cells (red spots), Scale bar: 100µm. (C) Quantification of fluorescence intensity (arbitrary units) of cells stained with Hoechst 33342 (nucleus marker), compared with control (no drug). These results represent the mean±S.E.M. of n=21-27 independent experiments; ***p<0.001, *p<0.05, compared with control; $^{\#}$p<0.05, compared with glutamate; One way ANOVA followed by Bonferroni's post-hoc test. (D) Quantification of TUNEL-positive cells (% of control). Cultured retinal cells were exposed to glutamate and treated with NPY (1h prior glutamate exposure), as indicated below bars. Data represent the mean±S.E.M. of n=5-6 independent experiments; *** p<0.001, *p<0.05, compared with control; #p<0.05, compared with glutamate; One way ANOVA followed by Bonferroni's post-hoc test. (E) Representative images of control, and cultures treated with NPY, glutamate or glutamate + NPY (1h before), showing TUNEL-positive cells (purple spots, indicated by white arrows) and cell nuclei stained with Hoechst 33342 (blue); Scale bar: 50µm.

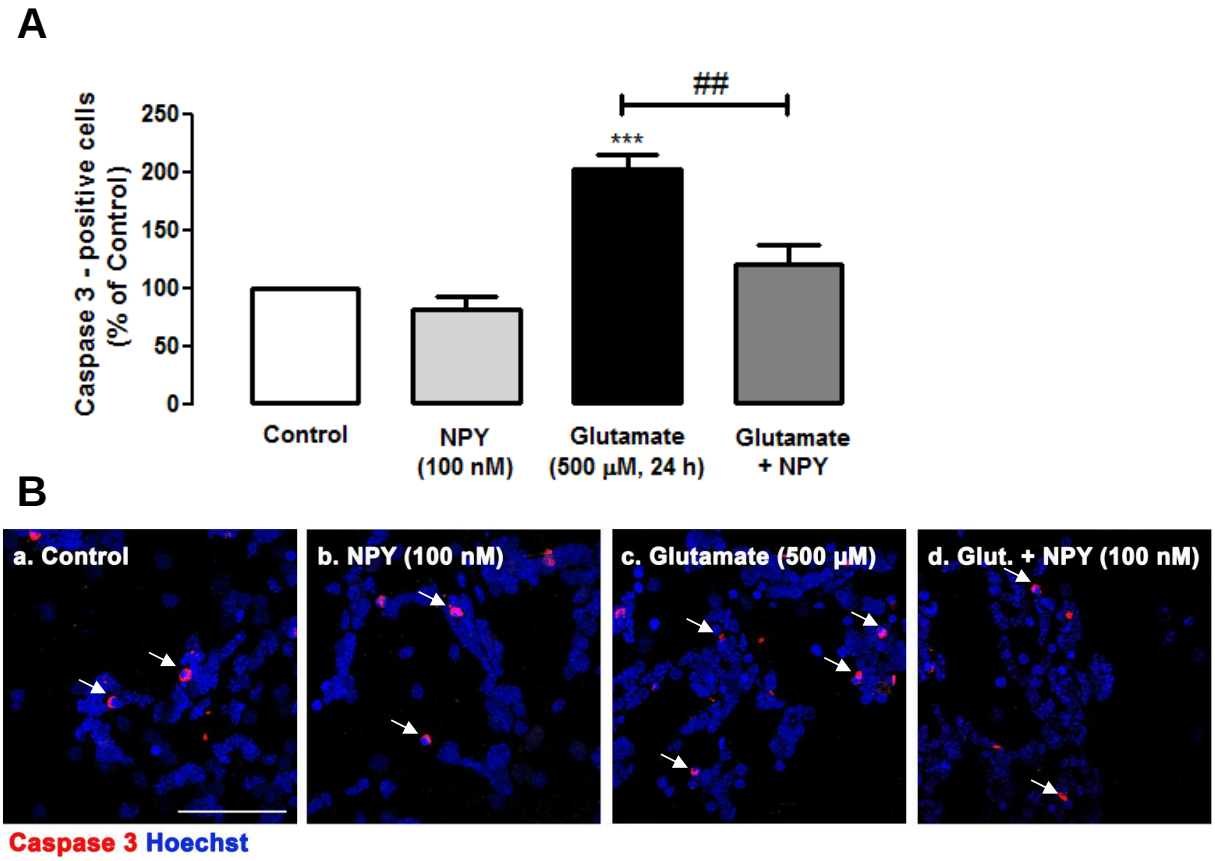


Fig. 3.2 - NPY inhibits the increase in the activation of caspase 3 in retinal neural cells induced by glutamate. Caspase 3-mediated apoptotic cell death was evaluated by immunocytochemistry against cleaved caspase 3 in rat retinal cells. Cells were exposed to NPY (100nM) 1 h prior to glutamate (500µM). (A) Quantification of cleaved caspase-3 positive cells (red) per field compared to control conditions (100%; no drug, Aa). The results represent the mean±S.E.M. of n=4-5 independent experiments; ***p<0.001, compared with control; $^{\#\#}$p<0.01, compared with glutamate; One way ANOVA followed by Bonferroni's post-hoc test. (B) Representative images of control, and cultures treated with NPY, glutamate or glutamate + NPY (1 h before), showing cleaved caspase 3-positive cells (purple spots). Cell nuclei were stained with Hoechst 33342 (blue). Scale bar: 50µm.

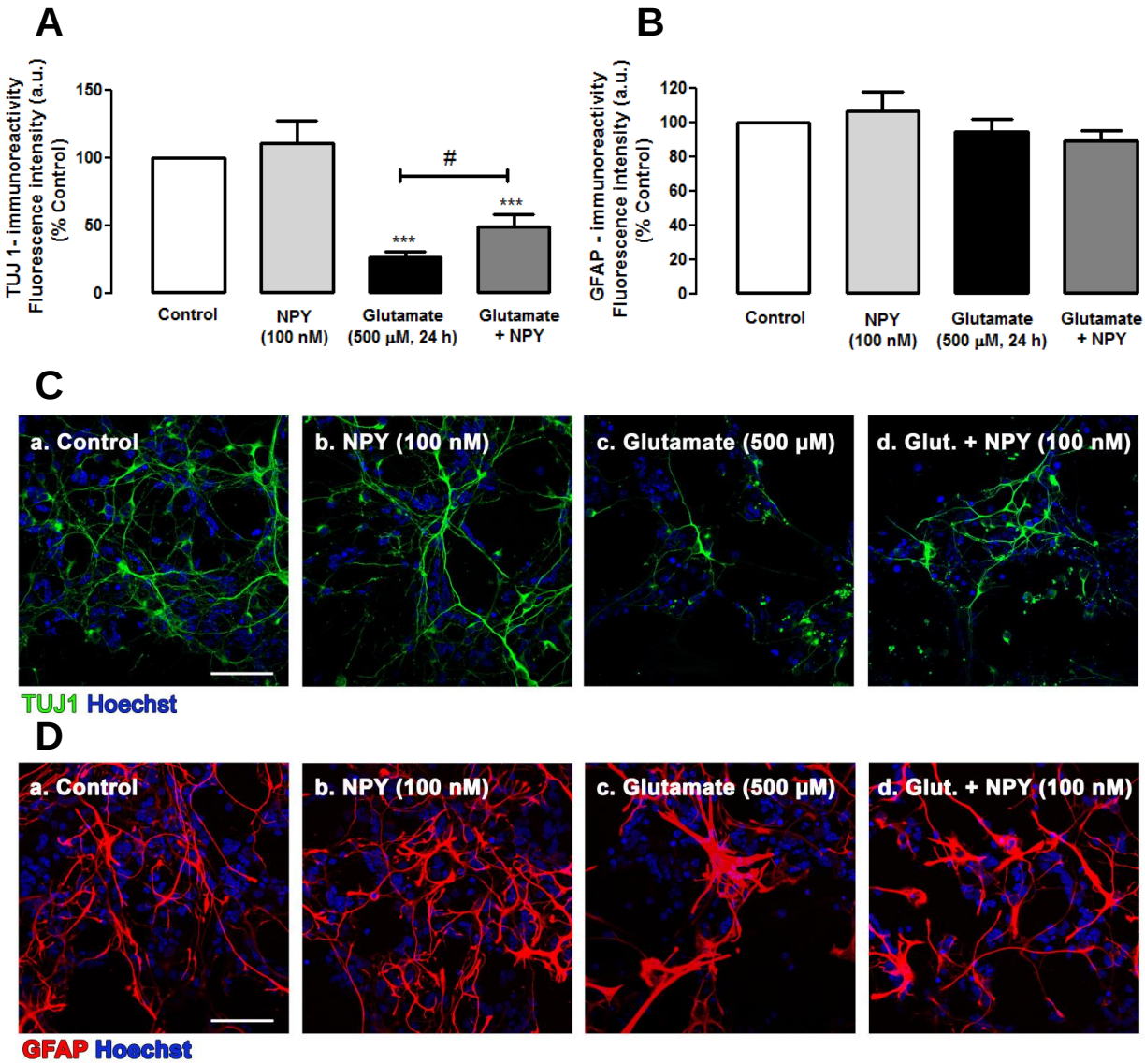


Fig. 3.3 - **NPY protects neuronal cell death induced by glutamate in rat retinal neural cell cultures.** Neurons and macroglial cells were identified with anti-TUJ1 (C - green) and anti-GFAP (D - red) antibodies, respectively. (A) Quantification of TUJ 1-immunoreactivity by fluorescence intensity (arbitrary units), compared to control conditions (100%; no drug, Ca). The results represent the mean±S.E.M. of n=4-8 independent experiments ***p<0.001, compared with control; $^{\#}$p<0.05, compared with glutamate; One way ANOVA followed by Bonferroni's post-hoc test. (B) Quantification of GFAP-immunoreactivity by fluorescence intensity (arbitrary units), compared to control conditions (100%; no drug, Da). (C) Representative images of control, and cultures treated with NPY, glutamate or glutamate + NPY, showing TUJ1 positive cells (green). Cell nuclei were identified by Hoechst 33342 staining (blue). (D) Representative images of control, and cultures treated with NPY, glutamate or glutamate + NPY, showing GFAP-positive cells (red). Cell nuclei were stained with Hoechst 33342 (blue). Scale bar: 50µm.

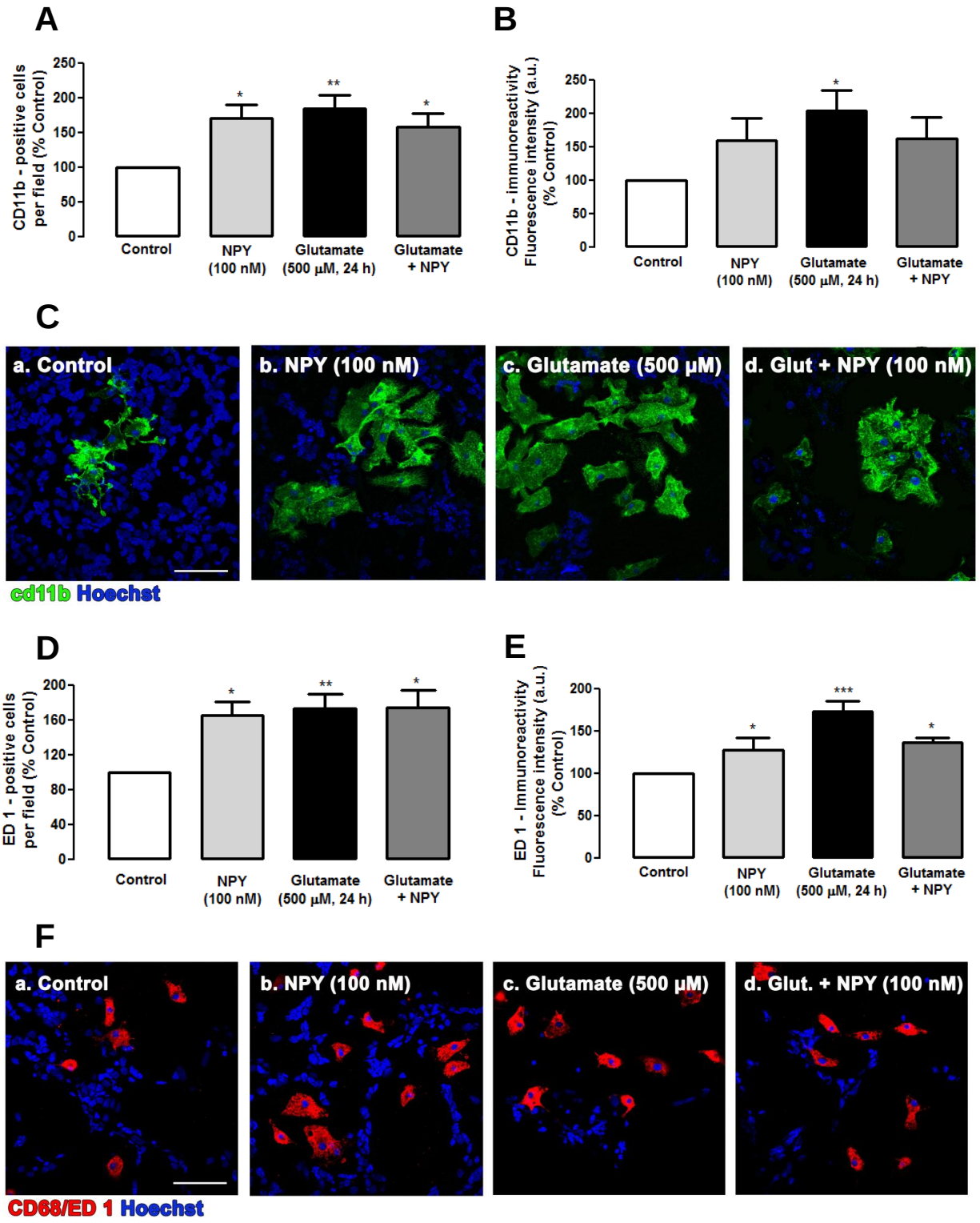
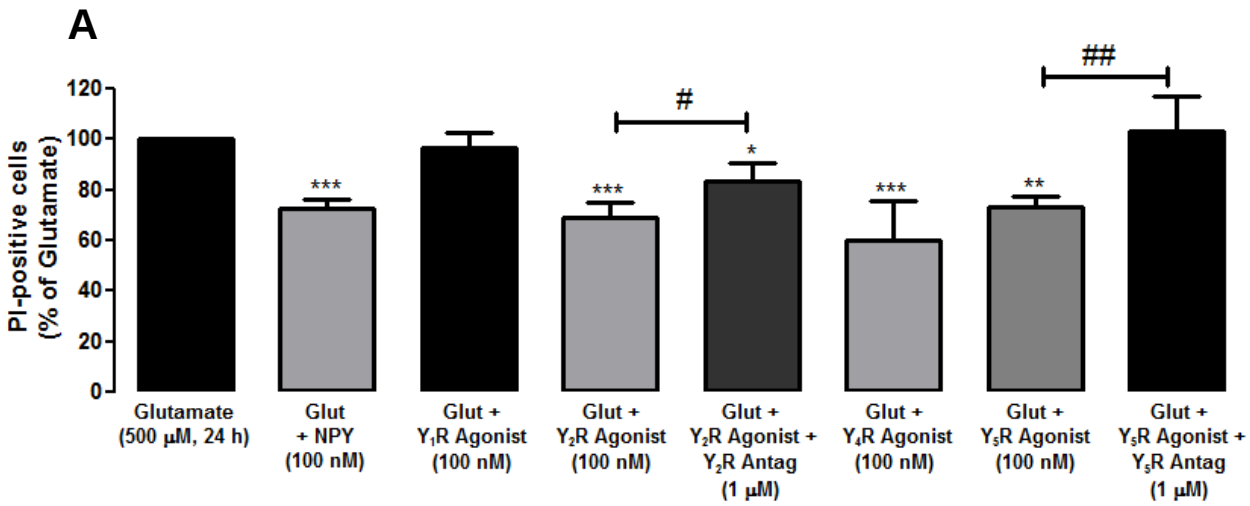

A
CD11b - positive cells per field (% Control)
250
200
150
100
50
0
Control
NPY (100 nM)
Glutamate (500 µM, 24 h)
Glutamate + NPY
B
CD11b - immunoreactivity Fluorescence intensity (a.u.) (% Control)
C
a. Control
b. NPY (100 nM)
c. Glutamate (500 µM)
d. Glut + NPY (100 nM)
cd11b Hoechst
D
ED 1 - positive cells per field (% Control)
E
ED 1 - Immunoreactivity Fluorescence intensity (a.u.) (% Control)
F
a. Control
b. NPY (100 nM)
c. Glutamate (500 µM)
d. Glut. + NPY (100 nM)
CD68/ED 1 Hoechst

Fig. 3.4 – Glutamate and NPY increase the proliferation and activation of retinal microglial cells. Microglial cells were identified by immunocytochemistry using anti-CD11b (C, green) and anti-CD68/ED1 (F, red) antibodies. (A) Quantification of CD11b-positive cells (green) per field, compared to control conditions (no drug, Ca). (B) Quantification of fluorescence intensity (arbitrary units) of CD11b-immunoreactivity, compared with control (100%; no drug, Ca). These results (A & B) represent the mean±S.E.M. n=8 of independent experiments, with **p<0.01, *p<0.05, compared with control; One way ANOVA followed by Bonferroni's post-hoc test. (C) Representative images of control, and cultures treated with NPY, glutamate or glutamate + NPY, showing CD11b- positive cells (green). Cell nuclei were stained by Hoechst 33342 (blue). Scale bar: 50µm. (D) Quantification of CD68/ED1-positive cells per field, compared to control (100%; no drug, Da). (E) Quantification of fluorescence intensity (arbitrary units) of CD68/ED1-immunoreactivity, compared with control (100%; no drug, Da). These results (D & E) represent the mean±S.E.M. n=5 of independent experiments; ***p<0.001, **p<0.01, *p<0.05, compared with control; One way ANOVA followed by Bonferroni's post-hoc test. (F) Representative images of control, and cultures treated with NPY, glutamate or glutamate + NPY, showing CD 68/ED1-positive cells. Cell nuclei were stained by Hoechst 33342 (blue). Scale bar: 50µm.


A
PI-positive cells (% of Glutamate)
120
100
80
60
40
20
0
***
***
*
***
**
#
##
Glutamate (500 µM, 24 h)
Glut + NPY (100 nM)
Glut + Y1R Agonist (100 nM)
Glut + Y2R Agonist (100 nM)
Glut + Y2R Agonist + Y2R Antag (1 µM)
Glut + Y4R Agonist (100 nM)
Glut + Y5R Agonist (100 nM)
Glut + Y5R Agonist + Y5R Antag (1 µM)

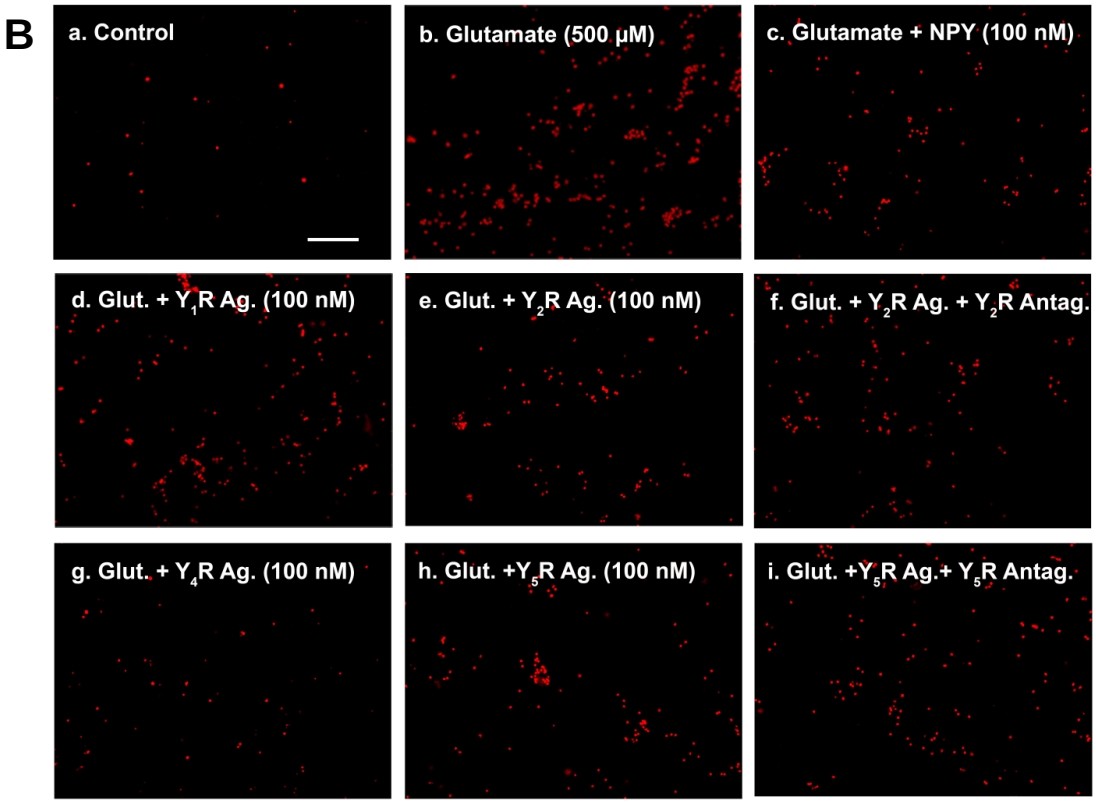

B
a. Control
b. Glutamate (500 µM)
c. Glutamate + NPY (100 nM)
d. Glut. + Y1R Ag. (100 nM)
e. Glut. + Y2R Ag. (100 nM)
f. Glut. + Y2R Ag. + Y2R Antag.
g. Glut. + Y4R Ag. (100 nM)
h. Glut. +Y5R Ag. (100 nM)
i. Glut. +Y5R Ag.+ Y5R Antag.

Fig. 3.5 - The activation of NPY Y_2, Y_4 and Y_5 receptors inhibits the necrotic cell death induced by glutamate. Necrotic cells were evaluated by Propidium Iodide (PI) incorporation assay. Cells were exposed to glutamate, and treated with NPY, or NPY receptor agonists and antagonists, indicated below bars. (A) Quantification of PI-positive cells (% of glutamate condition) per field in retinal cell cultures treated with NPY Y_1 receptor agonist ([Leu^{31},Pro^{34}]NPY; 100nM); NPY Y_2 receptor agonist (NPY_{13-36}; 100nM) and antagonist (BIIE 0246; 1µM); NPY Y_4 agonist receptor (r-PP, 100nM); NPY Y_5 receptor agonist ((Gly[1], $Ser^{3,22}$, $Gln^{4,34}$, Thr^6, Arg^{19}, Tyr^{21}, $Ala^{23,31}$, Aib^{32})PP) and antagonist (L-152,804; 1µM). (B) Representative images of control, and cultures treated with glutamate, glutamate + NPY, glutamate + Y_1R agonist, glutamate + Y_2R agonist, glutamate + Y_2R agonist + Y_2R antagonist, glutamate + Y_4R agonist, glutamate + Y_5R agonist, and glutamate + Y_5R agonist + Y_5R, showing PI-positive cells (red spots). Scale bar: 100µm. Values are expressed as the percentage of PI-positive cells per field compared to the glutamate condition. The results represent mean±S.E.M. n=4-11 of independent experiments; ***p<0.001, **p<0.01, compared with glutamate; $^{###}$p<0.001, $^{##}$p<0.01, compared with glutamate + NPY receptor agonist; One way ANOVA followed by Bonferroni's post-hoc test.

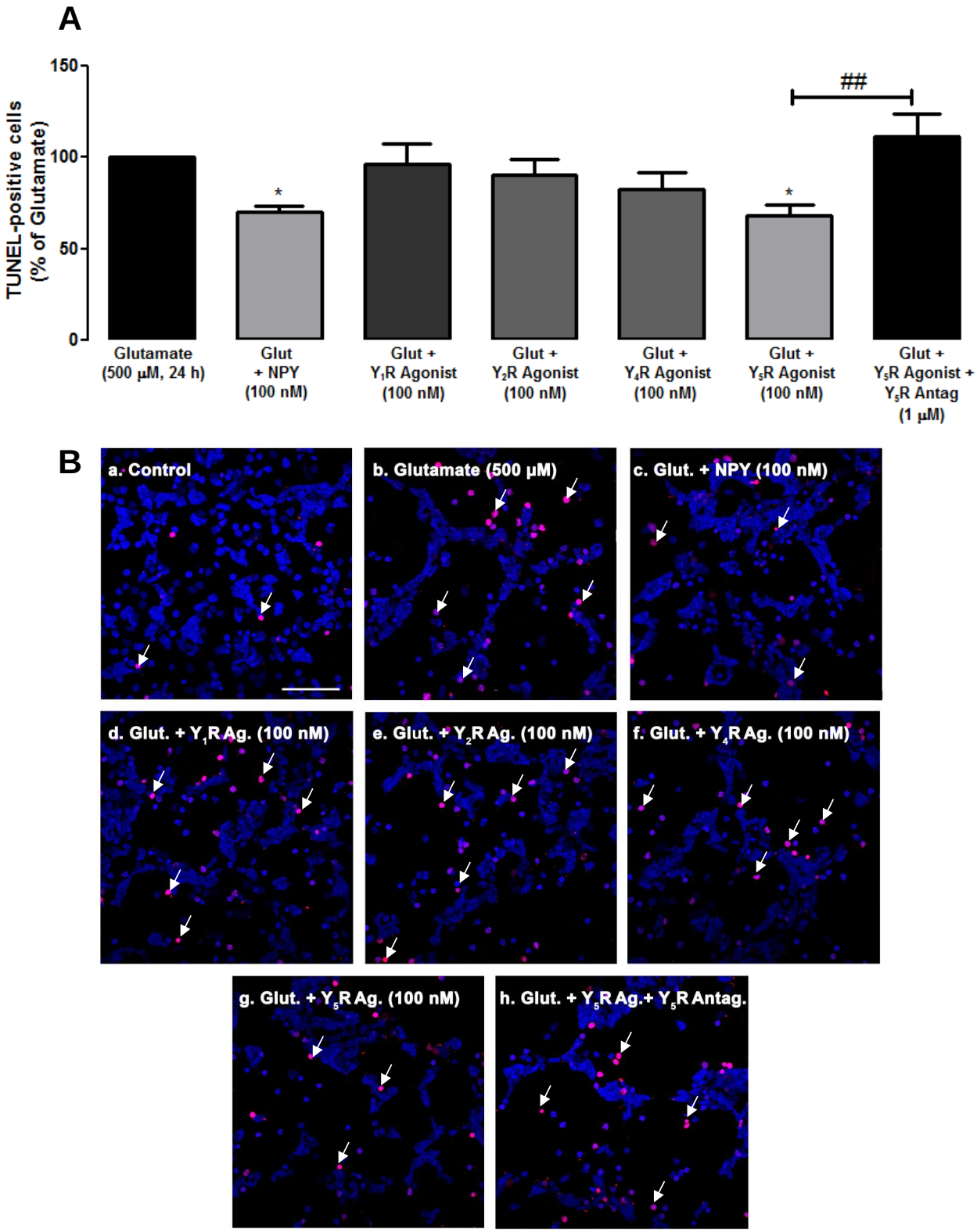

A
TUNEL-positive cells (% of Glutamate)
150
100
50
0
*
*
##
Glutamate (500 µM, 24 h)
Glut + NPY (100 nM)
Glut + Y1R Agonist (100 nM)
Glut + Y2R Agonist (100 nM)
Glut + Y4R Agonist (100 nM)
Glut + Y5R Agonist (100 nM)
Glut + Y5R Agonist + Y5R Antag (1 µM)
B
a. Control
b. Glutamate (500 µM)
c. Glut. + NPY (100 nM)
d. Glut. + Y1R Ag. (100 nM)
e. Glut. + Y2R Ag. (100 nM)
f. Glut. + Y4R Ag. (100 nM)
g. Glut. + Y5R Ag. (100 nM)
h. Glut. + Y5R Ag.+ Y5R Antag.

Fig. 3.6 – The activation of NPY Y_5 receptor inhibits the apoptotic cell death induced by glutamate. Apoptotic cells were assessed by TUNEL assay. Cells were exposed to glutamate, and treated with NPY, or NPY receptor agonists and antagonists, as indicated below bars. (A) Quantification of TUNEL-positive cells per field compared to glutamate condition (100%) in retinal cell cultures treated with NPY Y_1 receptor agonist ([Leu^{31},Pro^{34}]NPY; 100nM); NPY Y_2 receptor agonist (NPY_{13-36}; 100nM); NPY Y_4 agonist receptor (r-PP, 100nM); NPY Y_5 receptor agonist ((Gly^{1}, Ser^{3,22}, Gln^{4,34}, Thr^{6}, Arg^{19}, Tyr^{21}, Ala^{23,31}, Aib^{32})PP; 100nM) and antagonist (L-152,804; 1 µM). (B) Representative images of control, and cultures treated with glutamate, glutamate + NPY, glutamate + Y_1R agonist, glutamate + Y_2R agonist, glutamate + Y_4R agonist, glutamate + Y_5R agonist, and glutamate + Y_5R agonist + Y_5R antagonist, showing TUNEL-positive cells (purple spots – some examples are indicated by white arrows). Scale bar: 50µm. Values are expressed as the percentage of TUNEL-positive cells per field compared to the glutamate condition. The results represent mean±S.E.M. n=5-6 of independent experiments, with; $p<0.05$, compared with glutamate; ##$p<0.01$, compared with glutamate +Y_5R agonist; One way ANOVA followed by Bonferroni's post-hoc test.

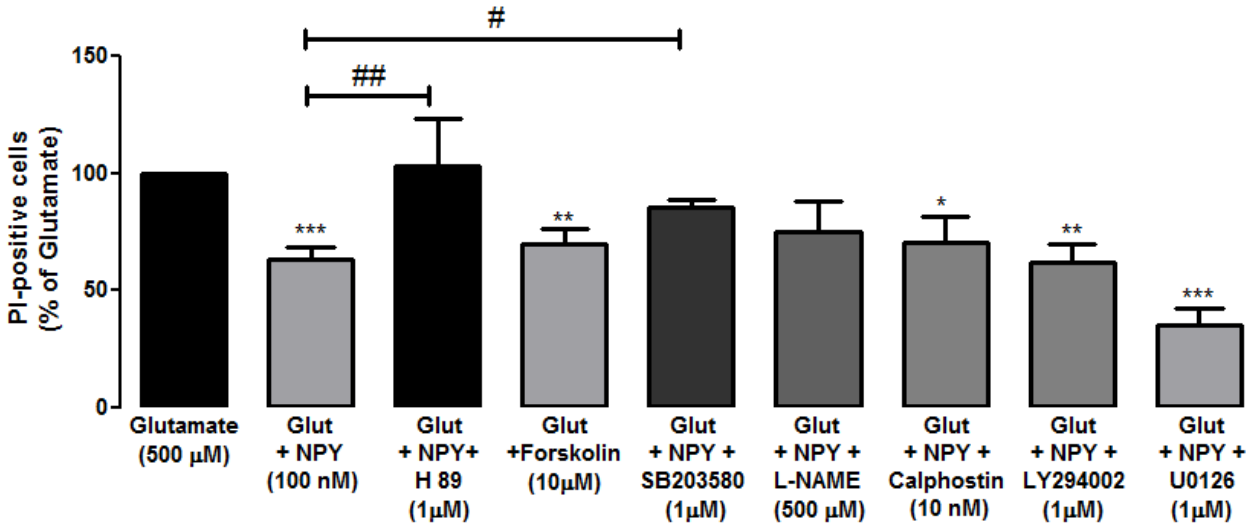


Fig. 3.7 – Protein Kinase A (PKA) and protein 38 Kinase (p38K) mediate the neuroprotective effect of NPY against retinal neuronal cell death triggered by glutamate. The involvement of different intracellular pathways in the neuroprotective effect of NPY against glutamate-induced excitotoxicity was assessed by Propidium iodide (PI) uptake (PI–positive cells), using different inhibitors of proteins involved on those pathways. Retinal cell cultures were exposed to NPY (100nM), glutamate (500µM) and the inhibitors indicated below bars. Quantification of PI-positive cells (compared to glutamate condition) in retinal cells treated with H89 (1µM; PKA inhibitor), forskolin (10µM; PKA activator), SD203580 (1µM; p38K inhibitor), L-NAME (500µM; nitric oxide synthase inhibitor), calphostin C (10nM; PKC inhibitor), LY294002 (1µM; PI3K inhibitor) and U0126 (1µM; MEK1/2 inhibitor). Values are expressed as the percentage of PI-positive cells (per field), compared to the glutamate condition. The results represent the mean±S.E.M. n=7-9 of independent experiments, with ***p<0.001, **p<0.01, *p<0.05, compared with glutamate; ##p<0.01, #p<0.05, compared with glutamate + NPY; One way ANOVA followed by Bonferroni's post-hoc test.

5. DISCUSSION

In this study, we investigated the protective role of NPY and NPY receptors against glutamate-induced neural cell death in rat retinal neural cell. Glutamate triggered necrosis and apoptosis in retinal cells, and NPY was able to inhibit both processes. Moreover, we have demonstrated that NPY Y_2, Y_4 and Y_5 receptors mediate the protective effect of NPY against necrotic cell death caused by glutamate, and that NPY Y_5 receptor mediated the NPY protective effect against apoptotic cell death induced by glutamate. Finally, we have shown that the neuroprotective effect of NPY is mediated by PKA and p38K. These findings suggest that NPY can be viewed as a potential new target to protect retinal cells in retinal corresponding degenerative diseases, such as glaucoma or diabetic retinopathy.

Previous studies have shown that NPY can exert neuroprotective effects against excitotoxicity triggered by glutamate or glutamate receptor agonists in various regions of the CNS, such as hippocampus and striatum (Silva et al., 2003b; Silva et al., 2005a; Xapelli et al., 2007; Smialowska et al., 2009; Decressac et al., 2012). In addition, we have previously shown that NPY protects against MDMA (ecstasy) toxicity in cultured rat retinal cells (Alvaro et al., 2008b). We extend this to show, for the first time, that NPY is able to protect necrotic and apoptotic cell death induced by glutamate in retinal cells. However, the neuroprotective effect of NPY only occurs when the peptide is applied before the excitotoxic stimulus, and is not present when it is added simultaneously or after glutamate. This is consistent with the majority of studies describing a protective role of NPY, where the peptide was applied prior to the toxic stimulus (Silva et al., 2003b; Thiriet et al., 2005; Alvaro et al., 2008b). However, some reports have also indicated that NPY is effective when is applied a few hours after the excitotoxic stimulus (Wu et al., 2005; Xapelli et al., 2007; Smialowska et al., 2009).

Glutamate excitotoxicity is characterized morphologically by a decrease in the number of neurons and a reduction in the length of neuronal processes (Mattson et al., 1988;

Doble, 1999). It induced a similar effect in rat retinal cells, which was partially prevented by NPY, specifically in neurons. Visually, glutamate exposure also decreases the number of macroglial cells. It was not possible quantify directly the number of GFAP-positive cells for technical reasons, and, instead, this was carried out using the immunoreactivity fluorescence intensity of GFAP-positive cells. Despite the visually observable effect of glutamate, the GFAP immunoreactivity fluorescence did not show this decrease, probably due to the increase in the thickness of GFAP-positive cells processes. In contrast to the results obtained with neurons in culture, NPY had minor effects in the prevention of this glutamate toxic effect. Some studies indicate that Müller cells play a dual role under toxic conditions. When threatened, these cells can be either neuroprotective or contribute to exacerbate the excitotoxic stimuli (reviewed in (Bringmann et al., 2011)). In the present study, glutamate dramatically changed the morphology of GFAP-positive cells, increasing the thickness of their processes, also causing the loss of cell processes and a decrease in the number of GFAP-positive cells.

The microglial cell response to glutamate exposure was completely different. Glutamate increased the number of CD11b- and CD68/ED1-positive cells, as well as the immunoreactivity of these two markers. Glutamate and glutamate receptor agonists are known to activate microglial cells in CNS, such as the hippocampus (Heppner et al., 1998; Christensen et al., 2006). In the present work, both glutamate and NPY increased microglial cell proliferation, as well as microglia activation. When the NPY and glutamate were present, the effect on microglia proliferation and activation was not enhanced. In contrast, other groups have reported inhibition by NPY of microglia phagocytosis and cell motility upon inflammatory challenge through the activation of NPY Y_1 receptor (Ferreira et al., 2011a; Ferreira et al., 2011b). In addition, NPY via Y_2 receptors, has a protective role against methamphetamine-induced microgliosis (Goncalves et al., 2012). In the early stages of neurodegenerative processes, the activation of microglia contributes to neuronal protection and tissue regeneration. However, continuous retinal microglial overactivation may lead to chronic inflammation, loss of autoregulatory mechanisms,

irreversible neuronal loss and photoreceptor apoptosis (Zeiss et al., 2004; Zeng et al., 2005; Hanisch et al., 2007; Langmann, 2007). Microglial activation is involved in the initiation and perpetuation of degenerative process in many diseases, such as retinal dystrophies (Langmann, 2007; Karlstetter et al., 2010).

Using pharmacological tools, we have shown that NPY protects retinal cells against necrotic cell death induced by glutamate through the activation of NPY Y_2, Y_4 and Y_5 receptors. In another study, using hippocampal slice cultures, the anti-necrotic effect of NPY was also seen to be mediated by Y_2 and Y_5 receptors. However, NPY Y_1 receptors contributed to the neuroprotective effect of NPY as well (Silva et al., 2003b). Although other studies have suggested that only the NPY Y_1 or Y_2 receptors are involved in the rescue of neurons from excitotoxic cell death (Xapelli et al., 2007; Xapelli et al., 2008), the involvement of NPY Y_4 receptor has not been evaluated in the majority of these.

In the present study, we show that only the NPY Y_5 receptor activation protects against glutamate-induced apoptotic cell death. Another study has linked the antiapoptotic effect of NPY in the hippocampus to the activation of NPY Y_2 and Y_5 receptors (Smialowska et al., 2009). The difference between ours and these results might be due to the differential expression of NPY receptors in retinal and hippocampal cultures, as well as to the involvement of different signaling pathways underlying the neuroprotective effects.

We have also found that three different NPY receptors are involved in the neuroprotective effect against necrotic cell death induced by glutamate in rat retinal cell cultures. Similarly, other groups have also shown that activation of different NPY receptors can induce the same biological effect (Xapelli et al., 2007; Alvaro et al., 2009; Smialowska et al., 2009; Son et al., 2011). For example, NPY inhibits KCl-evoked $[Ca^{2+}]_i$ increase in retinal neurons through the activation of NPY Y_1,Y_4, and Y_5 receptors. There are two possible main explanations for this: 1) the formation of homo- or hetero-dimers between different NPY receptors (Berglund et al., 2003b; Dinger et al., 2003; Silva et al.,

2003a; Movafagh et al., 2006); 2) heterogenous distribution of these receptors through the different cell types present in the culture.

To obtain a better understanding of the intracellular mechanisms underlying the NPY neuroprotective role against necrotic cell death induced by glutamate, we have looked at the possible involvement of various pathways. The NPY protective effect may be linked to its inhibitory effect on glutamate release, as found previously in hippocampus (Silva et al., 2003b; Silva et al., 2005b). In rat retinal cultures, NPY inhibits both the $[Ca^{2+}]_i$ increase induced by KCl (Alvaro et al., 2009), and the aspartate release in these cultures (unpublished observations). NPY neuroprotection has also been associated with the involvement of ERK1/2 and Akt pathways in a Parkinson's disease model (Decressac et al., 2012). In this study, we have suggested that the NPY neuroprotective role is mediated by PKA and p38K activation. The PKA inhibitor, H89, blocked the neuroprotective effect of NPY, while forskolin, a PKA activator, presented a similar protective effect to NPY, suggesting the involvement of this particular kinase in the neuroprotective effect of NPY against glutamate-induced necrotic cell death in rat retinal cells. PKA activation by NPY has been previously shown. For example, NPY has a biphasic modulatory effect on $[Ca^{2+}]_i$ increases induced by ATP, mediates the upregulated mRNA expression of gonadotropin-releasing hormone in a neuroblastoma cell line, and induces cathecolamine release in human adrenal chromaffin cells, through the activation of PKA (Soares Lemos et al., 1997; Dhillon et al., 2009; Rosmaninho-Salgado et al., 2009). However, there is also evidence showing that NPY inhibits PKA. The activation of NPY receptors inhibits both the axonal transport in sensory neurons and cell proliferation in vascular smooth muscle cells, with these effects being mediated by PKA inhibition (Michel, 1991; Pellieux et al., 2000; Hiruma et al., 2002; Pons et al., 2008; Son et al., 2011).

We also show that NPY activates p38K, and this enzyme, as PKA, appears to mediate, at least partially, the neuroprotective role of NPY against glutamate-induced cell death.

In retinal Müller cells, the activation of NPY Y_1 receptors activates p38 MAPK (Milenkovic et al., 2004). Moreover, p38K activation protects ARPE-19 cells (retinal pigment epithelium cells) against cell death triggered by pro-oxidant conditions (Pocrnich et al., 2009).

In conclusion, NPY can play a neuroprotective role against necrotic and apoptotic cell death induced by glutamate in rat retinal cells. NPY, by activating NPY Y_2, Y_4 and Y_5 receptors, protects retinal cells against glutamate-induced necrosis, and is also able to protect against retinal cell apoptosis by activating NPY Y_5 receptors. In addition, PKA and p38K mediate the neuroprotective effects of NPY. We believe these results might be useful to devise novel pharmacologic targets and therapies to treat retinal degenerative diseases such as glaucoma and diabetic retinopathy.

Chapter IV
Neuropeptide Y and NPY Y_1 receptor in retinal-induced pluripotent stem cells.

Chapter 4 – Neuropeptide Y (NPY) and NPY Y_1 receptor in retinal-induced pluripotent stem cells.

1. ABSTRACT

Induced pluripotent stem cells (iPS cells) were first generated via genetic reprogramming of dermal fibroblasts. Since then several techniques have been established and used to obtain iPS cells from different types of somatic cells avoiding the use of viral vectors for that purpose. Neuropeptide Y (NPY) is a 36-amino acid peptide with pro-neurogenic and proliferative properties in the Central Nervous System, inclusive in the retina. NPY induces proliferation of retinal glial (Müller) cells, neuronal progenitor cells and maintain human embryonic stem cells (hES cells) self-renewal and pluripotency. The aim of this work was to investigate the presence of NPY and NPY Y_1 receptors in the retinal-iPS (R-iPS) cells obtained from a reprogrammable mouse and to evaluate whether NPY affects the proliferation of these cells.

iPS cells were obtained from retinas of reprogrammable mice expressing the four reprogramming factors (OKSM) in the presence of doxycycline; these cells were immunostained to detect the expression of NPY and NPY Y_1 receptors. The mRNA expression of NPY and NPY Y_1 receptor was confirmed by qRT-PCR analysis. The proliferation effect of NPY in R-iPS cells was evaluated by EdU-Flow cytometry proliferation assay and immunocytochemistry of two proliferation markers: EdU and Ki-67.

The results show that Retinal-iPS cells express NPY and NPY Y_1 receptors. However, the exposure of R-iPS cells to NPY (100nM) did not increase the number of cells in S-phase, neither increase in cells expressing EdU and Ki-67 comparing to control. In conclusion, NPY and its receptors are expressed in R-iPS cells; however NPY was unable to induce proliferation of retinal-iPS cells.

2. INTRODUCTION

Induced pluripotent stem cells (iPS cells) were obtain for the first time by Yamanaka in 2006. Using transduction with four transcription factors, Oct4, Sox2, Klf4 and c-Myc, generated iPS cells via genetic reprogramming of dermal fibroblasts (Takahashi et al., 2006). Since then, much more cell types, like blood cells, stomach and liver cells, keratinocytes, melanocytes, pancreatic β cells and neural progenitors have been reprogrammed into iPS cells (reviewed in (Hochedlinger et al., 2009). Moreover, more and different protocols and delivery methods have been developed, as well as new reprogramming factors, with the goal of decreasing viral application and tumorigenic capacity, and increase efficiency.

In order to avoid several limitations of the viral vectors and secondary reprogramming systems of somatic cells, some groups started directly placing doxycycline-inducible polycistronic cassette encoding the four factors c-Myc, Klf4, Oct4 and Sox2 in the 3' untranslated region of a specific gene (Stadtfeld et al., 2010b). These groups generated chimeras and then reprogrammable mice which all cells are potentially capable to express pluripotency markers in the presence of doxycycline, i.e. give rise to iPS cells (Sommer et al., 2009; Stadtfeld et al., 2010b). As the iPS cells obtained from previous methods, these iPS check all the stringent criteria of pluripotency: form teratomas with all three embryonic germ layers and when injected into murine blastocysts, contribute to all tissues and form tetraploid blastocyst (Akiyama et al., 2008; Kim et al., 2008; Zhao et al., 2009).

NPY is a 36-amino acid peptide that has 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, 1982; Tatemoto et al., 1982). 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 following receptor subtypes: NPY Y_1, Y_2, Y_4, Y_5 and y_6 (Silva et al., 2005a; Xapelli et al., 2008).

In vitro and *in vivo* studies suggest that NPY has pro-neurogenic properties in olfactory epithelium, subventricular zone (SVZ) and subgranular zone (SGZ) of dentate gyrus (Hansel et al., 2001; Howell et al., 2005; Howell et al., 2007; Agasse et al., 2008; Rodrigo et al., 2010; Decressac et al., 2011; Thiriet et al., 2011; Baptista et al., 2012; Doyle et al., 2012). Moreover, in CNS, NPY also increases the number of cells in rostral migratory stream, olfactory blub and striatum (Decressac et al., 2009). These NPY effects on neuronal cell proliferation are mediated by the NPY Y_1-receptor activation (Hansel et al., 2001; Howell et al., 2003; Agasse et al., 2008; Doyle et al., 2008; Stanic et al., 2008; Rodrigo et al., 2010; Decressac et al., 2011). Moreover, the neurogenic effect of NPY requires ERK1/2 activation (Hansel, 2001; Howell et al., 2005; 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 at an equal level or even greater than other trophic/growth factors such as ciliary neurotrophic factor, vascular endothelial growth factor, and transforming growth factor (Jin et al., 2002; Emsley et al., 2003; Decressac et al., 2011).

Regarding the retina, others showed 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 (0.0234 and 0.234 nM) NPY decreases cell proliferation rate, while at higher concentration (23.4 nM) increases Müller cell proliferation (Milenkovic et al., 2004). In cultured rat retinal cells, we showed that NPY stimulates the proliferation of neuronal progenitor cells (BrdU+/nestin+ cells), which means that NPY promotes the proliferation of committed neural immature cells, being this effect mediated by the activation of the NOS–sGC and ERK 1/2 signaling pathways (Alvaro et al., 2008a). A recent study also showed that NPY, through Y_1 and Y_5 receptors activation, has the potential to maintain human embryonic stem (hES) cells self-renewal

and pluripotency (Son et al., 2011). NPY might be useful for the development of defined and xeno-free culture conditions for the large-scale propagation of undifferentiated hES cells (Son et al., 2011) or iPS cells. Thus, NPY system might be a putative target to develop new strategies to increase retinal progenitor cells proliferation.

Therefore, we aimed investigating the expression of NPY and NPY Y_1 receptor in retinal iPS cells and evaluate the potential proliferative role of NPY in these cells.

3. MATERIAL AND METHODS

Animals

Four-day-old reprogrammable mice were used as retinal cell progenitor donor (Stadtfeld et al., 2010b). These mice are heterozygous for a doxycycline-inducible polycistronic cassette encoding the four reprogramming factors Oct4, Klf4, Sox2 and c-Myc (OKSM) in the 3′ untranslated region of the collagen type I, alpha 1 gene (Col1a1).

These mice also express an optimized reverse tetracycline-dependent transactivator (M2-rtTA) from the ubiquitously expressed Gt(ROSA)26Sor (Rosa26) locus. The addition of doxycycline to any cell culture from the reprogrammable mice induces the expression of the four reprogramming factors (OKSM) and the reprogramming of the cells.

All experiments were conducted with the approval of the Schepens Eye Research Institute Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Generation of retina-derived iPS cells

Retinal-iPS (R-iPS) cells were generated from retinal progenitor cells, which were isolated from 4-day-old reprogrammable mice according to previously published protocol (Stadtfeld et al., 2010b). After isolation, retinal progenitor cells (about $1x10^5$) were seeded in retinal progenitor media on a mouse embryonic fibroblast (MEF) layer (ATCC, Manassas, Virginia, USA) inactivated by mitomycin C (Sigma-Aldrich, St. Louis, MO). Reprogramming was started in the next day,.

The cells were reprogrammed by adding pluripotency medium [DMEM F-12 (Gibco, Grand Island, NY, USA), 15% heat inactivated FBS (Gibco), 0.0008% β-mercaptoethanol (Sigma-Aldrich), 1% 100x Non-essential amino acids (NEAA) (Gibco), $1x10^6$ units/L of leukemia inhibitor factor (LIF/ESGRO, Milipore, Billerica, MA, USA), 1% penicillin/streptomycin (Gibco), and 0.2% Nistatin (Gibco)] containing 2 µg/mL of doxycycline. Fresh media containing doxycycline was replenished every 2 days. After 3

weeks of treatment the R-iPS cell colonies were manually picked, passaged and clonally expanded for further studies. To maintain pluripotency, iPS cells were cultured in pluripotency media without doxycycline on an inactive MEF feeder layer.

iPS Cell Culture

iPS cells were plated in plastic flasks or plates (Corning, New York, USA) with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) and mouse embryonic fibroblast (MEF) (ATCC), feeder layer. Cells were fed every two days with pluripotency media: DMEM F-12 (Gibco, Life Technologies Ltd**,** Paisley, UK), 15% heat inactivated FBS (Gibco), 0.0008% β-mercaptoethanol (Sigma-Aldrich), 1% 100x NEAA (Gibco), $1x10^6$units/L of leukemia inhibitor factor (LIF/ESGRO, Milipore, Billerica, MA), 1% penicillin/streptomycin (Gibco) and 0,2% Nistatin (Gibco). After confluence, cells were passed using trypsin 0.25% (Gibco) and seeded again.

Immunostaining

iPS cells on glass coverslips or 16-well chambers (Nunc, Roskilde, Denmark) were washed twice with PBS and fixed in 4% paraformaldehyde (20 min; room temperature - RT). Then, the cells were permeabilized with 1% Triton X-100 (Sigma-Aldrich) for 5 min, and blocked with 3% (w/v) fatty acid-free bovine serum albumin (BSA, Sigma-Aldrich) and 10% goat serum (Gibco), supplemented with 0.1% Triton X-100, to prevent nonspecific binding, for 1 h at RT. Cells were incubated with primary antibodies for 90 min at RT: sheep anti-NPY receptor Y_1 [1:500, AbD Serotec; (Ferreira et al., 2010; Baptista et al., 2012)]; rabbit anti-NPY receptor Y_2 [1:100, Alomone Labs, Israel (Baptista et al., 2012)]. All antibody solutions were prepared in 3% fatty acid–free BSA solution.
After washing, the cells were incubated for 1 h at RT with secondary antibodies: Alexa^{TM} 488 anti-sheep IgG, Alexa^{TM} 488 anti-rabbit IgG or Alexa^{TM} 594 anti-rabbit IgG (1:200,

Invitrogen, Eugene, Oregon, USA). Finally, after 5 min washing, cell nuclei were stained with Hoechst 33342 (1 mg/mL in PBS, Molecular Probes, Eugene, OR, USA) for 5 min, and upon rinsing twice with PBS, the coverslips were mounted on glass slides using Dako Fluorescent mounting medium (Dako Cytomation, Glostrup, Denmark). Cells were visualized using a fluorescent microscopic (Zeiss Axioshop 2 Plus) coupled to a digital camera (Axiocam HRc) and a laser scanning confocal microscope LSM 510 META (Zeiss, Jena, Germany). Images were analyzed using Adobe Photoshop or ImageJ.

RNA extraction

Total RNA was isolated from the iPS culture using RNeasy Mini-kit (Qiagen, Valencia, CA, USA) following the provided instructions. Samples were spun using RNeasy spin columns, washed and RNA was eluded using RNase-free water. RNA concentration and integrity were tested using Nanodrop device. Then 1 µg of RNA was reverse transcribed into cDNA using the iScript Select cDNA synthesis kit (Bio-Rad, Life Science, Portugal) after DNAse treatment. The reactions were done as follows: 25ºC for 5 min, 42ºC for 30 min, 85ºC for 5 min and 4ºC for 5 min. Samples were stored at -80°C.

Quantitative real time polymerase chain reaction (qRT-PCR)

Quantitative PCR was performed in an Step one Real-time PCR system (Apllied biosystems, Life technologies) using 96-well microtitre plates and the QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany). The primers for the target mouse genes (mouse NPY, NM_023456 - amplicon size 150bp and NPY Y_1 NM_010934 – amplicon size 134bp) and the reference gene (mouse HPRT1 - hypoxanthine guanine phosphoribosyl transferase 1, NM_013556 – amplicon size 168bp) were pre-designed and validated by QIAGEN (QuantiTect Primers, QIAGEN). The NPY Y_2 primers were obtained from Sigma. As positive controls, wildtype mouse retina cDNA and mouse adrenal gland cDNA were used. Negative controls were performed without RNA sample,

which was substituted by water A master mix was prepared for each primer set containing the appropriate volume of 2x QuantiTect SYBR Green PCR Master Mix and 10x QuantiTect Primer (both from QIAGEN). For each reaction, 8µl of master mix were added to 2 µl of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 10 µl per well. The reactions were performed in the following sequence of steps: 95 °C for 3 min. followed by 45 cycles at 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. The melting curve protocol started immediately after amplification. q RT-PCR products were run by electrophoresis on a 2% agarose gel containing SYBR® Safe DNA Gel Stain. Densitometrical analysis was performed on Versa-Doc Imaging System (Model 3000, Bio-Rad Laboratories, Hercules, CA, USA).

Drug Treatment

Retinal iPS cells were exposed to 100 nM NPY for 24 h or 48 h before harvesting.

Proliferation assay – EDU Flow Cytometry assay

Proliferation assay was performed with Click-iT® EdU Flow Cytometry Assay Kits (Invitrogen). iPS cells were incubated with 100 nM NPY for 48 or 24 h. iPS cells were exposed to 10 µM EDU for 2 h before harvest cells..Harvested cells were washed once with PBS with 1% BSA and then centrifuged. The pellet of cells was disaggregated and cells were fixed with 4% PFA for 15 min at RT and protected from light. After fixation, cells were washed, pelleted and then resuspended in a permeabilization solution (1% Triton X-100 in PBS) for 15 min. After washing, pelleted resuspension, the cells were incubated with Click-iT® reaction cocktail (including anti-EDU Alexa Fluor® 488 azide, for 30 min at RT and protected from light). Then cells were washed once and incubated, first with ribonuclease A and secondly with Cell Cycle 488-red (7-aminoactinomycin D - 7-AAD) for 30 minutes. Data acquisition was performed using the FAC Calibur flow

cytometer (Becton Dickinson and Company, NJ, USA). EDU was detected using a green emission filter (530/30-nm or similar) while 7-AAD was detected by a red emission filter (660/20-nm). Data was analyzed by CellQuest software.

Statistical analysis

All data are presented as mean ± S.E.M. Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni's post-test, as indicated in the figure legends.

4. RESULTS

Retinal-iPS cells express NPY and NPY receptors

Retinal-iPS cells in culture are cell colonies with refractional borders (Fig. 4.1A). Each iPS colonies came from a single R-iPS cell.

To evaluate the presence of NPY and NPY receptors in R-iPS cells, a qRT-PCR analysis was performed (Fig. 4.1B). NPY and NPY Y_1 receptor mRNA were detected in R-iPS cells.

In order to confirm the expression of NPY Y_1 receptor in R- iPS cells, we also analyzed expression of NPY Y_1 receptor in these cells by immunocytochemistry (Fig. 4.1C). We found NPY Y_1 receptor immunoreactivity (Fig. 4.1C) in the R-iPS colonies, especially around the nucleus stained with DAPI.

NPY does not increase R-iPS cells proliferation

In order to study the role of NPY in the proliferation of R-iPS cells, EdU and 7-AAD flow cytometry analysis was performed (Fig. 4.2A – H). Cells were incubated with 100 nM NPY for 24 or 48 hours.

The gates used to analyze these results were defined based on the non-specific fluorescence of EdU, namely the fluorescence detected when the cells were not treated with this proliferation marker (cell treated only with secondary antibody Alexa Fluor 488 - Fig. 4.2A). This basal fluorescence includes the auto fluorescence of the cells and the unspecific ligation of the secondary antibody. Therefore, two distinct peaks were observed to the several experimental conditions: control (no drug - Fig. 4.2B), cells treated with 100nM NPY for 24 h (Fig. 4.2C) and 48 h (Fig. 4.2D). The left peak, defined by M1, with lower fluorescence values represents non proliferating cells, cells that do not incorporate EdU (and also non-specific fluorescence). The other peak, on the right side (M2) represents the proliferating cells that incorporate EdU. No differences were

detected among the three experimental conditions tested, control (no drug), and exposure to NPY (100 nM) for 24 h and 48 h.

In order to better understand the NPY effect in the proliferation of R-iPS cells, double immunolabeling with EdU-Alexa Fluor® 488 azide and 7-AAD -Click-iT® CellCycle 488-red was analyzed (Fig. 4.2E - G). These graphs allow us to observe in a different manner the distribution of R-iPS cells in the cell cycle, observe the proliferating cells and define the percentage of cells in proliferation. The gates were defined similarly. The top gate shows proliferating cells (early and late S-phase) while the other phases of cell cycle (G1, G2) are included in the bottom gate. Then, two different populations of cells can be identified: one smaller, in the bottom gate and, another bigger, in the top gate (Fig. 4.2E - G). Again, no significant visual differences were detected among the three experimental conditions: control (red), NPY 24 h (blue) and NPY 48 h (orange).

Additionally, quantification analysis was performed. The percentage of proliferating and non-proliferating cells were analyzed and presented (Fig. 4.2H). In this graph, the grey bar represents the proliferating cells (S-phase mainly) while the white bar represents the non-proliferating R-iPS cells, cells out of S-phase (G1 and G2). Thus, the percentage of non- proliferating cells is around 33% while proliferating cells represent 66% of the cells analyzed for the three experimental groups, therefore no significant differences were observed among conditions. Furthermore, the phases of the cell cycle of the R-iPS cells were better evaluated. The percentage of events in G1, S and G2 was analyzed for the three experimental conditions (control -red, NPY 24 h - blue and NPY 48 h –orange; Fig. 4.2I). In line with the previous results, all conditions present a higher percentage of cells in S-phase. However, no significant differences were observed in the several phases of cell cycle to the different conditions studied.

To confirm these results, the expression of two proliferation markers, EdU and Ki-67, was evaluated by immunocytochemistry (Fig. 4.3). No differences were detected in the expression of EdU among the different conditions: control, NPY 24 h and NPY 48 h (Fig.

4.3 A-C). Similarly, as showed in figure 4.3, Ki-67 immunoreactivity was similar in all conditions tested (control; NPY 24 h and NPY 48 h).

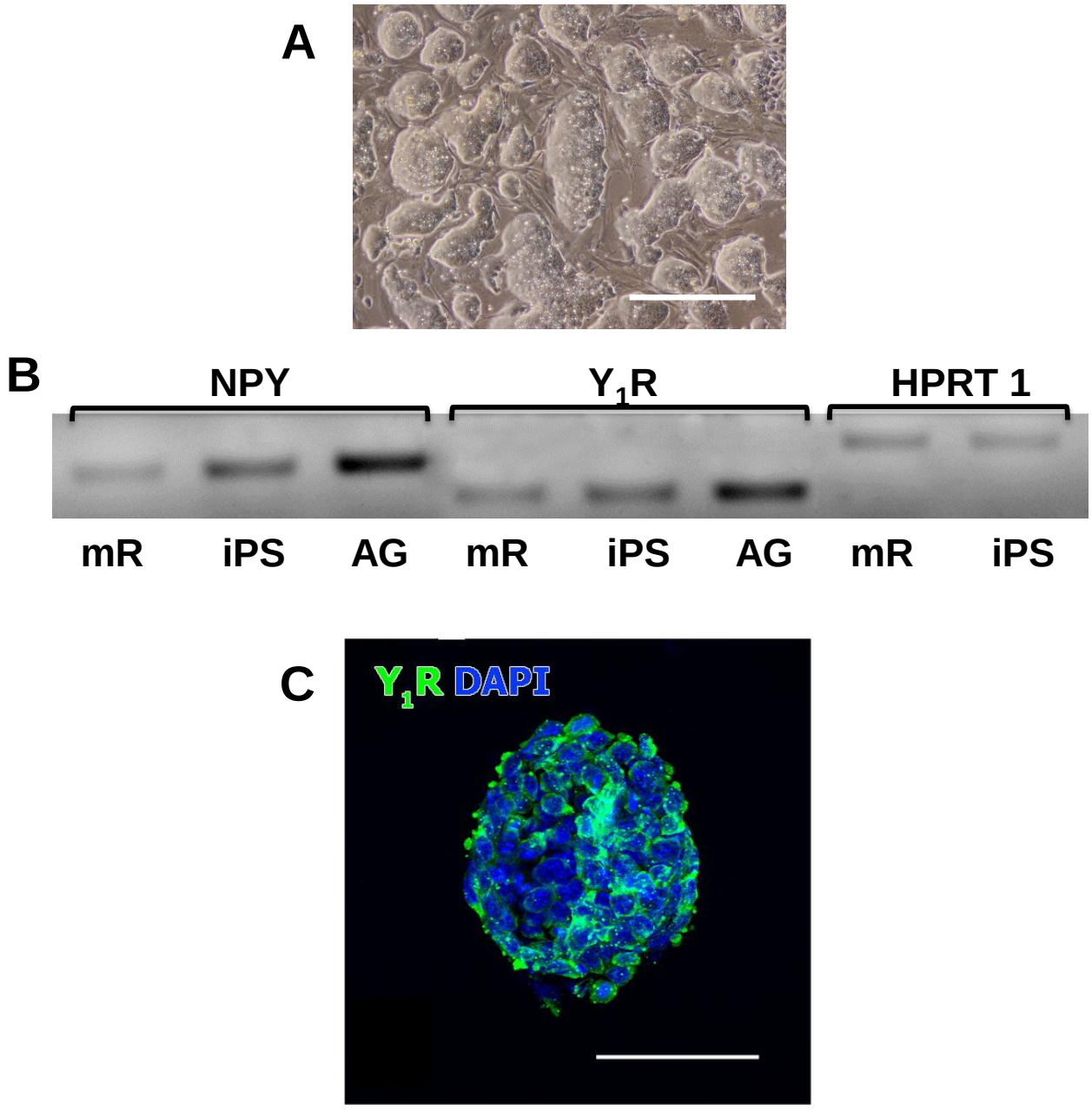


Fig. 4.1 – NPY and NPY Y_1 receptor in R-iPS cells. (A) Retinal-iPS cells. Transmission image of R-iPS cells colonies in culture. Scale bar: 500 µm. (B) NPY and NPY Y_1 receptor mRNAs in R- iPS cells. The mRNA expression of NPY and NPY receptors was assessed by qRT-PCR followed by agarose gel electrophoresis. PCR products used (base pairs (bp): NPY, 150 bp and NPY Y_1, 134 bp. HPRT1(168 bp) was used as reference gene. Mouse retina (mR) and Adrenal Gland (AG) were used as positive controls. Each figure is representative of three independent cell cultures. (C) NPY Y_1 receptor immunoreactivity (green) is present in R-iPS cells. NPY Y_1 receptor was detected with a sheep anti-NPY Y_1 antibody (1:500). Cell nuclei were stained with Hoechst 33342 (blue). The images are representative of three independent cell cultures. Scale bar: 50 µm.

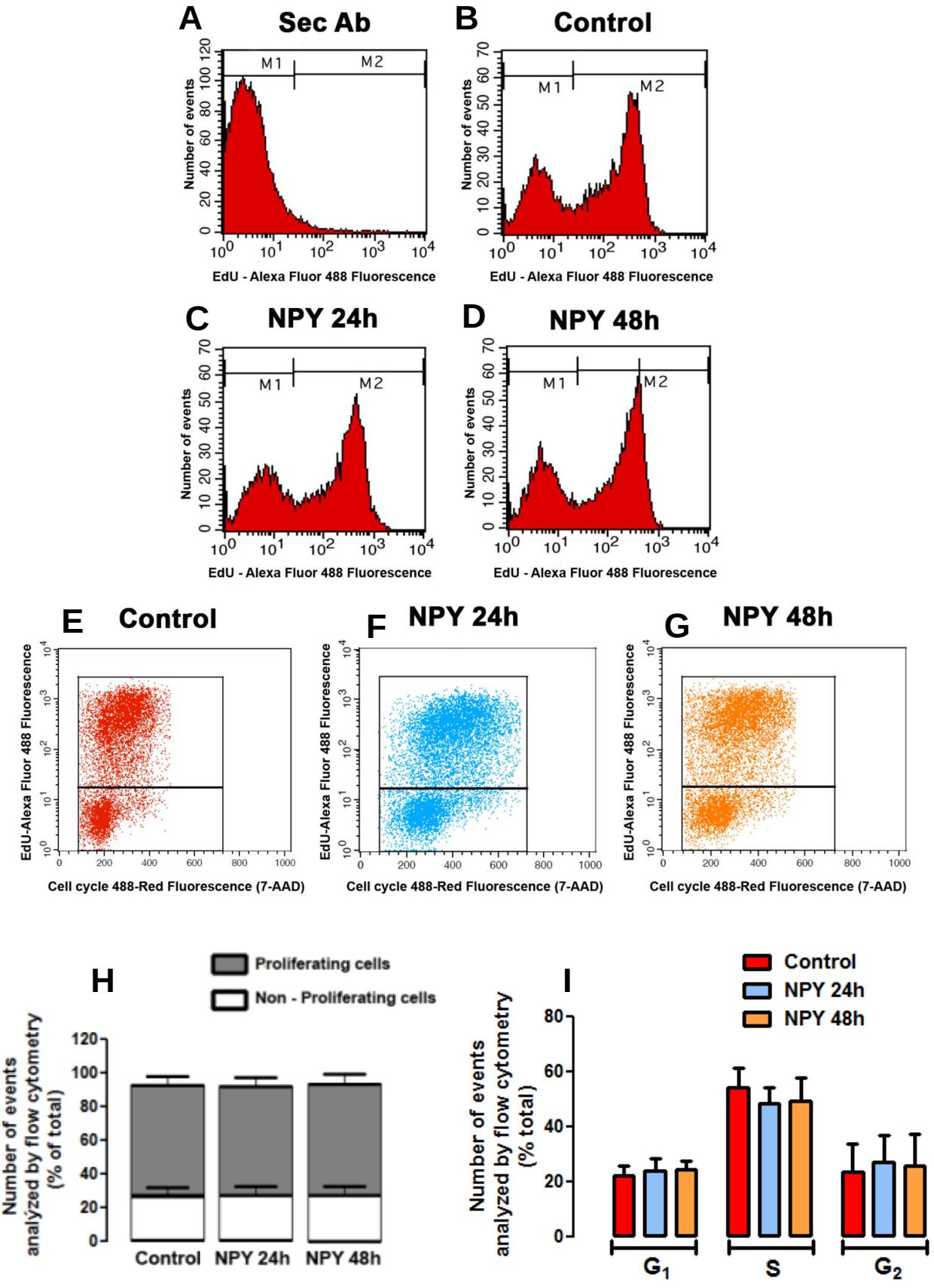

A
Sec Ab
B
Control
C
NPY 24h
D
NPY 48h
M1
M2
Number of events
EdU - Alexa Fluor 488 Fluorescence
E
Control
F
NPY 24h
G
NPY 48h
EdU-Alexa Fluor 488 Fluorescence
Cell cycle 488-Red Fluorescence (7-AAD)
H
Proliferating cells
Non - Proliferating cells
Number of events analyzed by flow cytometry (% of total)
Control
NPY 24h
NPY 48h
I
Control
NPY 24h
NPY 48h
Number of events analyzed by flow cytometry (% total)
G1
S
G2

Fig. 4.2 - NPY does not increase the proliferation of R-iPS cells. The effect of NPY in the proliferation of retinal iPS cells was evaluated by flow cytometry analysis by EdU incorporation. iPS cells were exposed to 10 µM EdU for 2 hours. (A – D) R-iPS cells were labeled with Alexa Fluor® 488 azide. The right peak refers to proliferating cells that had incorporated EdU whereas the left peak shows non proliferating cells. (A) Cells not exposed to EdU, only treated with secondary antibody, Alexa Fluor 488 azide; (B) untreated cells. Cells exposed to 100 nM NPY for 24 h (C) and 48 h (D). The M1 gate included non-proliferating cells while M2 gate indicates the proliferating cells.
(E – G) Double labeling of R-iPS cells with EdU-Alexa Fluor® 488 azide and 7-AAD - Click-iT® CellCycle 488-red: (E) Untreated cells; cells exposed to 100 nM NPY for 24 h (F) and 48 h (G). (H) Quantification of the percentage of proliferating R-iPS cells (early and later S-phase). As above, R-iPS cells were untreated (Control), or treated with 100nM NPY for 24 h or 48 h. (I) Quantification of the percentage of events in G1, G2 and S-phase in the three experimental conditions. These results represent the mean±S.E.M. of at least three independent experiments.

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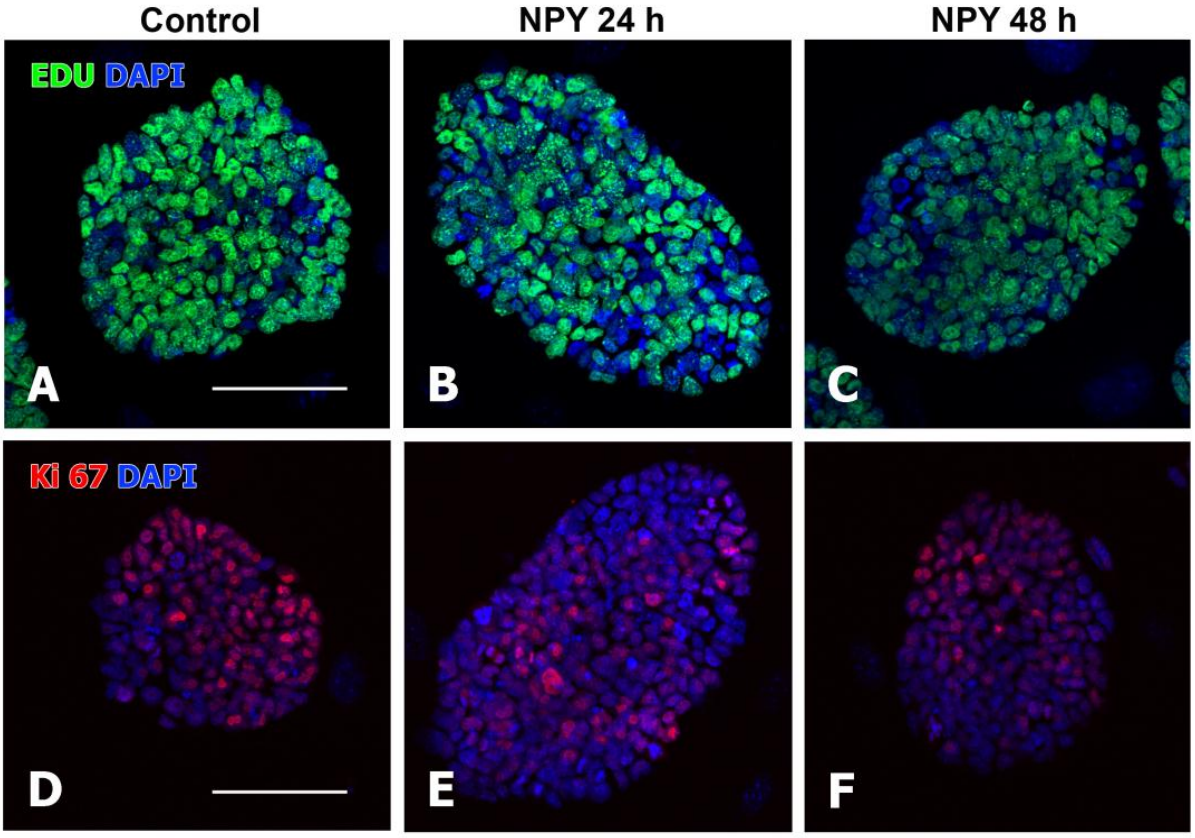

Fig. 4.3 – R iPS cells do not proliferate in the presence of NPY. Retinal-iPS cells were exposed to NPY 100 nM for 24 and 48h. (A – C) R-iPS cells were incubated for 2 h with 10µM EdU 10 µM. (D – F) R-iPS cells were immunolabeled for the Ki-67 proliferation marker. Scale bar: 50 µm.

5. DISCUSSION

NPY and NPY Y_1, Y_2, Y_4 and Y_5 mRNA are expressed in non-mammalian and mammalian retinas, not only in neurons, but also in glial and endothelial cells (Osborne et al., 1985; Bruun et al., 1986; Hutsler et al., 1994; Sinclair et al., 2001; Oh et al., 2002; Alvaro et al., 2007; Santos-Carvalho et al., 2012). NPY is a multifunctional peptide that has the ability to enhance cell proliferation. NPY promotes cell proliferation in olfactory epithelium, subventricular zone (SVZ) and subgranular zone (SGZ) of dentate gyrus (Hansel et al., 2001; Howell et al., 2005; Howell et al., 2007; Agasse et al., 2008; Rodrigo et al., 2010; Decressac et al., 2011; Thiriet et al., 2011; Baptista et al., 2012; Doyle et al., 2012) and also in the retina (Milenkovic et al., 2004; Alvaro et al., 2008a). NPY also has the potential to maintain human embryonic stem (hES) cells self-renewal and pluripotency (Son et al., 2011). In order characterize the R-iPS cells, we studied the presence of NPY and NPY Y_1 receptors in these cells obtain from reprogrammable mouse retinal cells (Stadtfeld et al., 2010b) and investigate the putative proliferative effect of NPY in this cells.

Previously, others showed that hES cells express NPY Y_1 and Y_5 receptors. In this study, we observed that R-iPS cells express NPY and NPY Y_1 receptor mRNA and, immunoreactivity to NPY Y_1 receptor. The same authors also suggested the implication of both NPY receptors in the maintenance of self-renewal and pluripotency of hES cells. On the contrary, in this work NPY (100nM) did not increase R-iPS cells proliferation. Nor shorter (24 h) or longer (48 h) incubations were effective. In fact, previously in our lab, 100-1000nM NPY was able to induce retinal neural cells proliferation (Alvaro et al., 2008a) through the activation of NPY Y_1, Y_2 and Y_5 receptors, the same was not observed in this work.

Stemness and pluripotency are characteristics of all stem cells, including iPS cells. Thus R-iPS cells also proliferate easily and, in our study, around 2/3 of these cells in control conditions were already proliferating (S-phase). Therefore, probably we did not observe

any enhancing effect on proliferation triggered by NPY, since these cells were already in a high rate of proliferation and a putative enhancing effect of NPY could be masked by the proliferative effect of all the other factors in the medium. In fact, several studies showed that NPY act as proliferating factor (Jin et al., 2002; Emsley et al., 2003; Decressac et al., 2011) however iPS cells are reprogrammable cells with unique characteristics that may in some way react differently.

Although R-iPS express NPY and NPY Y_1 receptors, NPY do not induce the proliferation. Nevertheless, other studies need to be done, such as investigating the presence of the other types of NPY receptors in R-iPS cells and unravel the role of NPY and its receptors in these cells. Additional experiments also need to be performed to understand whether epigenetic memory plays a role in NPY system in R-iPS cells. Namely, study the expression of NPY receptors in R-IPS cells in late passages.

In conclusion, we found for the first time that R-iPS cells express NPY and NPY Y_1 receptors. However, the role of NPY Y_1 receptors in these cells should be investigated.

Chapter V
The origin of induced pluripotent stem cells significantly effects their capacity for retinal differentiation

Chapter 5 – The origin of induced pluripotent stem cells significantly effects their capacity for retinal differentiation

1. ABSTRACT

Induced pluripotent stem (iPS) cells obtained from fibroblasts may provide a source of retinal photoreceptor cells for cell replacement-based therapies. One limitation of this approach is the low yield of photoreceptors obtained. Recently, it has been shown that iPS cells retain a transient transcriptional and epigenetic memory of their cell of origin that affects their differentiation capacity. The purpose of this study was to characterize the pluripotency and capacity for photoreceptor cell differentiation of iPS cells lines obtained from murine retinas (R-iPS) as compared to those obtained from fibroblasts (F-iPS) harvested from the same donor. Both R-iPS and F-iPS cells lines expressed the pluripotency markers c-Myc, Klf4, Oct4, Sox2, Nanog and SSEA1. Teratoma formation assays confirmed the presence of tissues of all three embryonic germ layers. R-iPS cells also expressed photoreceptor markers pre differentiation. Differentiation of R-iPS cells resulted in a higher number of photoreceptor precursor cells when compared to F-iPS cells. Fifty percent of the total number of cells showed positive staining for photoreceptor markers. In conclusion, we show that R-iPS cells differentiated into photoreceptors with a much higher efficiency then F-iPS cells. These results further indicate the importance of epigenetic memory in cellular reprogramming and differentiation. R-iPS cells are a new potential source of photoreceptors for cell-based therapies.

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2. INTRODUCTION

Induced pluripotent stem (iPS) cells were initially produced by Takahashi and Yamanaka via genetic reprogramming of dermal fibroblast to pluripotency via retroviral transduction of the four transcription factors Oct4, Sox2, Klf4 and c-Myc (Takahashi et al., 2006). More recently a variety of new protocols have been developed with different delivery methods and reprogramming factors aimed at reducing the need for virally-induced genetic insertion of the potentially tumorigenic factors c-Myc and Klf4 and at increasing the efficiency of reprograming (Shi et al., 2008; Feng et al., 2009). To date, iPS cells have been generated from cells of multiple tissues including blood, stomach, liver, keratinocytes, melanocytes, pancreatic β cells and neural progenitors (Stadtfeld et al., 2010a). Although all these iPS cell lines have been shown to express pluripotency genes and support differentiation into cell types of all three germ layers, recent studies have demonstrated that substantial molecular and functional differences among iPS and Embrionic Stem (ES) cells exist. Differences between iPS and ES cells have been noted in global gene expression (Yu et al., 2007; Chin et al., 2009), expression of imprinted genes (Pick et al., 2009), DNA methylation (Doi et al., 2009), and differentiation potential (Feng et al., 2010; Hu et al., 2010a). Some of these differences may be due to epigenetic regulatory mechanisms that lock characteristic gene expression patterns into place and do not allow for complete reprogramming to an ES cell-like state. Some iPS cells transiently retain a transcriptional and epigenetic memory of their cell of origin at early passages, which has been shown that affect their potential to differentiate into specific cell types (Hu et al., 2010b; Kim et al., 2010a; Polo et al., 2010). Therefore, there is a tendency for early-passage iPS cell lines to differentiate preferentially into the cell lineage of origin, a phenomena that could be exploited in to obtain specific cell types with high efficiency.

Reprogrammable mice which have a doxycycline-inducible polycistronic cassette encoding the four reprogramming factors Oct4, Klf4, Sox2 and c-Myc (*OKSM*) in the 3' untranslated region of collagen type I (*Col1a1*) were recently generated (Stadtfeld et al., 2010b). Virtually all the cells of these animals can be converted into iPS cells in the presence of doxycycline. These reprogrammable mice have been shown to be a useful tool in studies aimed at comparing cellular reprogramming in different cell types. Additionally it affords one the ability to compare the influence of epigenetic memory on differentiation potential without the concern of variations due to virally-induced genetic insertions or genetic background.

Retinal degenerative diseases such as Retinitis Pigmentosa (RP) and Age-related Macular Degeneration (AMD) are currently the leading cause of blindness in the western world (Chopdar et al., 2003; Mitchell et al., 2006; Earnshaw et al., 2007). These diseases are characterized by death of photoreceptor cells and no efficient treatment is available. Photoreceptor cell replacement therapy has the potential to restore visual function to these individuals, but many hurdles to clinical application remain, including cell source. Our lab and others have shown that fibroblast-derived iPS cells may provide a potentially unlimited source of photoreceptor cells for cell-based therapies (Lamba et al., 2010; Tucker et al., 2011). Fibroblast-derived iPS cells can be differentiated in several retinal cell types, including photoreceptors, which can engraft into the retina following delivery to the subretinal space (Meyer et al., 2009; Osakada et al., 2009; Lamba et al., 2010; Tucker et al., 2011). One limitation of this approach is the low yield of photoreceptor cells obtained using current differentiation protocols. Here we have exploited the epigenetic memory of iPS cells. We hypothesized that retina-derived iPS cells could be an efficient source of photoreceptors for cell based therapies.

The purpose of this study was to characterize the pluripotency and efficiency of retinal specific differentiation of iPS cells lines generated from the retina as compared to the skin of the reprogrammable mouse.

Cells obtained from mouse retina can be reprogrammed into iPS cells. These cells differentiate into photoreceptors with a significantly higher efficiency than those generated from fibroblasts.

3. MATERIAL AND METHODS

3.1 Animals

Four-day-old reprogrammable mice were used as retinal cell progenitor and tail tip fibroblast donors (Stadtfeld et al., 2010b). Reprogrammable mice were a kind gift of Konrad Hochedlinger (Harvard University, Cambridge, MA). These mice were heterozygous for a doxycycline-inducible polycistronic cassette encoding the four reprogramming factors Oct4, Klf4, Sox2 and c-Myc (OKSM) in the 3′ untranslated region of the collagen type I, alpha 1 gene (*Col1a1*). These mice also express an optimized reverse tetracycline-dependent transactivator (M2-rtTA) from the ubiquitously expressed Gt(ROSA)26Sor (Rosa26) locus. The addition of doxycycline to any cell culture from the reprogrammable mice induces the expression of the four reprogramming factors (OKSM) and the reprogramming of the cells.

Severe combined immunodeficient mice (SCID, Jackson Laboratory, Bar Harbor, ME) were used for assessment of teratoma formation.

All experiments were conducted with the approval of the Schepens Eye Research Institute Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3.2 Generation of retina- and fibroblast-derived iPS cells

F-iPS cells were generated from tail-tip fibloblasts. Tail tip fibroblast cultures were established from tail tip biopsies of 4-day-old reprogrammable mice and maintained in fibroblast media. Tail tip fibroblasts between passages 3-4 were used for reprogramming experiments.

R-iPS cells were generated from retinal progenitors cells. Retinal progenitor cells were isolated from 4-day-old reprogrammable mice according to our previously published protocol (Redenti et al., 2009). After isolation, retinal progenitor cells (about $1x10^5$ cells) were seeded in retinal progenitor media on a mitomycin C- (Sigma-Aldrich, St. Louis,

MO) inactivated mouse embryonic fibroblast (MEFs) layer (ATCC, Manassas, VA). The next day, reprogramming was begun.

The cells were reprogrammed by adding pluripotency medium [DMEM F-12 (Gibco, Grand Island, NY), 15% heat inactivated-FBS (Fetal Bovine serum, Gibco), 0.0008% β-mercaptoethanol (Sigma-Aldrich), 1% 100x NEAA (Non-essential amino acids, Gibco), $1x10^6$ units/L of leukemia inhibitor factor (LIF/ESGRO, Milipore, Billerica, MA), 1% penicillin/streptomycin (Gibco), 0.2% Nistatin (Gibco)] containing 2 µg/ml of doxycycline. Fresh media containing doxycycline was replenished every 2 days. After 3 weeks of treatment the R-iPS and F-iPS cell colonies were manually picked, passaged and clonally expand for further studies. To maintain pluripotency, iPS cells were cultured in pluripotency media without doxycycline on an inactive MEF feeder layer or low growth factors matrigel-coated plates (1:400, BD Bioscience, San Jose, CA).

3.3 Teratoma formation

IPS cells were collected by trypsinization and injected (1 µl containing $2.5x10^5$ iPS cells in PBS) into the eye of SCID mice. Injections were performed as previously described (Tucker et al., 2011). Twenty-one days after injection animals were euthanized and eyes were enucleated, fixed in 4% paraformaldehyde and embedded either in paraffin or OCT (Tissue teck, Japan) for H&E or immunofluorescence analysis, respectively. H&E staining was performed as per standard protocols.

3.4 In vitro differentiation

R-iPS and F-iPS cells were differentiated in vitro according to our previously published protocol (Tucker et al., 2011). Briefly, iPS cell colonies were passaged with type I Collagenase (Sigma-Aldrich) and resuspended in embryoid body (EB) formation media containing 1 ng/ml of Noggin, Dkk-1 and IGF-1 and 0.5 ng/ml of bFGF (R&D Systems, , Minneapolis, MN). After 5 days in culture, embryoid bodies were plated in fresh

differentiation media 1 (10 ng/ml of Noggin, Dkk-1and IGF-1 and 1 ng/ml of bFGF) in 6–well culture plates coated with poly-D-lysine, collagen (BD Bioscience, 25 µg/ml), laminin (Gibco, 50 µl/ml) and fibronectin (Sigma-Aldrich, 100 µg/ml). Cultures were fed every other day for 10 days with differentiation media 1, then every other day for an additional 6 days with differentiation media 2 (differentiation media 1 + 10 µM of Notch signaling inhibitor – DAPT (Calbiochem, Gibbstown, NJ), followed every other day for an additional 12 days with differentiation media 3 [differentiation media 2 plus 2 ng/ml of aFGF (R&D Systems)].

3.5 Immunostaining

Differentiated and undifferentiated R-iPS cells and F-iPS cells and teratoma sections were fixed with 4% PFA and permeabilized with 0.1% Triton-X-100 (Sigma-Aldrich). Cells and/or sections were stained with primary antibodies to β-III tubulin (Sigma-Aldrich), Recoverin (Chemicon, EMD Millipore, Billerica, MA), CRX (Santa Cruz Biotechnology, Inc., Santa Cruz, CA.), Rhodopsin (Chemicon), Pax6 (Santa Cruz), biotinylated OTX2 (R&D Systems), S-Opsin (Chemicon), Oct4 (Santa Cruz), c-Myc (Cell Signaling Tech, Danvers, MA), Klf4 (Santa Cruz), Sox2 (Chemicon), Nanog (Abcam, Cambridge, USA), NF160 (Chemicon), GFAP (Chemicon), α-smooth muscle actin and α fetoprotein (R&D Systems). Then, staining with the respective secondary antibodies conjugated to Cy2, Cy3 or Cy5 (Chemicon) was performed. Nuclei were counterstained with DAPI (Invitrogen). The samples were imaged using confocal microscopy (Leica TCS-SP5, Leica Microsystems, Buffalo Grove, IL). Cell counting on immunostained samples was performed by counting the total number of cells expressing the protein of interest in 10 randomly selected microscopic fields taken outside originally plated embryoid bodies. The results from 3 experiments were averaged. As such, analysis were based on counts from 30 microscopic fields.

3.6 Western blotting

Undifferentiated or differentiated F-iPS and/or R-iPS were homogeneized in lysis buffer (50 nM tris-HCl, pH 7.6, 150 mM NaCl, 10 mM CaCl2, 1% Triton X-100, 0.02% NaN (Sigma-Aldrich)), separated by SDS-PAGE, transferred to PVDF membranes (Millipore) and probed with the following antibodies: Recoverin (Chemicon), CRX (Santa Cruz), Rhodopsin (Chemicon), Pax6 (Santa Cruz), Oct4 (Santa Cruz), c-Myc (Cell Sign tech), Klf4 (Santa Cruz Biotechnology, Inc.), Sox2 (Chemicon), c-Myc, SSEA1 (Thermo Scientific, Waltham, MA), and β-actin (Abcam - as loading control). After conjugation with the respective secondary antibody, blots were visualized with ECL reagents (PerkinElmer, Waltham, MA) and exposed to X-Ray film (Kodak).

3.7 RNA isolation, RT-PCR

Total RNA was extracted using an RNeasy Mini-kit (Qiagen, Valencia, CA) according to the manufactures instructions. RNA was reverse transcribed into cDNA using the SuperScript III First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). All PCR reactions were performed in a 46 µL reaction containing 1x PCR Buffer, 1.5 mM $MgCl^2$, 0.2 mM dNTPs, 100 ng of cDNA, 1.0 U of Platinium Taq (Invitrogen) and 20 pmol of each gene specific primer (Table 1). All cycling profiles incorporated an initial denaturation temperature of 94ºC for 10 min through 35 amplification cycles (30 sec at 94 ºC, 30 sec at the annealing temperature (60, 61 or 62 ºC) of each primer and 1 min of 72 ºC) and a final extension at 72 ºC for 10 min. PCR products were separated by electrophoresis on 2% agarose gels.

Table 5.1. Gene specific primer list

TTGCCATCAACGACCCCTTC	GADPH Forward
TGTCATGGATGACCTTGGCC	GADPH Reverse
ATGCCCCTCAACGTGAACTTC	c-Myc Forward
CGCAACATAGGATGGAGAGCA	c-Myc Reverse
TCCTTTCCAACTCGCTAACCC	Klf4 Forward
CGGATCGGATAGCTGAAGCTG	Klf4 Reverse
TCTTCCTGGTCCCCACAGTTT	Nanog Forward
GCAAGAATAGTTCTCGGGATGAA	Nanog Reverse
AGTTGGCGTGGAGACTTTGC	Oct4 Forward
CAGGGCTTTCATGTCCTGG	Oct4 Reverse
ACGGATAAGGCGCTGGTACTA	SSEA1 Forward
GGAAGCCATAGGGCACGAA	SSEA1 Reverse
GACAGCTACGCGCACATGA	Sox2 Forward
GGTGCATCGGTTGCATCTG	Sox2 Reverse

4. RESULTS

4.1 Pluripotency of retina-and fibroblast-derived iPS cells

Retinal progenitor cells and tail tip fibroblasts from 4-day-old reprogrammable mice were cultured in the presence of doxycycline for three weeks to produce two different lines of iPS cells, retina- (R-iPS) and fibroblast-derived (F-iPS). We choose to reprogram retinal progenitors cells and not mature photoreceptor cells because reprogramming of cells post-differentiation is known to be less efficient (Gurdon et al., 2008; Eminli et al., 2009). Immunocytochemical (Fig. 5.1-A), RT-PCR (Fig. 5.1-B) and western blot analyses (Fig. 5.1-C) revealed that R- and F- derived iPS cell colonies expressed levels of pluripotency-associated markers Oct4, Sox2, Klf4, c-Myc, Nanog and SSEA1 that were comparable to those of mouse ES cells. To test for pluripotency, $2.5x10^5$ undifferentiated R- or F-iPS cells were injected into the eye of SCID mice. At 21 days post-transplantation, histological analysis revealed teratomas (Fig. 5.2 – a, a') containing tissues specific to each of the three embryonic germ layers (Fig. 5.2-b, b', c, c' neuroepithelia, neural rosettes-ectoderm; d, d' vascular structures-endoderm, e, e', f, f' chondrocytes and adipocytes-mesoderm). Similarly, immunohystochemical staining of the teratomas revealed neurofilament 160-, β-III tubulin- and GFAP-positive neural tissue demonstrating the presence of ectoderm germ layer (Fig. 5.2-g, g', h, h', i, i'), alpha-fetoprotein-positive cells specific of the endoderm germ layer (Fig. 5.2-l, l') and α-smooth muscle actin-positive arterial structures specific of the mesoderm germ layer (Fig. 5.2-m, m'). Collectively, these findings demonstrate that we produced retina- and fibroblast-derived iPS cell lines from the reprogrammable mouse. These lines possess the ability to produce cell types of all three embryonic germ layers, similar to embryonic stem cells.

4.2 Epigenetic analysis of the retina- and fibroblast derived iPS cells

To evaluate whether R-iPS cells retain gene expression patterns indicative of their origin we performed an immunofluorescence staining to confirm expression of retinal cell

progenitor markers in these cells (Fig. 5.3). Antibodies against an early retinal progenitor marker (Pax6) and against photoreceptor specific markers (CRX, Rhodopsin and Recovein) were used. Both iPS lines were analyzed between passages 4 and 5 since extensive passages have been shown to abrogate the epigenetic memory of the donor tissues (Polo et al., 2010). R-iPS cells express CRX, Rhodopsin, Recoverin and Pax6. Conversely, F- iPS cells do not express any of the photoreceptor markers analyzed. The data show that R-iPS cells have a transcriptional memory of their tissue of origin, in this case photoreceptor precursor cells.

4.3 Differentiation of retina- and fibroblast- derived iPS cells into photoreceptor precursor cells

Based on gene expression differences observed among R-iPS and F-iPS, we hypothesized that these differences might affect their capacity to differentiate into photoreceptors. We compared the ability of R-iPS and F-iPS cells to differentiate into photoreceptor cells in vitro. The protocol used for in vitro differentiation has been previously published (Tucker et al., 2011). This protocol induces iPS cells to differentiate first into embryoid bodies (5 days after the beginning of the protocol, D5) and then into photoreceptor cells over 33 days (D33). iPS cells between passages 4 and 5 were used for *in vitro* differentiation experiments.

Microscopically, at D33 clonal areas of differentiation were evident, i.e. clusters of differentiated and undifferentiated cells could be identified (Fig. 5.4-A). The areas of differentiated cells with photoreceptor shape were larger and higher in number in differentiated R-iPS compared to those in differentiated F-iPS cells (Fig. 5.4-A). Immunocytochemical staining was performed to study photoreceptor marker expression and compare the efficiency of photoreceptor differentiation of R-iPS and F-iPS cells (Fig. 5.4–A). This staining revealed that a high number of differentiated R-iPS cells expressed the photoreceptor markers, OTX2, Recoverin and S-Opsin. Conversely, significantly

fewer differentiated F-iPS cells expressed OTX2 when compared to the differentiated R-iPS cells (Fig. 5.4-A). The number of cells expressing Recoverin was even lower than that of cells expressing OTX2 while S-Opsin was expressed in very few differentiated F-iPS cells (Fig. 5.4-A).

To quantify the amount of R-iPS and F-iPS cells that differentiated into photoreceptor precursor cells the number of cells expressing the photoreceptor markers OTX2, Recoverin, and S-Opsin were counted (Fig. 5.4-B). Within the differentiated cell clusters approximately 70% of the differentiated R-iPS cells expressed OTX2, 50% expressed Recoverin and 20% expressed S-Opsin. For differentiated F-iPS cells the percentage of expression for each of these markers were significantly lower, 30%, 15%, and 1% respectively.

Western blot analysis on lysates from D0 undifferentiated R-iPS cells, D5 R-iPS embryoid bodies and D33 differentiated R-iPS cells showed that the expression of pluripotency markers (Oct4 and Nanog) is decreased over this period (Fig. 5.4-C). Oct4 and Nanog expression was higher in undifferentiated R-iPS cells (D0) and decreased in differentiated R-iPS cells (D33). However, at day 33 these pluripotency markers were still expressed. These data indicate that although substantial differentiation was achieved, an incompletely differentiated subpopulation remains as previously observed (Tucker et al., 2011).

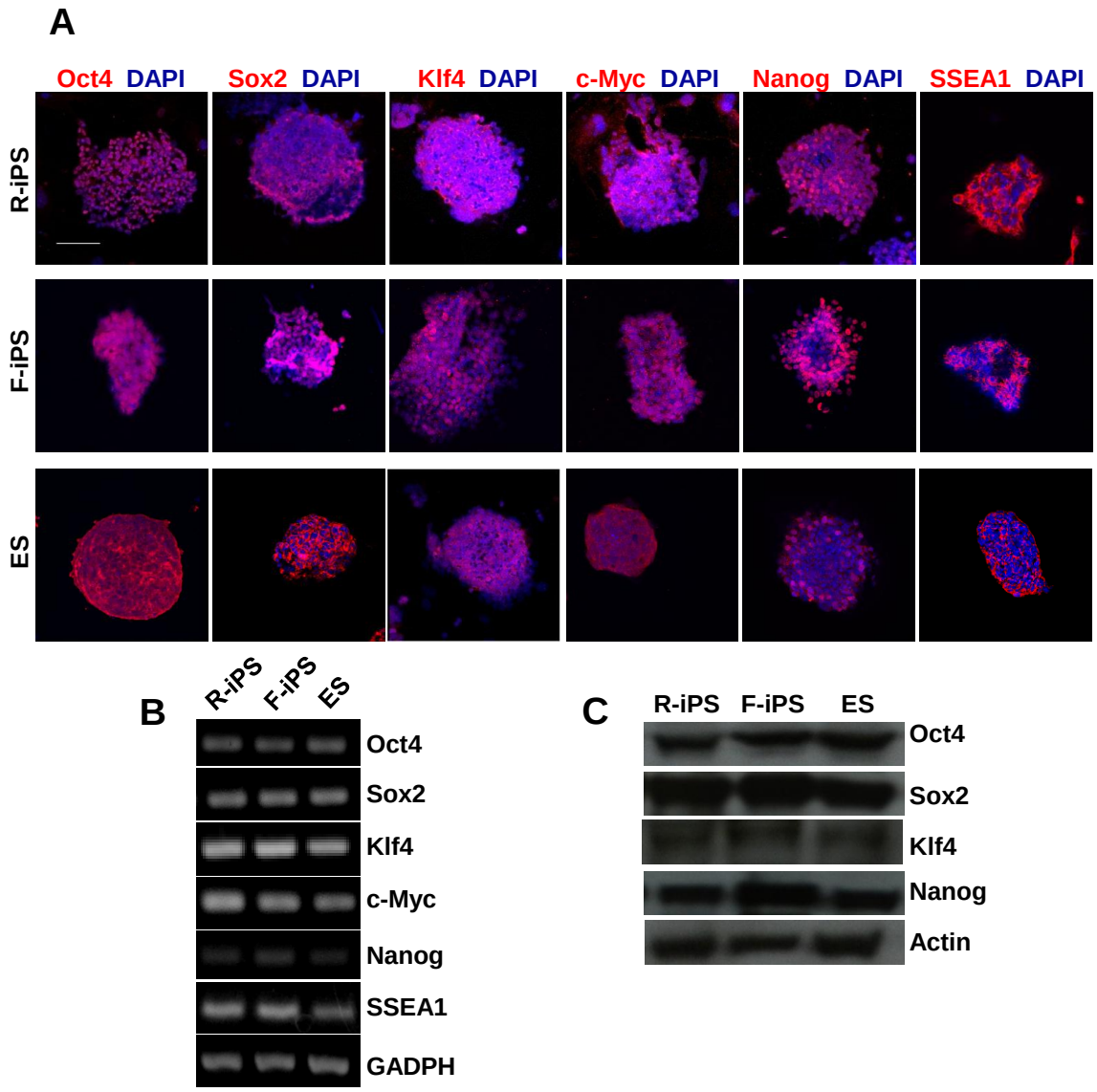

Fig. 5.1 - Expression of pluripotency markers in retina and fibroblast-derived iPS cells. Evaluation of the expression of the pluripotency markers Oct4, c-Myc, Sox2, Nanog, Klf4 and SSEA1 in retina (R-iPS)- and fibroblast (F-iPS)- derived iPS cells and ES cells by immunofluorescence (A), RT-PCR analysis (B), and (C) western blotting. Cells nuclei were stained with DAPI in (A), GADPH and actin were used as control markers in RT-PCR (B) and western blot analysis (C), respectively.

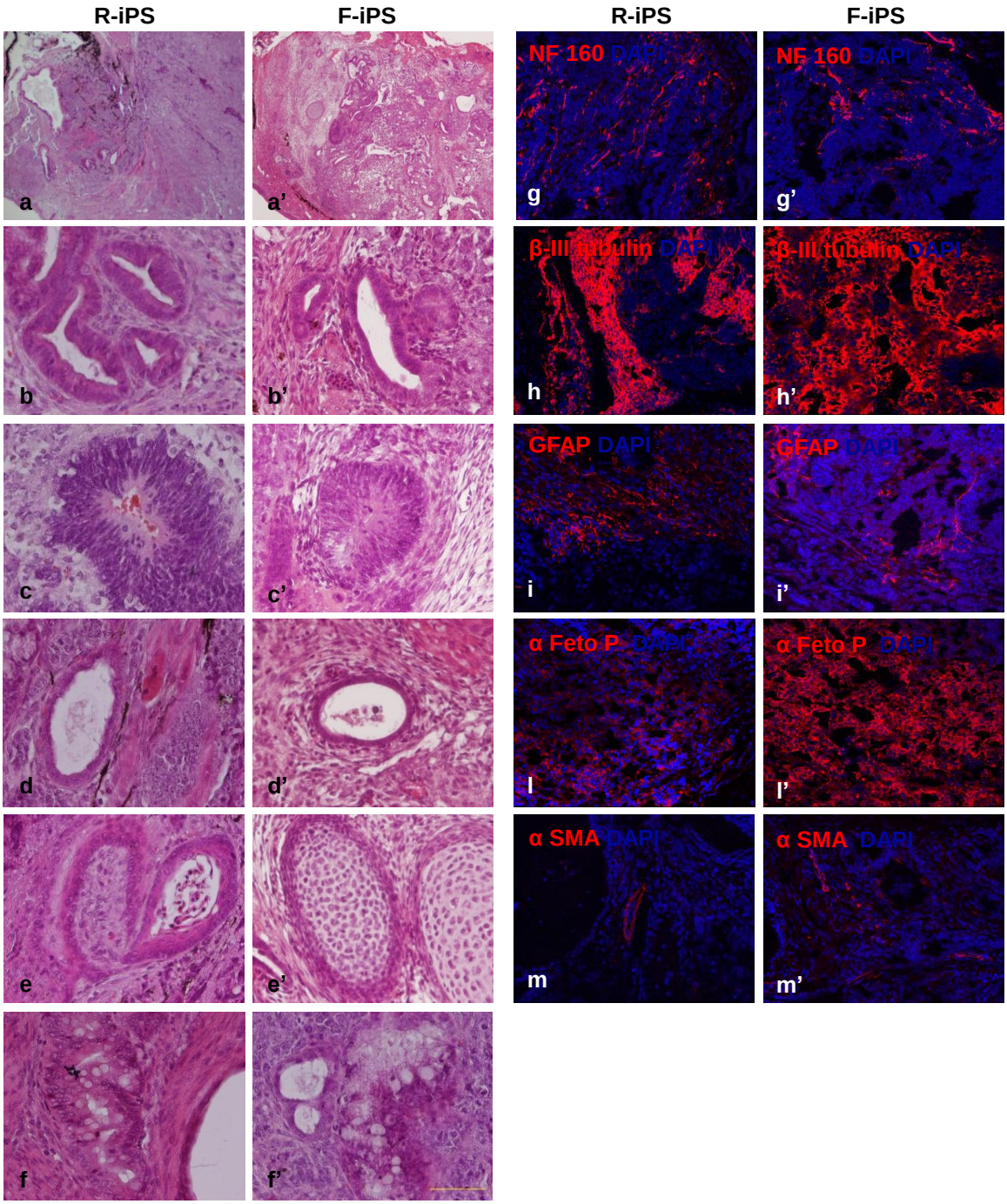

Fig. 5.2. - Analysis of R- and F-iPS cells-induced teratomas. Histological (a, b, c, d, e, f, a', b', c', d', e', f') and immunocytochemical (g, h, i, l, m, g', h ', i', l', m') analysis of teratomas (a, a') generated after subretinal injection of R- or F-iPS cells. The production of cells/tissues specific to ectododermal (b, c, g, h, i, b', c', g', h', i'), endodermal (d, l, d', l') and mesodermal (e, g, m, e', f', m') germ layers was analyzed: (b,b') neuroepithelium, (c, c') neurorosettes, (d, d') vascular-like structures, (e, e', f, f') chondrocytes and adipocytes (g, g', h, h') neural tissue neurofilament 160 (NF 160) and β-III tubulin positive, (i, i') glial cells GFAP positive, (l, l') yolk sac and fetal liver structures positive to α-Feto Protein (α Feto P) and (m, m') α-smooth muscle actin (α SMA)-positive cells, Scale bar for H&E: 100 µm; magnification for immunohistochemistry: 40x.

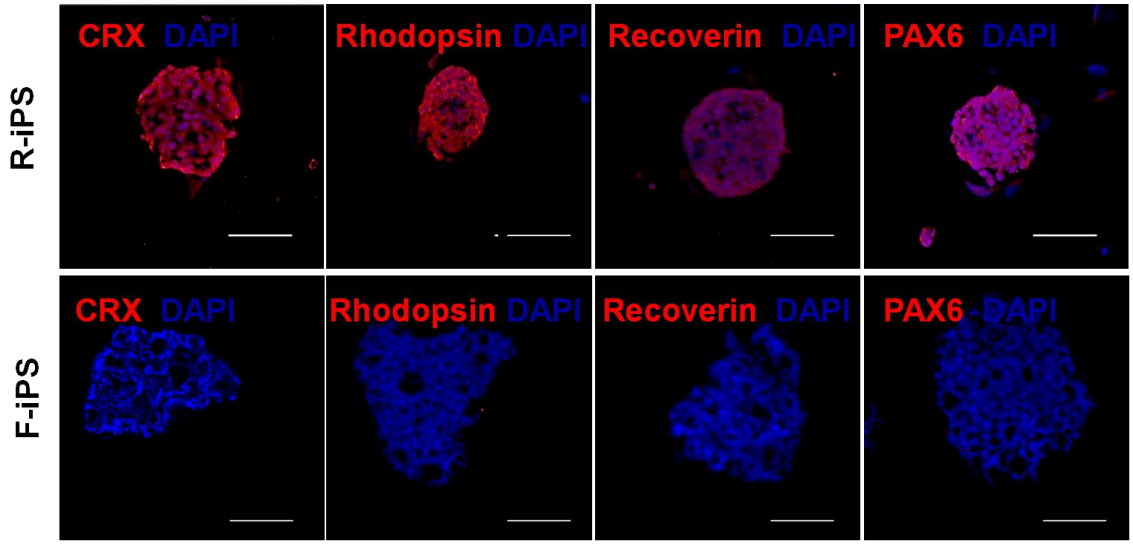

Fig. 5.3 - Epigenetic analysis of R- and F-iPS cells. (A) Evaluation of the expression of photoreceptor markers CRX, Rhodopsin, Recoverin and Pax6 in the R-iPS and F-iPS cells by immunocytochemical analysis. Scale bar: 100 µm.

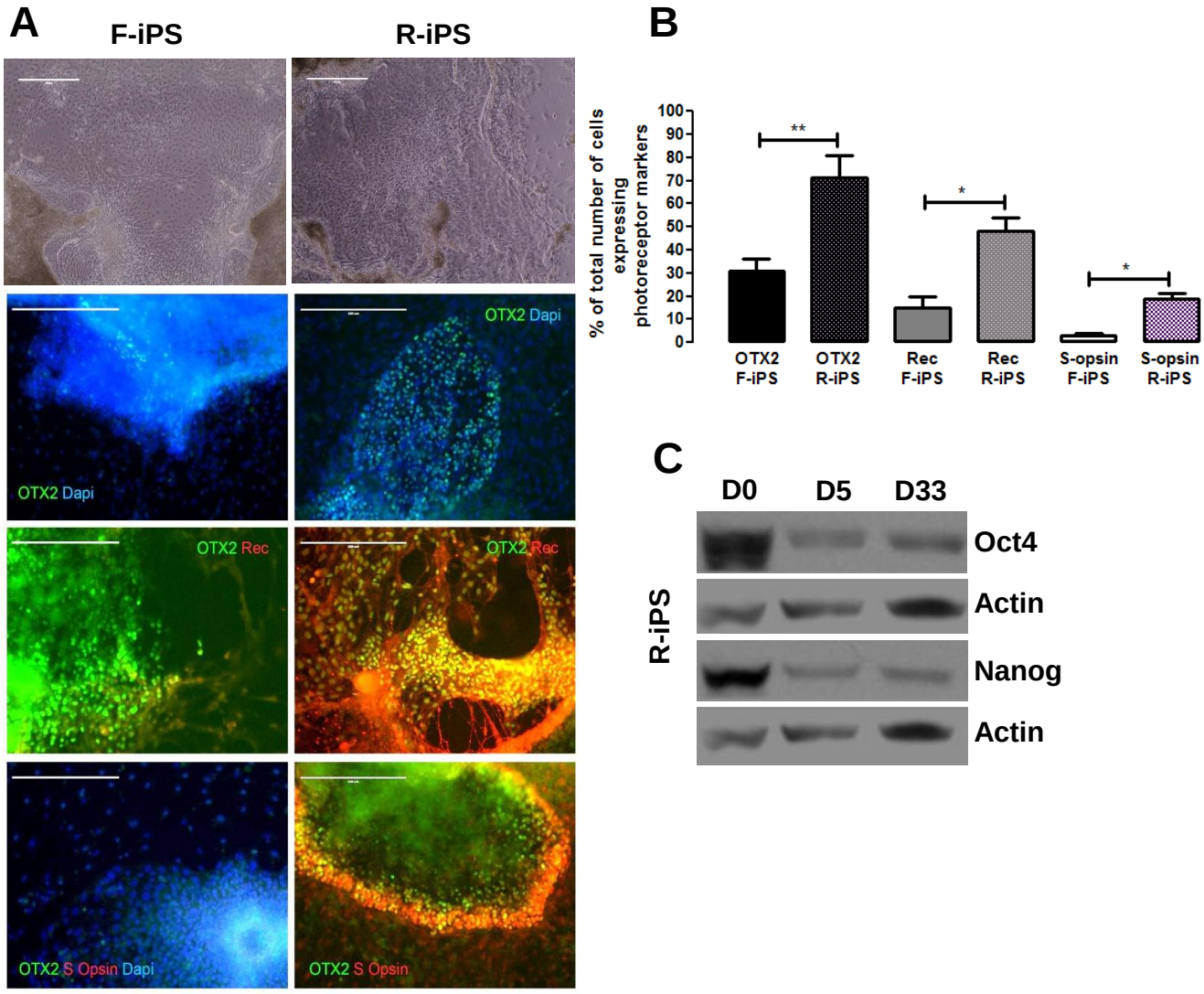

Fig. 5.4 - Differentiation of R- and F-iPS cells into retinal photoreceptors. (A) Microscopic/immunocytochemical analysis comparing R-iPS and F-iPS cells at differentiated stage day 33 (A) and the expression of the photoreceptor markers OTX2, Recoverin and S-Opsin in differentiated cells at day 33 (scale bar 400 µm). (B) Percentage of total number of cells expressing the photoreceptor markers OTX2, Recoverin (REC) and S-Opsin in R-iPS cells at day 33 post-differentiation. These results represent the mean±S.E.M. of n=3 independent experiments; **p<0.01, *p<0.05, compared with F-iPS. One way ANOVA followed by Bonferroni's post-hoc test. (C) Western blot analysis of D0 undifferentiated, D5 embryoid bodies and D33 differentiated R-iPS cells for pluripotency markers (Oct4 and Nanog). Actin was used as a loading control.

5. DISCUSSION

Collectively, these data demonstrate that mouse retinal progenitors cells can be reprogrammed into iPS cells with the four transcription factors Oct4, Klf4, Sox2 and c-Myc. This adds to the growing list of somatic cell types that can be reprogrammed to pluripotency (Stadtfeld et al., 2010a). The ability of human and mouse fibroblast to differentiate into photoreceptors has already been described (Meyer et al., 2009; Osakada et al., 2009; Lamba et al., 2010; Tucker et al., 2011). We demonstrate that retina-derived iPS cells differentiate into photoreceptor cells more efficiently than matched fibroblast-derived iPS cells. This ability is intrinsic to retinal cells because differences in genetic background have been eliminated by the use of the reprogrammable mouse as the donor of both cell types. We also show that this is likely due to the persistence of epigenetic memory from their tissue of origin. Although it is beyond the focus of this study, it is yet to be determined whether epigenetic memory is due to a methylation signature, differences in histone marks, or to other kinds of epigenetic imprinting. Nevertheless, the existence of epigenetic memory has been established in several studies including those focused on iPS cells derived from fibroblasts (Chin et al., 2009; Kim et al., 2010a; Polo et al., 2010), neural cells (Marchetto et al., 2009), retinal pigment epithelium cells (Hu et al., 2010b), hematopoietic progenitors (Kim et al., 2010a), splenic beta cells, bone marrow-derived granulocytes and skeletal muscles precursors (Polo et al., 2010). These studies show that epigenetic memory manifests itself as differential gene expression and altered differentiation capacity that favors differentiation toward the cell of origin. Our study supports these data adding an additional cell type to the list, the retinal progenitor cell. All groups, including ours, characterized early passage iPS cells. Polo et al. found that epigenetic memory and functional differences were absent in late passage cells (p10-p16) suggesting that residual methylation and incomplete reprogramming results in the

transient epigenetic memory (Polo et al., 2010). This is not true for iPS cells derived from human retinal pigment epithelium cells that retain memory after extensive passaging (Hu et al., 2010b). However, it is currently unknown if R-iPS cells loose their epigenetic memory in late passages. Photoreceptor precursors, obtained in vitro from dermal fibroblast –derived iPS cells with the same protocol we used in this study, have been transplanted in degenerative mice by our lab (Tucker et al., 2011). The percentage of differentiated iPS cells expressing early photoreceptor markers in that study was similar to the percentage obtained in this study with F-iPS. Although a low percentage of photoreceptor precursor cells were transplanted in those studies, partial morphological and functional improvement was obtained. Transplantation of a higher number of photoreceptor precursor cells produced in vitro by using R-iPS cells may result in further rescue and represent a new tool for cell-based therapies. One concern regarding the use of R-iPS cell derived photoreceptor precursors is that, unlike retinal progenitor cells, residual undifferentiated iPS cells are tumorigenic. We previously demonstrated that remaining pluripotent cells can be successfully eliminated in iPS cell populations through SSEA selection (Tucker, et al., 2011). Further refinement of such selection strategies will aid in the development of safe and effective transplantation approaches.

6. CONCLUSION

We demonstrate that mouse retinal progenitor cells can be reprogrammed to iPS cells that differentiate into photoreceptors with a very high efficiency compared to the fibroblast-derived iPS cells generated from the same donor. These data confirm that iPS cells from different origins may exhibit distinct differentiation biases. This could be exploited to obtain specific cell types. The retina-derived iPS cells represent potentially a new source of photoreceptors for cell-based therapies of inherited photoreceptor diseases.

Chapter VI
Concluding remarks

Chapter 6 - Concluding remarks

In order to unravel the localization and distribution of NPY receptors in the retina, rat retinal neural cell cultures and pure Müller cell culture were used as *in vitro* models. Additionally, rat retinal slices were also analyzed. As reports in the second chapter of this thesis, several types of cells were detected in this rat retinal neural cell culture: neurons (photoreceptors, bipolar, horizontal, amacrine and ganglion cells), macroglial cells (astrocytes and Müller cells) and microglial cells. By immunocytochemistry, the expression of NPY Y_1 and Y_2 receptors was detected in all these type of cells. Moreover, these two NPY receptors were detected in all retinal layers in rat retinal slices. Therefore, as has previously been suggested (D'Angelo et al. 2002; Oh et al. 2002), the localization of NPY in several types of retinal neurons may indicate a neuromodulatory role of this peptide in the retina. Though, to the best of our knowledge, there are few studies that have demonstrated the participation of the NPY receptors in the physiology of Müller cells and microglial cells. Hence, the presence of NPY Y_1 and Y_2 receptors in microglial cells suggests the involvement of these receptors in the immune responses occurring in the retina under pro-inflammatory conditions found in retinal degenerative diseases. Further studies are needed to dissect the role of NPY receptors in glial cells. However, in spite of our findings regarding NPY Y_1 and Y_2 receptors, further studies are needed to characterize the localization of the remaining NPY receptors in rat retinal cells.

In fact the NPY receptors are present in the retina; their functions are not well known. In order to investigate this question, the neuroprotection role of NPY against glutamate excitotoxicity was evaluated in rat retinal cells. Some retinal diseases are characterized by glutamate excitotoxicity (Kowluru et al., 2003; Santiago et al., 2008) therefore is critical to reduce or prevent this phenomenon. In line with previous studies that have shown the neuroprotective effect of NPY in several parts of the CNS, necrotic and apoptotic cell death trigged by glutamate in rat retinal cells were evaluated in the presence of NPY. In the third chapter of this thesis, we showed the protective effect of

NPY against glutamate excitotoxicity. Likewise the NPY receptors involved in this effect was also investigated. NPY Y_2, Y_4 and Y_5 receptors mediate the protective effect of NPY against necrotic cell death caused by glutamate, and NPY Y_5 receptor mediated the NPY protective effect against apoptotic cell death induced by glutamate. Finally, it has been shown that the neuroprotective effect of NPY is mediated by PKA and p38K.

These results are consistent with the majority of previous studies describing a protective role of NPY, where the peptide was applied prior to the toxic stimulus (Silva et al., 2003b; Thiriet et al., 2005; Alvaro et al., 2008b). NPY prevented glutamate retinal neuronal death, but it had minor effects in the prevention of this glutamate toxic effect in GFAP-positive cells. Furthermore, NPY, in the presence of glutamate, did not enhance the microglia proliferation and activation in contrast with other groups' reports (Ferreira et al., 2011a; Ferreira et al., 2011b; Goncalves et al., 2012). The anti-necrotic effect of NPY, in hippocampal slice cultures, was previously associated with NPY Y_2 and Y_5 receptors. However, NPY Y_1 receptors also contributed to the neuroprotective effect of NPY (Silva et al., 2003b). Although other studies have suggested that only the NPY Y_1 or Y_2 receptors are involved in the rescue of neurons from excitotoxic cell death (Xapelli et al., 2007; Xapelli et al., 2008); the involvement of NPY Y_4 receptor has not been evaluated in the majority of those. The simultaneously activation of different NPY receptors to play the same biological effect was previously described (Xapelli et al., 2007; Alvaro et al., 2009; Smialowska et al., 2009; Son et al., 2011). Additionally NPY roles, including neuroprotection, mediated by PKA and p38K activation have been previously shown(Michel, 1991; Soares Lemos et al., 1997; Pellieux et al., 2000; Hiruma et al., 2002; Milenkovic et al., 2004; Pons et al., 2008; Dhillon et al., 2009; Pocrnich et al., 2009; Rosmaninho-Salgado et al., 2009; Son et al., 2011).

It was previously described that, in cultured rat retinal cells, NPY stimulates the proliferation of neuronal progenitor cells (BrdU+/nestin+ cells) (Alvaro et al., 2008a).

Moreover, NPY, through NPY Y_1 and Y_5 receptor activation, has the potential to maintain human embryonic stem cells self-renewal and pluripotency (Son et al. 2011). Consequently, NPY system may be a putative target to develop new strategies to increase retinal progenitor cell proliferation. In line with these studies, the presence and distribution of NPY and NPY receptors in R-iPS cells, as well as, the proliferative effect of NPY in these cells was evaluated in the fourth chapter of this thesis. We observed that NPY and NPY Y_1 receptor are expressed in R-iPS cells. Further studies are needed to characterize the localization of the remaining NPY receptors in R-iPS cells. However NPY had no effect in the proliferation of R-iPS cells. These receptors should play other role in these cells but further studies are required. Nevertheless it was detected for the first time the presence of NPY system in R-iPS cells.

The retina is trigged by several degenerative diseases (such as, age related macular degeneration, retinitis pigmentosa, etc.) without an effective treatment. Neuroprotection and cell based therapies are two different approaches to treat these diseases. In the fifth chapter of this thesis, mouse retinal progenitor cells from reprogrammable mouse were reprogrammed into iPS cells with the four transcription factors Oct4, Klf4, Sox2 and c-Myc. The retina-derived iPS cells differentiate into photoreceptor cells more efficiently than matched fibroblast-derived iPS cells. This is likely due to the persistence of epigenetic memory from their tissue of origin. More studies need to be performed to understand whether R-iPS cells lose their epigenetic memory in late passages. In fact, a high yield of photoreceptors was obtained; however the big concern regarding the transplantation of R-iPS cells-derived photoreceptors in the presence of tumorigenic residual undifferentiated iPS cells is real. Cell sorting techniques need to be improved. Nevertheless, R- iPS cells represent potentially a new source of photoreceptors for cell-based therapies of inherited photoreceptor diseases.

In summary, NPY receptors are present in the retina. They play a important neuroprotective role against glutamate excitotoxicity and may be used as putative targets in the prevention of several retinal degenerative diseases. Additionaly R-iPS cells may be used in cell based therapies as a alternative and effective treatment in retinal degenative diseases.

Chapter VII
References

Chapter 7 - References

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