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THE ROLE OF NEUROPEPTIDE Y IN ARTICULAR CHONDROCYTE FUNCTIONS

Dissertação de Mestrado em Investigação Biomédica, orientada pela Professora Doutora Alexandrina Ferreira Mendes e pela Doutora Cláudia Maria Fragão Pereira e apresentada à Faculdade de Medicina da Universidade de Coimbra

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Digo: o real não está na saída nem na chegada:
ele se dispõe para a gente é no meio da travessia
Guimarães Rosa

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Sumário

List of abbreviations

Acp65	Acetyl-lysine 310 NFkB-p65
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ANOVA	Analysis of Variance
BCA	Bicinchoninic acid
BMCs	Bone marrow cells
BSA	Bovine serum albumin
CHUC	University and Hospital Center of Coimbra
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECF	Enhanced chemifluorescence
ECL1	Extracellular loop-1
ECL2	Extracellular loop-2
ECL3	Extracellular loop-3
ECM	Extracellular matrix
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FBS	Fetal bovine serum
GPCR	G-coupled protein receptor
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-18	Interleukin-8
iNOS	Inducible nitric oxide synthase
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa B
NPY	Neuropeptide Y
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PP	Pancreatic peptide
PYY	Peptide YY
PVDF	Polyvinylidene fluoride

RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOX-9	SRY (sex determining region Y)-box 9
TBS	Tris-buffered saline
TBS-T	Tris-Buffered Saline and Tween 20
TNF-α	Tumor necrosis factor alpha
Y1	Neuropeptide Y receptor Y1
Y3	Neuropeptide Y receptor Y3
Y4	Neuropeptide Y receptor Y4
Y5	Neuropeptide Y receptor Y5
Y6	Neuropeptide Y receptor Y6
Y7	Neuropeptide Y receptor Y7
Y8	Neuropeptide Y receptor Y8

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Abstract

Neuropeptide Y (NPY) has been suggested as a possible therapeutic target in aging-related diseases due to its ability to inhibit inflammation and stimulate cell proliferation, differentiation and survival. Different studies show its role in inhibiting inflammatory cytokines in cells of the immune system. Moreover, it has beneficial effects on bone remodelling, promoting cellular homeostasis. In the synovial tissue, this peptide has been detected in sympathetic nerve fibers in patients with osteoarthritis (OA) and recently studies have shown that patients with advanced degrees of osteoarthritis have high levels of NPY in the synovial fluid and those levels correlated with the intensity of pain, suggesting a role for NPY as a putative regulator of pain transmission and perception in OA.

OA is a disease that causes damage throughout the joint tissues and cartilage is the most severely affected. The causes of the disease are not well understood; however, it is accepted that synovial inflammation can be an aggravating and/or initiating factor, promoting changes in the human chondrocytes that begin to produce enzymes that degrade the cartilaginous matrix. One of the major risk factors for OA development is aging. Due to the increase in life expectancy worldwide and the damage caused by OA, leading to disability and pain, and the lack of effective disease-modifying therapies, the associated socio-economic costs are very high and keep increasing which led the World Health Organization to designate OA as a priority disease for which it is urgent to find new therapeutic targets and therapeutic strategies.

Due to the potential of NPY to promote homeostasis by inhibiting aging-related cell and tissue damage and loss of function and to previous studies in our group that have shown the presence of NPY receptors on human chondrocytes and that NPY is able to reduce inflammatory responses in these cells, we hypothesised that it may be effective in stopping OA development and/or progression. Thus, this study aims to understand the role of NPY in OA by studying its ability to modulate human chondrocyte anabolic, catabolic and inflammatory functions. For this, we used human chondrocytes isolated from the knee cartilage of cadaver tissue donors, collected at the Bone and Tissue Bank of the University and Hospital Center of Coimbra. First, we tried to identify the NPY receptor subtypes that mediate its anti-inflammatory effects in human chondrocytes. The results obtained suggest that the three main NPY receptor subtypes, Y1, Y2 and Y5 receptors, are required to mediate NPY anti-inflammatory responses, yet Y5 appears to play a more significant role. Furthermore, we have shown that NPY does not directly modulate the NF-κB activation pathway to promote the reduction of inflammation, but appears to act on other regulatory proteins indirectly leading to inhibition of NF-κB/p65 transcriptional activity.

The cartilage extracellular matrix is actively remodelled by chondrocytes under inflammatory conditions. Thus, we also evaluated the ability of NPY, alone and under inflammatory conditions, to modulate the expression of Matrix Metalloprotease (MMP)-13, which is a major collagenase involved in OA, and of collagen type 2, the major protein component of the articular cartilage ECM. NPY did not decrease the protein levels of MMP-13, either under basal or inflammatory conditions mimicked by treatment of the cell with the pro-inflammatory cytokine, Interleukin-1β (IL-1β). However, it significantly decreased basal MMP-13 mRNA levels and also reduced the increase induced by IL-1β, although in this case, the difference did not reach statistical significance due to the large variability in cell extracts obtained from different donors. On the other hand, NPY was

found to significantly increase collagen type 2, both at the mRNA and protein levels, and also to significantly decrease the inhibitory effect of IL-1β, although statistical significance was reached only for MMP-13 protein levels, suggesting that NPY plays an important pro-anabolic role, either under basal or inflammatory conditions. Nonetheless, a larger number of samples will have to be analysed to determine more conclusively whether NPY has anti-catabolic and pro-anabolic effects in human chondrocytes.

In summary, this study shows that NPY has anti-inflammatory, anti-catabolic and pro-anabolic effects in human chondrocytes, suggesting that it can play a protective role in human joints which are the basis for additional studies to determine the efficacy of NPY and its receptors as potential therapeutic targets for OA.

Keywords: NPY, osteoarthritis, aging, chondrocytes.

Resumo

O neuropeptídeo Y (NPY) tem sido sugerido como possível alvo terapêutico em doenças relacionadas ao envelhecimento, devido à sua capacidade de inibir a inflamação e estimular a proliferação, diferenciação e sobrevivência celular. Diferentes estudos mostram seu papel na inibição de citocinas inflamatórias em células do sistema imunológico. Além disso, tem efeitos benéficos na remodelação óssea, promovendo a homeostase celular. No tecido sinovial, esse peptídeo foi detectado em fibras nervosas simpáticas em pacientes com osteoartrite (OA) e estudos recentes mostraram que pacientes com graus avançados de osteoartrite têm níveis elevados do NPY no líquido sinovial e esses níveis se correlacionam com a intensidade da dor, sugerindo um papel para o NPY como um regulador putativo da transmissão e percepção da dor na OA.

OA é uma doença que causa danos ao longo dos tecidos articulares e a cartilagem é mais gravemente afetada. As causas da doença não são bem compreendidas; entretanto, aceita-se que a inflamação sinovial pode ser um fator agravante e / ou desencadeante, promovendo alterações nos condrócitos humanos que passam a produzir enzimas que degradam a matriz cartilaginosa. Um dos principais fatores de risco para o desenvolvimento da OA é o envelhecimento. Devido ao aumento da expectativa de vida em todo o mundo e aos danos causados pela OA, levando à incapacidade e dor, e à falta de terapias modificadoras da doença, os custos socioeconômicos associados são muito altos e continuam aumentando, o que levou a Organização Mundial da Saúde a designar a OA como uma doença prioritária para a qual é urgente encontrar novos alvos terapêuticos e estratégias terapêuticas.

Devido ao potencial do NPY em promover a homeostase pela inibição do dano e perda de função celular relacionada ao envelhecimento e aos estudos anteriores em nosso grupo que mostraram a presença de receptores NPY em condrócitos humanos e que o NPY é capaz de reduzir respostas inflamatórias nessas células, nós levantamos a hipótese que poderia ser eficaz em parar o desenvolvimento e / ou progressão da OA. Assim, este estudo tem como objetivo compreender o papel do NPY na OA estudando sua capacidade de modular as funções anabólicas, catabólicas e inflamatórias dos condrócitos humanos. Para isso, foram utilizados condrócitos humanos isolados da cartilagem do joelho de dadores post mortem, coletados no banco de ossos e tecidos do centro hospitalar e universitário de Coimbra. Primeiro, tentamos identificar os subtipos de receptores do NPY que medeiam seus efeitos anti-inflamatórios nos condrócitos humanos. Os resultados obtidos sugerem que os três principais subtipos de receptores do NPY, os

receptores Y1, Y2 e Y5, são necessários para mediar as respostas anti-inflamatórias do NPY, no entanto Y5 parece desempenhar um papel mais significativo. Além disso, mostramos que o NPY não modula diretamente a via de ativação do NF-κB para promover a redução da inflamação, mas parece agir indiretamente sobre outras proteínas reguladoras, levando à inibição da atividade transcricional do NF-κB / p65.

A matriz extracelular da cartilagem é ativamente remodelada por condrócitos sob condições inflamatórias. Assim, também avaliamos a capacidade do NPY, isoladamente e sob condições inflamatórias, de modular a expressão da metaloproteinase da matriz (MMP) -13, que é uma das principais colagenases envolvidas na OA, e do colágeno tipo 2, o principal componente proteico do ECM da cartilagem articular. O NPY não diminuiu os níveis da proteína MMP-13, seja em condições basais ou inflamatórias, mimetizadas pelo tratamento das células com a citocina pró-inflamatória Interleucina-1β (IL-1β). No entanto, diminuiu significativamente os níveis basais do mRNA da MMP-13 e também reduziu o aumento induzido pela IL-1β, embora, neste caso, a diferença não tenha alcançado significância estatística devido à grande variabilidade nos extratos celulares obtidos de diferentes doadores. Por outro lado, descobriu-se que o NPY aumenta significativamente o colágeno tipo 2, tanto os níveis proteicos como do mRNA, assim como também diminuiu significativamente o efeito inibitório da IL-1β, embora a significância estatística tenha sido alcançada apenas para os níveis de proteína MMP-13, sugerindo que o NPY desempenha um importante papel pró-anabólico, seja sob condições basais ou inflamatórias. No entanto, um número maior de amostras terá que ser analisado para determinar mais conclusivamente se o NPY tem efeitos anti-catabólicos e pró-anabólicos em condrócitos humanos.

Em resumo, este estudo mostra que o NPY tem efeitos anti-inflamatórios, anti-catabólicos e pró-anabólicos em condrócitos humanos, sugerindo que ele pode desempenhar um papel protetor nas articulações humanas, que são a base para estudos adicionais para determinar a eficácia do NPY e seus receptores como potenciais alvos terapêuticos para OA.

Palavras-chave: NPY, osteoartrite, envelhecimento, condrócitos.

Chapter 1: Introduction

1.1. NPY general considerations

Neuropeptide Y (NPY) is a 36-amino acid peptide originally isolated from porcine brain in 1982, by a technique developed in 1978, that detects biologically active peptides with an amidated carboxy-terminus (1). NPY belongs to the same peptide family as tyrosine-tyrosine peptide (PYY) and pancreatic polypeptide (PP) to which it exhibits 70-80% and 50% homology, respectively (2).

Larhammar, et al (3) reported that, during evolution, NPY an extremely well conserved sequence throughout vertebrate evolution, sharing between cartilaginous mammals and fish 92% identity. When comparing to the sequence between different species of mammals, only 2 to 3 amino acids are variable (4). On the other hand, PYY exhibits eight varying amino acids between different orders of mammals and as PP evolved very quickly, it is one of the least conserved peptides known (5).

In humans, the NPY gene is located in chromosome 7 at the locus 7-15.1, composed of four exons and results in the synthesis of a 97 amino acid pre-pro NPY (6). NPY processing occurs through cleavage of pre-pro NPY by different enzymes, such as peptidases, prohormone convertase, carboxypeptidases and peptidyglycine alpha amidating monooxygenase, resulting in various peptide fragments (7). Pre-pro NPY cleavage produces 3 fragments one of which, the NPY 1-36, is referred as biologically active, later being truncated by enzyme dipeptidyl peptidase 4 (DPP-4) to produce $NPY_{3-36,}$ which is an inactive fragment (8).

NPY is abundantly expressed in numerous cells of the nervous system, acting as a neurotransmitter and promoting an increase in food intake and energy balance, facilitating learning and memory via the modulation of hippocampal activity (9) (10). It also regulates endocrine function (11), blood pressure (12), body weight (13) and the circadian rhythm (14). Recent studies reported that NPY is further expressed in many cells such as macrophages (15), mature osteoblasts (16), osteocytes (17), megakaryocytes (18) and leukocytes (19). In peripheral tissues, NPY plays a role in bone homeostasis (20), regulates immune cell functions (21), is a potent vasoconstrictor and angiogenesis promotor (8) and regulates differentiation, proliferation and apoptosis in different cell types (22) (23).

All the neuropeptides interact with the NPY family of G-protein coupled receptors (GPCRs) with distinct degrees of affinity and potency. The functional receptors in mammals are NPY Y1, NPY Y2, NPY Y4 and NPY Y5 (24).

1.1.1. Subtypes of NPY receptors

The GPCR activation, alters the G protein conformation, causing GTP-Gα disassociation in beta and gamma subunits, thus transmitting cellular signal (figure 1) (25). NPY receptors in most cases are coupled to pertussis toxin-sensitive G protein (Gi / Go). Signal transduction mechanisms related to NPY activation are not fully understood. However as shown in figure 1 one of the commonly observed signaling responses in various cell types is inhibition of adenylyl cyclase after binding of an agonist to NPY receptors (26). Activation of NPY receptors promotes a decrease in cyclic adenosine monophosphate (cAMP), through the activation of an inhibitory Gα protein (8). NPY was also reported to be able to stimulate genes containing cAMP response element (CRE) in human neuroblastoma cell lines, suggesting that NPY could promote the production of cAMP or inhibit its degradation by phosphodiesterases (27) (14). NPY can also regulate calcium levels by activating or blocking calcium channels at the plasma membrane (14). Moreover, NPY may activate potassium transient current (8), or inhibit as observed in vascular smooth muscle cells (28), showing that potassium channels are also targets of NPY receptors. Several studies suggest that NPY can activate MAPK and ERK pathway, leading to cellular proliferation via Y1 (20) (29). Another molecule that NPY is involved in is nitric oxide (14). NPY mediates the activation of NO in retinal neural cells and progenitor cells, which can also activate ERK1 / 2 and has been implicated in inducing proliferation in these cells (20). The Gβγ protein subunits, in turn, activate different kinase cascades that are the foundation for the physiological effects of NPY.

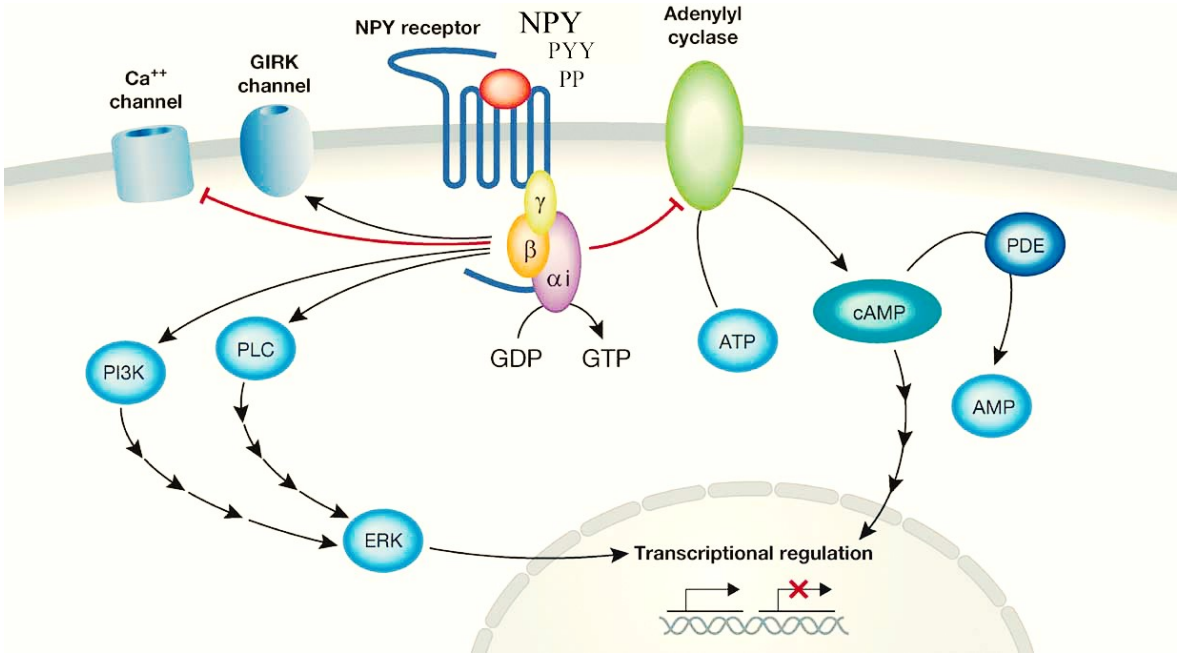

Figure 1. Intracellular signaling cascades for NPY receptors. *Adapted with permission from Brothers and Wahlestedt* (25)*., 2010.*

1.1.2. NPY Y1

NPY Y1 (Y1) was cloned in immortalized cells derived from human neuroblasts (SKN-MC) in 1992 (30). It is composed of 384 amino acids and can be activated by NPY and PYY agonists, showing low affinity towards PP. Y1 was already described as being expressed in the brain (31), kidney (32), heart (8), lung (33), bone marrow and bone tissue (34), among others. It mediates various NPY functions in peripheral tissues (table 1), including anti-inflammatory responses (35), vasoconstriction (36), proliferation, differentiation and survival of different cell types (22) (37), and plays a great role in bone homeostasis maintenance (38).

1.1.3. NPY Y2

NPY Y2 (Y2) was cloned from immortalize kidney cells (COS-7) in 1995. It is composed of 381 amino acids and can be activated by NPY and PYY agonists with similar degrees of affinity and very low affinity for PP (39). The Y_2 receptor is principally expressed in hippocampal neurons, in the thalamus, hypothalamus, and in the peripheral nervous system (40). Moreover, it is expressed by adipose cells, macrophages, neutrophils, cardiomyocytes and endothelial cells (41) (42). In general, it mediates functions such as the anti-inflammatory response (43), angiogenesis (44), bone formation (45), cell proliferation and differentiation (46).

1.1.4. NPY Y5

NPY Y5 (Y5) was also cloned in COS-7 cells in 1996 and is composed of 371 amino acids. The affinity for NPY is huge compared to PYY, and for PP is slightly lower (47). The Y5 receptor is preferentially expressed in the central nervous system, in high density in the hippocampus and hypothalamus (48). Y5 receptors have been reported in peripheral cells, such as macrophages (49), bone marrow stromal cells (BMCs) (46), human embryonic stem cells (50), rat granulocytes (51), and endothelial cells (52). Some of the functions of Y5 are to promote revascularization after ischemia (53), to stimulate proliferation and differentiation (52), and to modulate inflammation (51).

1.1.5. NPY Y3, NPY Y4 and NPY Y6

NPY Y3 (Y3) is characterized by high affinity for NPY compared to PYY. The existence of the Y3 receptor has been suggested by pharmacological studies in human, rat, and rabbit tissues. However, since all attempts to clone Y3 were unsuccessful, it is suggested that this receptor may be a multimerization of one or more NPY receptors (25). NPY Y4 (Y4) contains 375 amino acids, with the highest affinity for PP. PYY and NPY also activate this receptor, but with much lower potency. It is mainly expressed in the gastrointestinal tract, but can also be found in the pancreas, prostate and brain and plays a role in colonic transit, circadian ingestion, energetic homeostasis and regulation of feeding (24)

The gene NPY Y6 (Y6) is a pseudogene in humans, monkeys and pigs, because it contains a frameshift mutation corresponding to the third intracellular loop that results in a truncated and non-functional receptor protein. Nevertheless, this 371 amino acid receptor is present and has been cloned in rabbits, mice, and chickens (54).

1.1.6. NPY Y7 and NPY Y8

Concerning the NPY Y7 (Y7) receptor, only pharmacological information in zebrafish exists indicating that it can bind NPY, PYY and PP. In zebrafish, it is expressed in the brain, eye and intestine. According to evolutionary studies, it is suggested that the gene of this receptor was lost long before the divergence of the marsupial and placental mammals (55). As Y8 seems to be absent in mammals and birds, studies consider that this receptor may have been lost in the amniote ancestor (56).

Table 1: Receptor subtypes of the NPY family

Receptors	Native ligand	cDNA cloned (years)	Comments/Function
NPY Y1	NPY>PYY	1992	Regulation of bone homeostasis, anti-inflammatory function, vasoconstriction, maintenance of cellular self-renewal and pluripotency.
NPY Y2	NPY=PYY	1995	Bone formation, anti-inflammatory function, angiogenesis .
NPY Y3	NPY=PYY	never	It may be a multimerization form of one or more NPY receptors .
NPY Y4	PP > PYY > NPY	1995	Y4 receptors appear to be the principal receptor for pancreatic polypeptide.
NPY Y5	NPY > PYY	1996	Stimulates the proliferation of all bone marrow cells, endothelial cell proliferation and migration and tube formation
NPY Y6	NPY=PYY > PP	1996	Inactive in primates due to a frameshift mutation.
NPY Y7	N/A	N/A	Appears to have lost its function in mammals.
NPY Y8	N/A	N/A	Appears to have lost its function in mammals.

Table 1: *Adapted from Brothers, Shaun P. and Claes Wahlestedt* (25). *"Therapeutic potential of neuropeptide Y (NPY)*

1.2. Functions of NPY and its receptors

Inflammation is critical in many aging-related diseases. Many studies describe that chronic low-grade inflammation is a risk factor for disease development and mortality in the elderly (57). The harmful condition of low-grade inflammation is the persistent one that results in responses that lead to damage and tissue degeneration (58). Understanding the role of NPY and its receptors in inflammatory conditions may be a tool for the treatment of low-grade inflammatory diseases. NPY also regulates cell

proliferation, differentiation and survival, which are factors that may be deregulated in aging-related diseases (23) (59).

1.2.1. Role of NPY in inflammation

An anti-inflammatory role of NPY has been demonstrated through the signaling of Y1 and Y5 receptors, which suppressed paw edema in adult and elderly rats, but not in young rats, showing that NPY action in paw edema is age-dependent (60). Y1 receptor-deficient intraperitoneal macrophages isolated from obese rats have an increased inflammatory response upon stimulation, with increased secretion of pro-inflammatory proteins; however macrophages isolated from wild-type obese mice that were treated with NPY showed an attenuation of the inflammatory effects and this effect was blocked by a selective Y1 antagonist (35).

NPY is produced at basal levels by macrophages and dendritic cells (DCs) and pro-inflammatory stimuli increase NPY production. In addition, inhibition of NPY receptors promotes the maturation of DCs cells and the production of Interleukin-6 (IL-6) and TNF-α suggesting an anti-inflammatory function of NPY in DCs cells (15). Moreover, NPY stimulates the migration of immature dendritic cells and the polarization of these cells to a Th2 profile (61).

Several studies have shown that NPY can suppress microglial reactivity and also act to inhibit phagocytosis and motility caused by inflammatory stimuli through Y1 receptor signaling (62) (63) (64). However, it has been shown that NPY stimulated phagocytosis and chemotaxis of peritoneal macrophages from adult mice and decreased phagocytosis and the respiratory burst of peritoneal macrophages from old mice (65).

The oxidative burst in peritoneal macrophages is regulated by NPY and can be stimulated via Y1 and Y2 receptors or suppressed via Y2 and Y5 receptors (49) . Finally, NPY also appears to inhibit LPS and concanavalin A-induced lymphoproliferation in adult rats, however the effects in old rats were only observed with higher NPY doses (66).

1.2.2. Role of NPY in proliferation, differentiation, and cell survival.

Different studies reveal that NPY plays a fundamental role in neurogenesis, in pathological and non-pathological conditions . Howell et al (67), found that NPY regulates neurogenesis in normal dendritic hippocampal cells via Y1 receptor (68). Moreover, mice under the conditions of induced convulsion and knock-out by Y1 receptor

($Y1^{-/-}$), showed a decrease in the proliferation of dentate gyrus cells, caudal subventricular zone and subcallosal zone (69).

Wu et al (23), investigated the role of NPY in the proliferation of BMSCs. The results revealed that NPY promotes proliferation of bone marrow stromal cells by protecting BMSCs from apoptosis induced by starvation, showing the role of NPY in cell survival (23). Park et al (70), showed that NPY deficiency affected bone marrow microenvironment and $Y1^{-/-}$ mice survival, leading to apoptotic destruction of sympathetic nervous system fibers (SNS) and endothelial cells. Furthermore, treatment with NPY of a mouse model of SNS injury induced by chemotherapy, was able to significantly reduce bone marrow lesions (70). The role of NPY in proliferation and survival has been shown to have the ability to rejuvenate the growth characteristics of mature BMSCs, as a result of overexpression of the Y5 receptor, indicating an anti-aging role (34). In addition, in embryonic stem cells, NPY promotes self-renewal maintenance and pluripotency via Y1 and Y5 (50).

It was reported that NPY has a mitogenic effect in adipose tissue cells, because it stimulates proliferation via Y1. Furthermore, Yang et al (71), suggested that NPY contributes to increase visceral adiposity in lean rats and showed that NPY expression in primary rat preadipocytes is subject to up-regulation by insulin and glucocorticoids, which are important hormones involved in obesity. Kuo1 et al (72), showed that NPY promotes the growth in preadipocytes and endothelial cells and these effects were blocked by a Y2 antagonist. Moreover, after injection with an Y2 antagonist, a decrease in adipose tissue weight and volume in obese and lean mice was observed and the authors concluded that NPY stimulated in situ adipogenesis and angiogenesis via Y2 (72).

NPY plays a critical role in bone remodeling, being produced by osteoblasts, osteocytes and chondrocytes (16) (17). Yahara et al (73), showed in vitro that after knockdown of the Y1 receptor with a siRNA, the osteoblastic cells presented high levels of bone proteins, such as osteocalcin, collagen I and bone sialoprotein, and increased levels of Runx2 and osterix mRNA, which are essential transcription factors in osteoblastic differentiation and bone formation (73). Moreover, BMCs from $Y1^{-/-}$ mice, under osteogenic conditions, formed more mineralized nodules (74) and mesenchymal osteoprogenitor cells isolated from the bone of $Y1^{-/-}$ mice, had enhanced ability to differentiate into osteoblasts (46). Thus, NPY appears to play a suppressive role in the differentiation of osteoprogenitor cells (74). Teixeira et al (75), reported that chronically elevated NPY levels are a negative regulator of Y1 receptor expression on osteoblasts,

having an osteogenic potential (75). NPY via Y1 receptor regulates bone uptake through osteoclasts. It has been shown that Y1 receptor deficiency in osteoclasts stimulates the formation of multinucleated cells with reduced resorption capacity. In addition, it has been suggested that the increase in bone mass observed in $Y1^{-/-}$ mice is a result of a deficiency on resorbing allied to increased osteoblast activity (76). However, the differentiation effect of NPY via Y1 receptors on osteoblasts is still controversial, since NPY has been reported to promote osteoblastic differentiation in human cells, rats and mesenchymal stem cells (46).

1.3. NPY functions in bone and cartilage

NPY is present in the bone through fibers of the sympathetic nervous system and is also expressed by bone cells such as osteoblasts, osteoclasts and also chondrocytes (77) (78) (79). Studies have also identified that NPY fibers of sympathetic nerves are also present in the articular structure as in the the subchondral bone marrow and osteophytes in osteoarthritis disease (80).

1.3.1. Role of NPY in bone

The presence of NPY was confirmed in the bone, nerve fibers, megakaryocytes of the bone marrow and the membrane that covers the outer surface of the bones (periosteum) (81) (77). As these NPY fibers are commonly found on the walls of blood vessels of various animals, it was first thought that NPY would play a role in the bone as a vasoregulator (82). However, other studies have found the presence of NPY in other cells of the bone microenvironment (16) (20).

Osteoclasts play a role in bone remodeling, where damaged old bone is reabsorbed followed by the formation of a new bone (83). Exacerbation of reabsorption activity is observed in metabolic diseases such as rheumatoid arthritis and osteoporosis (84) (85). Sousa et al (76), showed that the morphology of the knockout osteoclasts for the Y1 receptor is altered, and these osteoclasts increase in size and have a deficiency in their reabsorption activity. This deficiency is a consequence of a downregulated of MMP-9 expression, which consequently decreases the C-terminal telopeptide (CTX-I), which is degradation products from collagen I. Moreover, after treatment of cells with BIBP 3226 a Y1 receptor antagonist, showed a decrease in resorption (76). A mice model of osteoporosis caused by ovariectomy was used to study the involvement of NPY in bone

remodelling. Female mice underwent ovariectomy and treated for 21 days with NPY, results showed that the number of osteoclasts was reduced after the treatment by mobilization of hematopoietic stem/progenitor cell (HSPC), as well as by the increase in the number of osteoblasts via Y1 receptor (86). Furthermore, using transthyretin (TTR) knockout mice as a model of increased NPY levels, it was observed a decrease in the number of osteoclasts (87). NPY also inhibited the formation of osteoclast-like cells of BMCs following treatment with isoprenaline, a β-adrenergic receptor agonist (β-AR) which promotes osteoclastogenesis. The study suggests that the interaction between NPY and β-adrenergic stimulation in the neural regulation of bone resorption occurs via Y1 and β2-AR (88).

1.3.2. NPY role in cartilage

Healthy cartilage does not contain blood vessels and is not innervated (89). However, Suri et al.(80), showed that innervation of articular cartilage and osteophytes is associated with invasion by blood vessels and nerves in tibiofemoral osteoarthritis disease. Moreover, NPY-positive sympathetic nerve fibers were found in the osteoarthritic synovial joint in the vascular channels of cartilage (79) (80). The sympathetic nerves positive for NPY appear in both the early and advanced stages of OA, nonetheless Wang et al. (90), showed that the concentration of NPY in synovial fluid may vary depending on the degree of osteoarthritis.

NPY concentrations were increased in the middle and advanced degrees of knee OA, with pain in patients being directly proportional to NPY concentration (90). The authors point out that the pro-inflammatory mediators released in the joint could sensitize afferent neurons of the joint, leading to the sensation of pain. NPY is a neuromodulator that is stored in the terminal branches of the Aδ and C fibers, where its release at the joint decreases the activation threshold of the snowy endings, which probably contributes to the chronic and sensitized pain (91) (92).

This recent study points to NPY as a possible regulator of transmission and perception of pain in knee OA. Furthermore, studies have shown that NPY can modulate pain by decreasing membrane Ca^{2+} conductance in dorsal root ganglion neurons and peripheral inflammation also increases NPY and its Y1 and Y2 receptors in the dorsal corpus of the spinal cord, reinforcing that NPY participates in nociception (90).

1.4. NPY as therapeutic target

NPY promotes homeostasis in various tissues and under pathological conditions its level is altered in different age-related diseases such as neurodegenerative (93) (Alzheimer's and Parkinson's), metabolic disease (40) (dyslipidemia, diabetes and hypertension) and osteoarthritis (94).

1.4.1. NPY in neurodegenerative diseases

NPY is decreased in Alzheimer's disease in the hippocampus and cerebral cortex of patients. In Parkinson's disease, NPY levels are increased in the striatum which may be due to a mechanism trying to neutralize the hyperexcitability induced by excess glutamate in the disease. Studies have shown that NPY appears to have a therapeutic potential in neurodegenerative diseases, attenuating pathological mechanisms such as protein aggregates, inflammation and neuronal death. NPY has been described as a stimulator of neural survival and proliferation and an inducer of autophagy and attenuates neuroinflammation by decreasing inflammatory factors (93).

In an in vitro model of Alzheimer in which cortical neurons from rats are exposed to amyloid β peptide, NPY decreased neuronal death by restoring nerve growth factor (NGF) levels (95). Moreover, NPY induces an up-regulation of BDNF that has the function of maintaining established neurons and stimulates the proliferation and differentiation of neurons and synapses (96). In other models of neurodegenerative diseases such as Machado-Joseph, NPY increased the levels of BDNF in the striatum (97). In a Huntington's disease model in mice, NPY improved the motor phenotype and the survival of these mice, and it was suggested that NPY also promotes neuronal survival in pathological condition (98). Autophagy, is an intracellular degradation system where unwanted cargo, such as old or damaged organelles and misfolded proteins, are digested and the macromolecular contents from the digestion are released back into the cytosol (99). In neurodegenerative diseases, such as Alzheimer and Parkinson, for example, there is an accumulation of proteins promoting dysregulation in the cellular microenvironment (100). A recent study showed that NPY stimulates autophagy in neural cells of the hypothalamus via Y1 and Y5 receptors. By using an antagonist for NPY receptors, the authors showed that caloric restriction-induced autophagy was inhibited (101). NPY is able to inhibit the release of pro-IL-1β and also inducible nitric oxide synthase (iNOS), as well as phagocytosis and microglial motility, all of these mechanisms are activated via

the Y1 receptor (102). NPY prevents excessive production of IL-1β and TNF-α by inhibiting microglia reactance through the reduction of N-methyl-D-aspartate current (NMDA) in rat cortical neurons, preventing excitotoxicity (62). It can act in neuroinflammation that accompanies neurodegenerative diseases.

1.4.2. NPY in metabolic diseases

In metabolic diseases, such as diabetes and dyslipidemia, NPY and its receptors may play a role in accentuating pathophysiological aspects of these diseases. Dyslipidemia is characterized by an elevation of triglycerides due to hepatic secretion of very low-density lipoproteins (VLDL). Studies have reported that increased NPY in the neural circuits may result in dyslipidemia. The injection of NPY in the third lateral ventricle increases the secretion of VLDL in the liver of lean and fasted rats, via Y1 receptor. Type 2 diabetes is characterized by insulin resistance that is related to islet cell dysfunction. NPY has an influence on glucose metabolism in adipocytes, inhibiting adenylate cyclase in conjunction with the reduction of cyclic adenosine monophosphate levels, and calcium influx via Y1 receptor (40). NPY plays an important role in the regulation of insulin sensitivity in adipocytes. It has been observed that NPY inhibited the uptake of glucose stimulated by insulin via the AKT signaling pathway. NPY impairs the phosphorylation of AKT through the Y1 receptor, which this leads to inhibition of glucose uptake induced by insulin secretion (103). In addition, Y2 receptor inhibitors has been implicated as a therapeutic target for obesity and diabetes because it has slowed the progress of diabetes and improved glucose tolerance . In hypertension, NPY appears to play a regulatory and beneficial role. In a rodent model of over-expression of the NPY gene, the elevation of blood pressure after central nitric oxide synthase (NOS) inhibition was decreased and the opposite was shown with the Y1 antagonist (40). NPY is expressed in the brainstem (the nucleus of solitary tract, the area postrema, and the dorsomotor nucleus of the vagus), sympathetic and perferonic nerves that play critical roles in blood pressure regulation (40). Microinjection of NPY into the nucleus of the rat solitary tract has markedly reduced blood pressure and heart rate, showing that NPY has central hypotensive effects (104). These findings have shown that NPY and its receptors may be a pharmacological target for the treatment of metabolic diseases. Further studies are needed to understand the role of NPY in these metabolic diseases.

1.4.3. NPY in osteoarthritis

Osteoarthritis, an age-related disorder and progressive disease that affects the entire joint, is an active process characterized by destruction of articular cartilage, subchondral bone sclerosis and chronic low-grade inflammation, leading to biomechanical failure (105). The World Health Organization (WHO) has included OA in a group of priority diseases for which new therapeutic targets and therapies are urgent (106). The available drugs only treat the symptoms of the disease and to date there are no drugs that stop the progression of OA. Studies have shown that NPY is detected in human synovium in sympathetic nerve fibers (79). NPY immunoreactivity in synovial tissue is found in several species in the joint cleft indicating that NPY may contribute to diseases associated with joints (107). In agreement, NPY is found in the arthritic temporomandibular joint of animals (108). In an OA model induced by monoiodoacetate there is an increase in NPY expression in the dorsal root ganglion (109). Immunoreactivity against the C flanking peptide of NPY is a sympathetic marker and has been detected in vascular cartilage channels in OA, however it is unclear how these fibers contain NPY and the role of NPY in this environment (80). Wang et al. (90) , showed that patients with knee OA had higher levels of NPY in synovial fluid compared to controls and NPY may be involved in pain perception. Moreover, they showed that the concentration of NPY in middle and advanced OA patients were significantly higher compared to early OA patients. In cartilage, little is known about the function of NPY (94). Fernandes, et al. shown that NPY receptors are present in human cartilage, and receptor expression is influenced by age and gender. Wang et al. (90), showed that NPY may be a possible biomarker in OA, because it has high levels in the advanced stages and pointed out that further studies are necessary to understand its therapeutic potential in the disease. NPY may be a possible therapeutic target in OA, however, it is essential to understand whether this peptide has a protective or destructive role in OA.

1.5. Osteoarthritis

OA is a heterogeneous and progressive disease that leads to damage in the articular structure such as joint cartilage degradation, formation of osteophytes, thickening of the subchondral bone, inflammation of the synovium and degeneration of ligaments and menisci (110).

1.5.1 Epidemiology

Osteoarthritis is a common cause of disability in older adults, promoting injury of the entire joint. In Europe alone, about 40 million people are affected by OA, 80% of whom have a movement limitation and 25% cannot perform daily activities (111) (112). According to the WHO, the prevalence of OA is increasing due to population aging (112). In 2050, the number of people aged over 60 will account for more than 20% of the world's population (112). Moreover, the United Nations showed that in 2050 around 130 million people will suffer from OA worldwide of which 40 million will be severely disabled by the disease (113). In the classification of the main causes of disability, OA ranks 9th in low- and middle-income countries and 5th in high-income countries (112). The differences in classification are due to the increase in life expectancy in developed countries, which leads to a larger number of older adults in these countries, with aging being a major risk factor for OA development (114).

In Portugal, it is estimated that 21% of people have already undergone long-term medical treatment for muscle, bone or joint problems. The estimated prevalence of OA in the Portuguese population (50-64 years) is 29.7%, and knee OA is more prevalent with 18.6% compared to OA hand with 12.6% and hip with 3.6 %. In addition, the prevalence of OA in the Portuguese population correlates with the loss of years of working life, about 58% asked for early retirement, 35% were unemployed and 7% were retired due to disability. The study analyzed more women with OA about 80% and only 20% were men with OA (115).

1.5.2. Risk factor for OA development

The major risk factor for OA development is aging, accompanied by others such as obesity, trauma, gender and hormones and genetic predisposition.

1.5.3. Aging

The mechanisms of aging performance in joint damage are poorly understood. Studies suggest that there is not only one mechanism by which aging predisposes to OA, but it is likely to be multifactorial (116). Aging alone does not cause osteoarthritis, studies show that aging promotes a systemic and local pro-inflammatory state, which may contribute to the progression of OA and to pain (117). Greene et al. (118), showed that in

aging there is production of pro-inflammatory proteins such as IL-6 in elderly people that may increasing the risk of knee OA progression (119).

1.5.4. Obesity

The biomechanical factor of obesity was the most common association to predispose to OA. There is evidence that the load is just one of the mechanisms that contributes to predispose to OA, because obesity is also associated with the development of OA in joints that do not support load as the hands (120). Mechanical stress can lead to the production of pro-inflammatory mediators from the joint tissue contributing to the pathology of OA (121).

1.5.5. Trauma

For knee OA, the trauma is common among athletes, specifically found that anterior cruciate ligament injury in soccer players has increased the prevalence of knee osteoarthritis (122). In the joint trauma the mechanisms that usually predispose to osteoarthritis is the promotion of acute inflammation after the injury that can perpetuate itself and initiate critical pathological changes in OA (123).

1.5.6. Gender and hormones

Women have a higher prevalence of knee, hands and hip OA compared to men, with the incidence increases after menopause. It is hypothesized that the hormonal factor could influence the progression of OA (124).

Estrogen has been observed to decrease the risk of knee OA in individuals who self-report taking this hormone (125). Ingestion of estrogens in postmenopausal women suggested a protective effect on radiographs, with these women having a reduced risk of developing OA (126).

It has been observed that the acute loss of estrogen increases the levels of reactive oxygen species and the production of nuclear factor-kB (NF-κB) and pro-inflammatory cytokine production. Moreover, deletion of estrogen receptors in female mice results in damage to the joint structure, suggesting that estrogen may play a protective role in joint homeostasis (127).

1.5.7. Genetic predisposition

Stecher et al, demonstrated that the presence of Heberden nodules on the fingers of osteoarthritis on the hand was three times more likely to occur in twins compared to the general population (128). After years, there are still extensive studies in twins who present with rare genetic diseases like chondrodysplasias (124). Genetic factors account for 60% of the hand and hip OA and 40% of the knee (129). Many studies have tried to understand the affected genes to try to understand more about OA and thus to identify new therapeutic targets. The genes that are often affected are for the vitamin D receptor, the insulin-like growth factor, type 2 collagen and the growth differentiation factor 5 (124).

1.5.8. Pathology

The damage generated in the cartilage structure is an obvious pathological feature of joint dysfunction (114). For a healthy articular cartilage, it is essential to maintain the structure and composition of the extracellular matrix (ECM) which are functions under the control of chondrocytes (89). The ECM is a structure of macromolecules and tissue fluid. The ECM fluid is composed mainly of water, small proteins, metabolites and cations that balance the negatively charged proteoglycans (130). The proteins that comprise ECM are collagen, large proteoglycans, namely aggrecan, and non-collagenous glycoproteins. In the ECM structure, the predominant collagen is type 2 that provides the cartilage with tensile and shear strength, having a low turnover in healthy cartilage (131). Proteoglycans can be classified into 2 types, the polysaccharide-protein conjugates which are large aggregating molecules called aggrecan and non-aggregating, such as decorin, biglycan and fibromodulin. Aggrecan in the ECM fill the voids and are trapped within the structure of three-dimensional collagen, providing compressive stiffness. In a normal ECM the rate of turnover of the aggrecan is relatively high (132) (133).

Chondrocytes are incorporated into this network of collagen fibers and proteoglycans in ECM. The function of articular chondrocytes is to maintain the physical function of cartilage, synthesizing and degrading matrix components in response to changes in the microenvironment as growth factors, cytokines and biomechanical forces. The survival of the chondrocytes is dependent on the diffusion of nutrients and metabolites, since the articular cartilage is an avascular tissue with low oxygen and metabolic turnover (134).

Chondrocytes in healthy cartilage are quiescent and have little renewal of the cartilaginous matrix, maintaining basal levels of activity (135). Osteoarthritis is responsible for modifying the cartilage environment and "activating" chondrocytes, which begin to have a more proliferative profile, form clusters and increase the production of enzymes that degrade matrix proteins and increase the production of non-native collagens (136). In human OA cartilage, there is an increase in the number of catabolic proteins such as matrix metalloproteinases (MMP), especially MMP-1, MMP-9, MMP-13 and MMP-14, and also aggrecanases (disintegrin and metalloproteinases with thrombospondin-like motifs, ADAMTS), namely ADAMTS5, ADAMTS4, and ADAMTS9. These extensive changes are accompanied by expression of inflammatory cytokines, like IL-1β (IL-1), IL-6, IL-8, IL-18 and TNF-α, vascular endothelial growth factor (VEGF), alarmins, iNOS, COX-2 and PGE2 (137). Human OA articular chondrocytes also show upregulation of runt-related transcription factor 2 (RUNX-2), which increases the production of non-articular cartilage specific proteins, like type I and X collagens, and leading chondrocytes to a hypertrophic phenotype (138) (131). There is a decrease in type 2 collagen, aggrecan, lubricin, tissue inhibitors of metalloproteinases (TIMPs) because of the MMPs and by downregulation of the chondrocyte-specific transcription factor, SRY (sex determining region Y)-box 9 (SOX9) (139). SOX9 is a critical factor for the production of the cartilage-specific ECM components (140). Lubricin is a glycoprotein secreted by chondrocytes that leads primarily to lubrication of the joint surfaces, being a chondroprotective protein (141). TIMPs are proteins responsible for inhibiting the activity of metalloproteinases (142). The loss of homeostasis in OA articular chondrocytes is followed in the articular tissue by a subchondral bone vascularization promoting subchondral sclerosis, an increase of the calcified matrix giving rise to the formation of osteophytes, degeneration of the synovium, degeneration of ligaments and menisci (figure 2) (143).

Inflammation of synovial tissue has been considered a pathogenic factor in OA and appears to intensify the disruption of homeostasis in the chondrocytes (figure 3) (144). The pathology of OA has for years been classified as non-inflammatory arthritis and wear-related of joint structures (145). However, epidemiological studies have found a strong link between synovial inflammation and cartilage degradation. Ayral et al. (146), showed that synovial inflammation may be a predictive factor for the increased degradation of medial chondropathy, using pre-radiography and arthroscopy analysis. Synovial inflammation has been observed in early and late phases of OA (147). The

synoviocytes in OA (fibroblasts and macrophages) are responsible for actively synthesizing proteases and cytokines, such as IL-6, IL-8, TNF and others that negatively affect articular cartilage. Chondrocyte functions are deregulated by pro-inflammatory cytokines, promoting a positive feedback in the activation of inflammatory pathways such as activation of NF-κB (145).

The NF-kB classical pathway contributes to cartilage degradation. Briefly, five members of the RelA family (p65), RelB, c-Rel, p50 / p105 (NF-κB1) and p52 / p100 (NF-κB2) can form different NF-κB dimers. In unstimulated cells, p65 and p50 form a trimer with the NF-kB inhibitory protein, IκBα. Following a stimulus, for example a cytokine such as IL-1β, the IκB kinase (IKK) complex is activated by phosphorylation, promoting the phosphorylation of IκBα after which it is ubiquitinated signaling its proteasomal degradation. Free from the complex, the NF-kB dimer migrates to the nucleus, where it can activate genes to produce matrix-degrading proteins such as MMP-1/3/7/13 and inflammatory proteins, like IL-1 β, TNF-alpha and iNOS (148). On the other hand, articular chondrocytes stimulated with IL-1β or TNF-α activate the NF-κB pathway (149). Moreover, NF-κB activation was increased in human chondrocytes stimulated with IL-1β in an age-dependent manner (150). Furthermore, increased NF-κB activity was found in unstimulated human OA chondrocytes relative to non-OA chondrocytes (151). In primary human chondrocytes, the ablation of IKKα or IKKβ expression has dramatically increased the deposition of type 2 collagen, glycosaminoglycans and proteoglycans. Furthermore, there was a blockade of hypertrophy and endochondral ossification in chondrocytes, these effects were independent of SOX-9 levels, and RUNX-2 factor suppression was responsible for increasing ECM proteins and blocking hypertrophy (152). Oxidative stress in the articular chondrocytes leads to a positive regulation of NF-κB, contributing to the production of iNOS (153) (154).

Nitric oxide (NO) is a small molecule that has an extremely short half-life and plays important roles in the pathology of OA (155) (156) (157). It is synthesized by the conversion of L-arginine to NOH-arginine and ultimately to L-citrulline plus NO, this reaction being catalyzed by nitric oxide synthases of which the inducible isoform (iNOS) is expressed in response to a variety of stimuli, including pro-inflammatory cytokines and alarmins (158). Human articular chondrocytes have an overexpression of iNOS after inflammatory stimuli, resulting in an excess of nitric oxide that is responsible for perpetuating inflammation and catabolic processes (158). Pelletier et al. (159), showed

an increase of iNOS and NO in OA dog model. Furthermore, treatment of OA chondrocytes with an inhibitor of iNOS reduced the incidence of osteophytes, decreased synovial inflammation, MMPs, IL-1β and PGE2 (159). Moreover, elevated levels of iNOS in OA chondrocytes result in an excess of NO which suppresses the synthesis of proteoglycans and collagen in chondrocytes and enhances MMPs, accelerating the catabolism induced by IL-1β or TNF-α (155). In summary, iNOS increases levels of NO, which enhances the degradation of the cartilage by promoting inflammation, oxidative stress and catabolic products in OA.

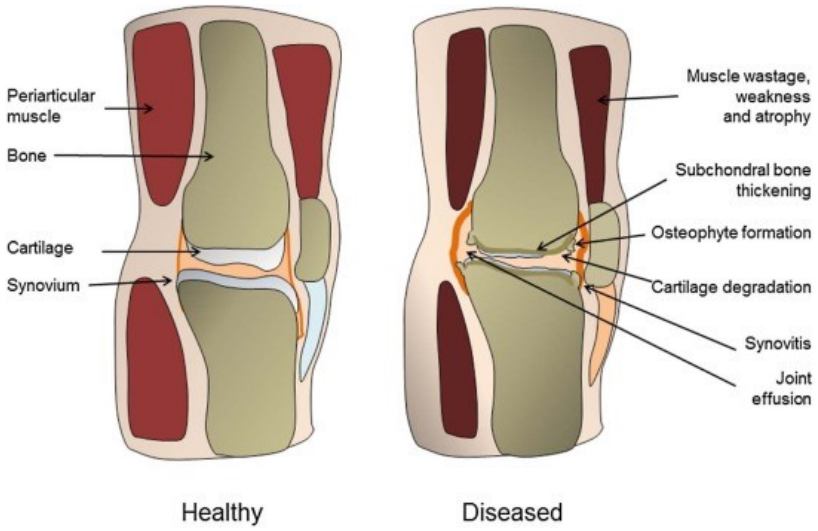

Figure 2. Healthy and OA joints. *Adapted with permission from Johnson et al.* (160)*, Vet J, 209, 40-49 (2016)*

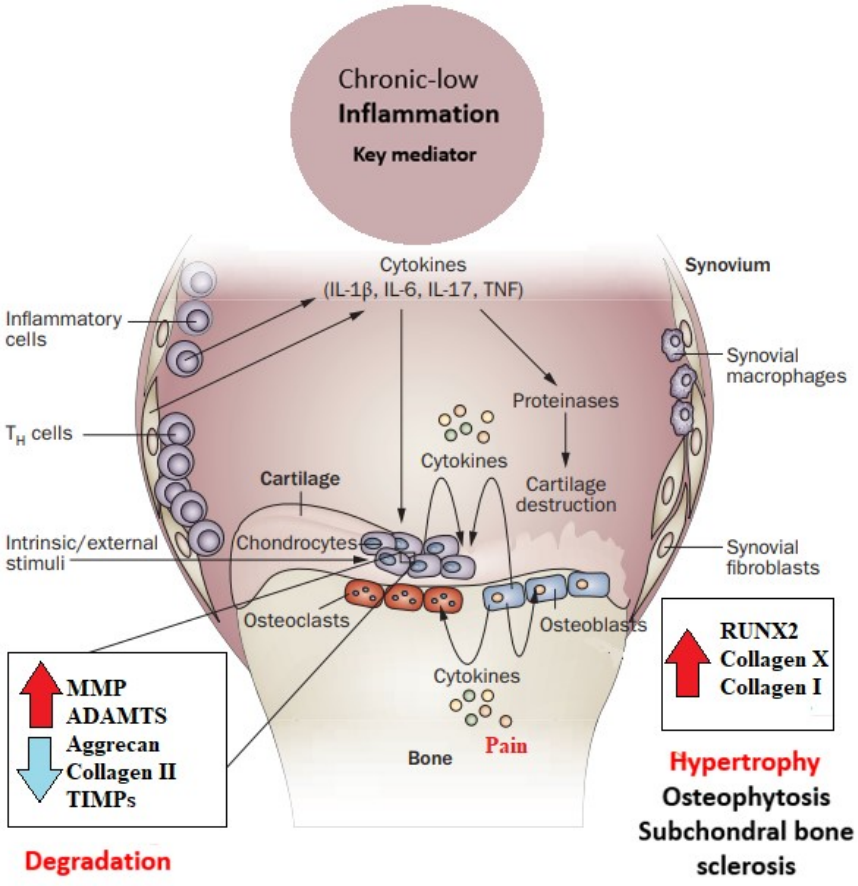

Figure.3. Role of proinflammatory cytokines in the pathophysiology of OA. *Adapted with permission from Kapoor, M. et al* (161). *Nat. Rev. Rheumatol. 7, 33–42 (2011)*

1.5.9. OA treatments

In 2013, in the WHO commissioned report "Priority medicines for Europe and the world", OA was considered as one of the priority diseases for which are urgently needed new preventive strategies and therapeutic targets that effectively limit the progression of the disease and decrease the number of patients with severe pain and functional disability (106). Symptoms in OA include pain, stiffness, joint instability, reduced movement (162). Guidelines for the treatment of these symptoms define that in the therapy can be used: non-steroidal anti-inflammatory drugs (NSAIDs) that include ciclooxigenase-2 (COX-2) inhibitors, analgesics like paracetamol, corticosteroids and, in cases of severe pain, the total replacement of the joint (163). OA drugs act only by remedying the pain and arthroplasty is an invasive surgery, being indicated in cases in which all the treatments fail, and the patient presents severe pain. Therapies are insufficient to stop the progression of the disease (164). Although the disease affects a large part of the aged population, causing high economic costs and loss of quality of life, the treatment is still performed in

a non-specific manner. Understanding the pathophysiologic mechanisms underlying the disease can provide insights into new treatments that directly act on the progression and development of the disease (165). Identifying signaling pathways relevant in OA may help in the identification of target molecules amenable to therapeutic intervention and, thus, in a future perspective, lead to the development of therapies that can reduce economic costs and restore OA patients' quality of life.

1.6. Aims

Detailed molecular mechanisms of initiation and development of OA remain poorly understood and currently there are no interventions available to restore degraded cartilage or slow joint destruction and disease progression. Therefore, it is necessary to elucidate the molecular mechanisms that promote joint degradation to accelerate the development of new curative therapeutic strategies.

NPY appears to have a relevant anti-aging role documented in several studies for its potential for maintaining cellular homeostasis. Evidence showed that NPY is present in the articular microenvironment. NPY-positive sympathetic nerve fibers were detected in joint and NPY was found in synovial fluid of patient with OA, which strongly suggests that NPY may be important in controlling joint homeostasis. However, the functions of NPY in articular tissues and its role in OA pathogenesis are poorly elucidated.

The purpose of this study was to understand whether NPY plays a destructive or protective role in OA. NPY is present at high levels in the inflammatory environment of the synovial joints of OA patients. Therefore, first objective of this thesis was to evaluate the role of NPY in the inflammatory response of human chondrocytes. For this, human chondrocytes were isolated from healthy and osteoarthritic human articular cartilage. Treatment with IL-1β was used to mimic the inflammatory environment to which chondrocytes are exposed in the osteoarthritic joint. Receptors responsible for the effect of NPY on inflammation were studied using agonists and antagonists of its receptors. To further understand the role of NPY in chondrocyte functions, the role of NPY in modulating the expression of MMP-13 and type 2 collagen was evaluated in primary human chondrocyte cultures stimulated or not with IL-1β to determine whether NPY has pro or anti-catabolic and/or pro- or anti-anabolic effects.

Chapter 2: Materials and Methods

2.1. Materials and reagents

Penicillin, Streptomycin, Chloroquine, and Nutrient Mixture F-12 were purchased from Sigma Chemical Co., St. Louis, MO, USA. The Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Thermo Fisher Scientific. NPY was purchased from Phoenix Pharmaceutical, Burlingame, CA, USA. Recombinant human IL-1β was purchased from Peprotech, Rocky Hill, NJ, USA. The protein marker II was purchased from NZYtech genes & enzymes, Lisbon, Portugal and the substrate for western blot, ECF was purchased from GE Healthcare,UK. The enzymes for cartilage digestion, collagenase A from *Clostridium hystoliticum* and pronase from *Streptomyces griseus* were purchased from Roche, Indianapolis, IN, USA. The agonists for NPY Y1($Leu^{31}Pro^{34}$) , NPY Y2 (NPY 13-34) and NPY Y5 (Peptide YY) were purchased from Bachem, Bubendorf, Switzerland. NPY Y1 (BIBP 3226), NPY Y2 (BIIE 0246) and NPY Y5 (L-152,804) antagonists were purchased from Tocris Bioscience, Bristol, UK.

2.2 Cartilage samples and isolation of primary chondrocytes

Cartilage was removed from the femoral condyles of non-osteoarthritic and osteoarthritic human knees from 10 multi-organ donors of which 7 were women (mean age: 57,85 ± 12,11 years) and 3 were men (mean age: 59,7± 7,5 years), at the Bone and Tissue Bank, University and Hospital Center of Coimbra (CHUC), with approval by the Ethics Committee. Cartilage samples were classified according to the macroscopic degree of degradation using the Outerbridge classification system (166), in which grade 0 corresponds to normal undamaged or only slightly damaged cartilage, grade 1 presents mild damage with cartilage softening and swelling, grade 2 presents superficial damage with fibrillation, grade 3 shows partial thickness erosion with fissures to the level of the subchondral bone and grade 4 presents severe damage with exposure of the subchondral bone, representing advanced OA.

Cartilage slices underwent sequential digestion with 2% pronase for 1 h 30 min and then 1% collagenase for 20-22 h. Cultures were setup by plating $1x10^6$ chondrocytes/ml in Ham F-12 medium containing 1% antibiotic/antimycotic solution and 5% fetal bovine serum (FBS) and allowed to recover for 24 h at 37 °C in a humidified

atmosphere supplemented with 5% CO_2. Prior to any treatments, the cells were serum-starved overnight and thereafter maintained in culture medium with 1% antibiotic/antimycotic solution, without serum.

Table 2: Donors used for the isolation of human articular chondrocytes.

Multi-organ donors: gender	Age (years)	Outerbridge classification
Female	41	Grade 0-1
Female	57	Grade 0
Female	45	Grade 0
Male	67	Grade 0
Female	58	Grade 2
Female	77	Grade 4
Female	64	Grade 4
Female	63	Grade 4
Male	60	Grade 4
Male	52	Grade 4
Females: mean age 57,85 ± 12,11years Males: mean age 59,7± 7,5		

2.2.1 Experimental conditions

To assess the role of NPY in modulating anabolic, inflammatory and catabolic responses and to its ability to counteract the effects of IL-1β in the same responses, chondrocyte cultures were treated with 50 nM NPY alone or pre-treated with same concentration for 3 hours before the addition of 10 ng/mL IL-1β and then further incubated for the time periods indicated in Chapter 3: Results. To identify the NPY receptor subtypes that mediate its effects in human chondrocytes, 100 nM of Y1, Y2 and Y5 agonists were added to the chondrocyte cultures in conditions identical to those used for NPY. Furthermore, additional cultures were setup and treated with 1 μM of Y1 (BIBP 3226), Y2 (BIIE 0246) and Y5 (L-152,804) antagonists in presence of NPY and IL-1β to determine which ones were able to prevent NPY effects.

2.3 Western Blot

Following treatments, the cells were lysed in RIPA buffer containing NaCl, 50 mM Tris HCl, 5 mM EGTA, 1% Triton, 0,5% sodium deoxycholate, 0,1% SDS with protease and phosphatase inhibitor cocktails (Roche). The cells were placed on ice for 30 min with lysis buffer, then removed from the plate by scraping and centrifuged for 15 min at 13.2 g at 4 °C. The supernatants were collected and stored at –20°C until further analysis.

2.3.1 Preparation of total cell extracts

The articular chondrocytes were treated with NPY or Agonist of receptors NPY Y1, NPY Y2 and NPY Y5 for 3 h, antagonists for 3 h 30 min and IL-1β for 24h. Following treatments, the cells were lysis in a RIPA buffer containing 150mM NaCl, 50 mM Tris HCl, 5mM EGTA, 1% Triton, 0,5% sodium deoxycholate, 0,1% SDS with protease and phosphatase inhibitor (Roche). Cells were placed on ice for 30 min with lysis buffer, then removed from the plate by scraping and centrifuged 15 min at 13.2 g at 4°C, collected supernatant and stored at –20°C

2.3.2. Isolation of nuclear and cytoplasmic extract

Nuclear and cytoplasmic extracts were prepared using the Nuclear Extract kit (Active Motif, La Hulpe, Belgium) according to the manufacturer's instructions. Chondrocyte cultures were washed with ice-cold phosphate-buffered saline (PBS), pH 7,4 and phosphatase inhibitor and then scraped and centrifuged for 5 min at 300 g at 4 °C. The supernatants were discarded, and the pellet was resuspended in 125 µl of 1x hypotonic buffer and incubated on ice for 15 min. Then, 6.25 µl of detergent were added to each tube, vortexed and centrifuged for 30s at 14 g, at 4 °C. The cytoplasmic fraction (supernatants) was transferred to new microtubes and stored at -80 °C until further use. The nuclear fraction was resuspended in 18 uL of lysis buffer and vortexed for 10 sec, incubated in ice for 30 min and afterwards centrifuged at 14 g for 10 min. The supernatants (nuclear fraction) were transferred to new microtubes and stored at -80 °C until further use.

Total protein concentration of the cellular extracts was determined by the bicinchoninic acid assay (Pierce Biotechnology). 25 µg protein were loaded in SDS-polyacrylamide gels (SDS/PAGE) and subjected to electrophoresis under reducing conditions. Then, the proteins were transferred onto polyvinylidene fluoride (PVDF)

membranes (Bio-Rad). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), at room temperature, for 2 h, and then incubated overnight, at 4 °C with either of the following primary antibodies: anti-IL-1β rabbit polyclonal antibody (dilution 1:500; sc-7884, Santa Cruz Biotechnology, INC., Texas, USA), anti-iNOS mouse monoclonal antibody (dilution 1:500; MAB9502, R&D Systems, Minneapolis, MN, USA), anti-Acetyl-NF-κB p65 rabbit polyclonal antibody (dilution 1:750, 3045S, Cell Signaling, Leiden, Netherlands), anti-Lamin B1 rabbit polyclonal antibody(diluted 1:1000, ab16048, Abcam, Cambridge, UK),anti- NF-κB p65 rabbit polyclonal antibody (dilution 1:1000, #3034, Cell Signaling, Leiden, Netherlands) anti- IkB-α rabbit polyclonal antibody (diluted 1:1000, 9242S, Cell Signaling, Leiden, Netherlands). Following incubation, the membranes were washed in TBS-T buffer for 30 min and incubated with anti-rabbit or anti-mouse secondary antibodies diluted 1:20.000 (Ge Healthcare), for 1 h, at room temperature. Following extensive washing in TBS-T for 30 min, the proteins of interest were detected with Enhanced ChemiFluorescence reagent (GE Healthcare,UK) in the imaging system Thyphoon™ FLA 9000 (GE Healthcare Life Sciences, Uppsala, Sweden). The membranes were reprobed with a mouse monoclonal anti-β-Tubulin I antibody (dilution 1:20.000, Sigma-Aldrich Co.), as a loading control, for 1 h, at room temperature. Image analysis was performed with TotalLab TL120 software (Nonlinear Dynamics Ltd).

2.4. Nitrite quantification

NO production was measured as the amount of nitrite accumulated in the culture supernatants using the colorimetric method based on the Griess reaction (167). Optical density was measured at 540 nm using the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc. USA).

2.5. Resazurin reduction assay

Cell viability was evaluated with the resazurin reduction assay. This method is based on the ability of metabolically active, viable cells to reduce resazurin (a nonfluorescent dye) into resorufin (a pink-coloured compound). The magnitude of dye reduction, evaluated as the increase in absorbance, positively correlates with cell number. 2 h before the end of the treatment periods indicated in figure legend, resazurin solution was added to each well to a final concentration of 50 μM. Two hours later, the

absorbances of each well were read at 570 nm and 620 nm (reference wavelength) in the Synergy™ HT Multi-Detection Microplate Reader (Instruments, Inc. USA). Each sample was tested in duplicate and the average of both readings was used to evaluate the corresponding cell viability.

2.6. ELISA

2.6.1. MMP-13

Culture supernants were collected for MMP-13 quantification with the SensoLyte® Plus 520 MMP-13 AnaSpec kit , according to the manufacturer's instructions. To each well of a monoclonal anti-human MMP-13-coated microplate, 100 µl/well of each sample, MMP-13 standards or blank control were added. The plate was incubated for 2 h under shaking (50-100 rpm) at room temperature. After extensive washings, 100 µl of 1 mM APMA were added to each well and the microplate incubated at 37 °C, for 40 min. After washing, 100 µl/well MMP-13 substrate solution were added to each well and the microplate was incubated at room temperature, in the dark, for 16 h. Fluorescence intensity was measured in the microplate reader, BioTek (Synergy HT, Inc. USA) with excitation and emission wavelengths of 485 ± 20 nm and 528 ± 20 nm, respectively. MMP-13 concentration is directly proportional to the fluorescence intensity.

2.6.2. Type II collagen

Type II collagen was measured in culture supernatants using the type II collagen detection kit by Chondrex, Inc (Redmond, USA), according to the manufacturer's instructions. Supernatants and type II collagen standard dilutions were added to a 96-well plate pre-coated with the capture antibody. Upon addition of the detection antibody, the plate was incubated for 2 h at room temperature and then washed and incubated with streptavidin-peroxidase for 1 h. After washing, OPD solution was added for 1 h at room temperature. The reaction was stopped with 2 N sulfuric acid and the concentration of type II collagen in each sample was measured by reading the absorbance at 490 nm and 630 nm, using the plate reader BioteK, Synergy HT, Inc. USA. Each sample was analyzed in duplicate and both readings averaged.

2.7. Total RNA isolation and real-time RT-PCR

Chondrocytes (1×10^6/well of 6-well plates) were pre-treated with NPY or Y1, Y2 or Y5 agonists for 3 h, before addition of 10 ng/ml IL-1β and further incubated for 24 h. Total RNA was isolated by mechanical disruption directly in 1 ml of NZYol (Nzytech) following the manufacturer's instructions. Upon precipitation in isopropanol and washing in ethanol, total RNA was dissolved in RNAss (Ambion™, Life Technologies), quantified in a Nanodrop ND-1000 spectrophotometer at 260 nm and stored at -80 °C until further use. RNA purity was assessed by analysis of 260/230 and 260/280 absorption ratios.

The cDNA was reverse-transcribed using NZY First Strand cDNA Synthesis Kit (NZYTECH), beginning with 2 µg of total RNA. qRT-PCR was performed, in duplicate for each sample, using NZYSpeedy qPCR Green Master Mix (2x) (NZYTECH) on CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The efficiency of the amplification reaction for each gene was calculated using a standard curve of a series of diluted cDNA samples, and the specificity of the amplification products was assessed by analysing the melting curve generated in the process. Gene expression changes were analysed using the built-in CFX Manager software which enables the analysis of the results by the Pfaffl method, a variation of the ΔΔCT method corrected for gene-specific efficiencies (Pfaffl, 2001). The results were normalized using Hprt1 as the housekeeping gene. This gene was experimentally determined with Genex software using NormFinder and geNorm algorithms (MultiD Analyses AB, Göteberg, Sweden) as the most stable for the treatment conditions used. Specific sets of primers for each gene were designed using Beacon Designer software version 8 (Premier Biosoft International, Palo Alto, CA, USA). The sequences of the specific sets of primers used for each gene, including housekeeping genes, are summarized in table 3.

Table 3: Sequences of the Primers designed for each gene

Gene name	Forward	Reverse
Collagen II	5'-GGCAGAGGTATAATGATAAGG-3’	5'-ATTATGTCGTCGCAGAGG-3’
TIMP-1	5'-TGTTGCTGTGGCTGATAG-3’	5'-CTGGTATAAGGTGGTCTGG-3’
TIMP-3	5'-CCATACACTATCCAC -3'	5'-TAACAGCATTGAACA -3'
Aggrecan	5'-CAATGTAAGTGGAGAATC-3'	5'-ATAGTTGGTTCAGTAACA-3'
Collagen X	5'-CTATCAGACCAACAAACC-3'	5'-AACATAGCAGGACTTCTT-3'
Collagen I	5'-GGAGGAGAGTCAGGA-3’	5'-GCAACACAGTTACAC-3’
MMP-13	5’-GTTTCCTATCTACACCTACAC-3’	5'-CTCGGAGACTGGTAATGG-3’
MMP-1	5’-GAGTCTCCCATTCTACTG-3’	5'-TTATAGCATCAAAGGTTAGC-3’
GADPH	5’'-ACAGTCAGCCGCATCTTC-3’	5'-GCCCAATCAGACCAAATCC-3’

2.8. Data Analysis

Results are presented as mean ± SEM. Statistical analysis using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) was performed by one-way analysis of variance (ANOVA), followed by the Dunnett’s post hoc test for multiple comparisons. Differences were considered significant at $p<0.05$.

Chapter 3: Results

3.1. Assessment of human chondrocyte viability upon treatment with NPY receptor antagonists, NPY and IL-1β

To exclude eventual cytotoxic effects, the viability of chondrocytes treated with each NPY receptor subtype antagonist in the presence of NPY and IL-1β was evaluated with the resazurin reduction assay. As shown in Figure 4, neither treatment under these experimental conditions affected cell viability compared to control, NPY or IL-1β alone.

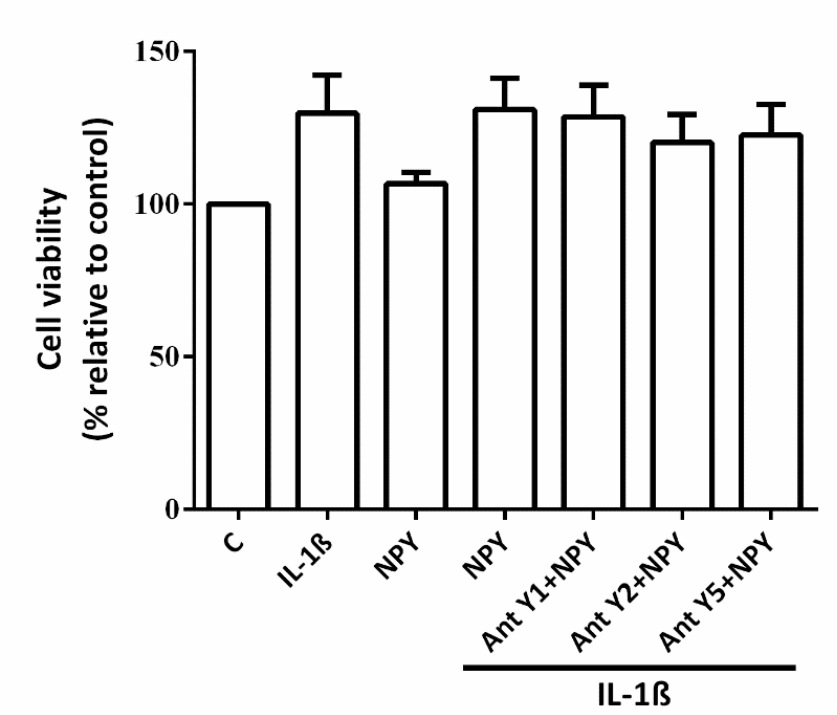

Figure 4. Effect of treatments with antagonist, NPY and IL-1β on cell viability*. Human chondrocytes were pre-treated with 1 μM of each NPY receptor antagonist for 30 min, followed by addition of 50 nM NPY for 3h and then, addition of 10 ng/ml IL-1β for 24h, as described in the methods section. The resazurin assay was performed to assess cell viability and results are expressed in percentage relative to control. Each bar represents the mean ± SEM of 3 independent experiments.*

3.2 Role of NPY in modulating pro-IL-1β levels in human chondrocytes stimulated with IL-1β

NPY plays an immunomodulatory role in cells of the immune system decreasing the expression of inflammatory proteins, such as IL-1β and iNOS (168). Previous studies in our laboratory have shown that NPY receptors are expressed in human chondrocytes in OA and healthy donors (169). To investigate the role of NPY and its receptors in the regulation of IL-1β expression, human chondrocytes from healthy and OA donors were treated with NPY or Y1, Y2 or Y5 agonists for 3 h, followed by addition of 10 ng/ml IL-1β for 24 h under serum deprivation conditions. Pro-IL-1β levels were analyzed by western blot. Pro-IL-1β is a mature form of IL-1β, with 31 kDa and is processed to the active of IL-1β by the interleukin-1 converting enzyme.

Figure 5 shows that NPY alone does not affect pro-IL-1β levels in human chondrocytes, but significantly reduces the levels of this protein induced by treatment with IL-1β.

Agonists and antagonists of the major NPY receptors, Y1, Y2 and Y5, were used to investigate which receptor(s) are responsible for the decrease caused by NPY at pro-IL-1β levels. The results obtained show that none of the Y1, Y2 or Y5 agonists alone was able to reverse this inflammatory effect induced by IL-1β (Fig. 5).

To further understand which NPY receptor subtypes mediate NPY effects on IL-1β expression, human chondrocytes were pre-treated with Y1, Y2 or Y5 antagonists for 3 h 30 min in the presence of NPY for 3 h and then stimulated with IL-1β for 24 h. Interestingly, Y1 and Y2 antagonists did not counteract the inhibitory effect induced by NPY treatment while the Y5 antagonist elicited a smaller decrease of pro-IL-1β levels than NPY alone (Figure 6).

The results in figures 5 and 6 suggest that the Y5 receptor may be of greater importance in mediating NPY effects in chondrocytes than the other two subtypes, since none of the agonists alone was able to mimic the effects of NPY while only blockade of the Y5 receptor was effective in counteracting the inhibitory effect of NPY. Nonetheless, since the Y5 agonist was unable to reduce IL-1β induced effects, it is possible that two or more NPY receptor subtypes cooperate to reduce levels of pro-IL-1β.

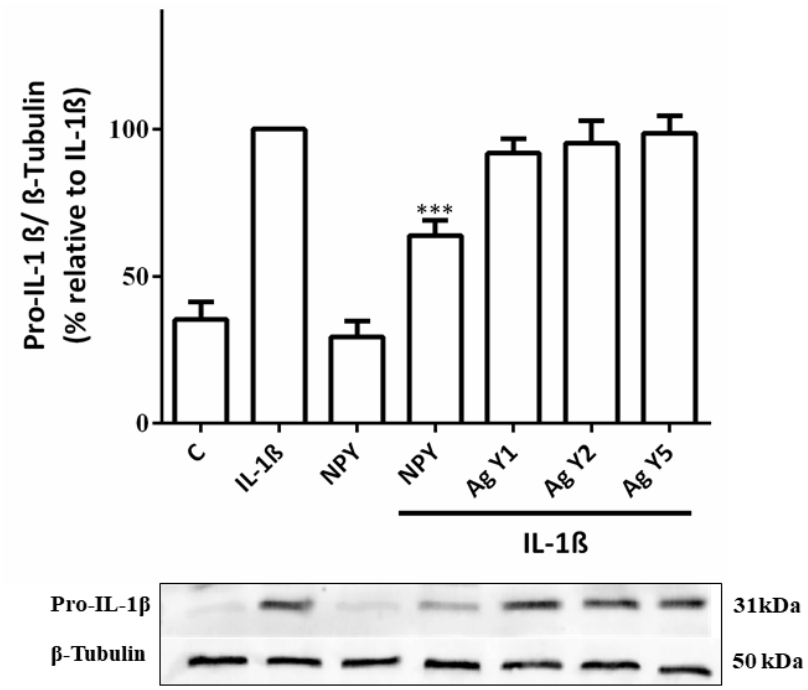

Figure 5*. *Role of NPY and its receptors in reducing pro-IL-1β levels. *Protein levels of pro-IL-1β were measured by Western Blot. Human chondrocytes were treated with NPY (50 nM), Y1, Y2 or Y5 agonists (100 nM) for 3 h, followed by addition of IL-1β for 24 h (10 ng/mL). Each column represents the mean ± SEM of the results obtained with chondrocytes isolated from 10 different donors. ***p< 0.001 relative to IL-1β.*

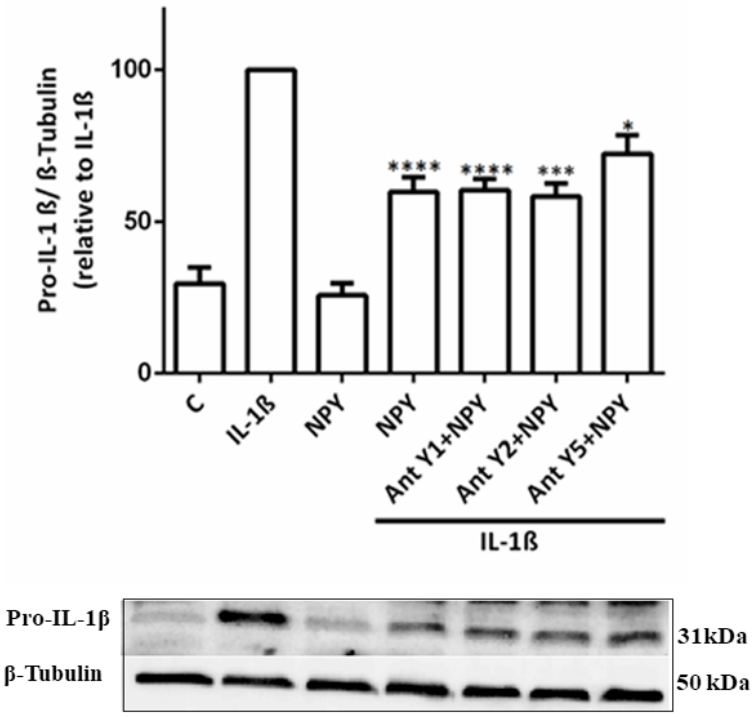

Figure 6. Evaluation of role of NPY and its receptors in reducing of pro-IL-1β levels, after blockade with antagonists. *Pro-IL-1β expression was measured by Western Blot. Human chondrocytes were treated with NPY (50 nM) for 3 h and Y1, Y2, Y5 antagonists (1 μM) for 3 h 30 min and IL-1β for 24 h (10 ng/mL). Each column represents the mean ± SEM of 8 independent experiments, *p< 0.05, ***p< 0.001 and p****< 0.0001 relative to IL-1β.*

3.3. Evaluation of cooperative action between of Y1, Y2 and Y5 receptors in the interaction with NPY, promoting the regulation of pro- IL-1β

NPY appears to interact with 3 receptors to decrease pro-IL-1β levels. To test this hypothesis, human chondrocytes were incubated with combinations of Y1, Y2 and Y5 agonists. Figure 7 shows that the three agonists together did not reverse the inhibitory effect of NPY on pro-IL-1β expression. Combinations of two agonists were performed and the combination of Y1 and Y5 agonists shows a tendency to decrease pro-IL-1β levels although it did not reach statistical significance (Figure 7).

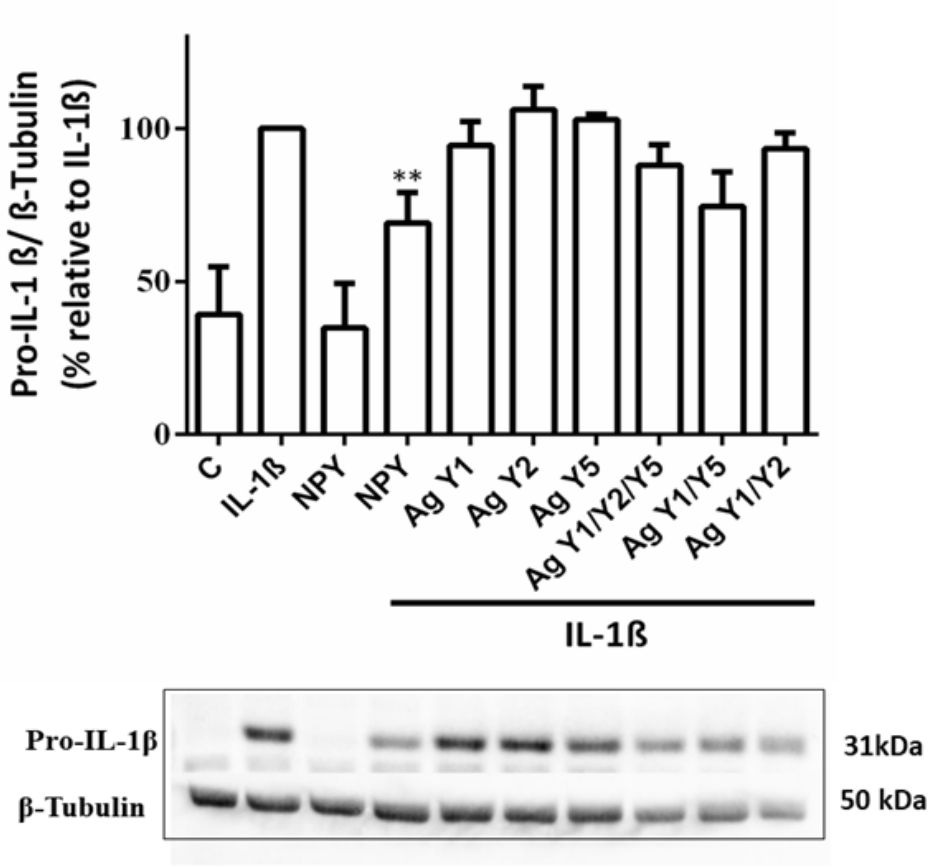

Figure 7. Evaluation of potential cooperative interactions between NPY receptor subtypes. *Human chondrocytes were treated with NPY (50 nM) or different combinations of Y1, Y2 and Y5 agonists (100 nM) for 3 h before addition of 10 ng/ml IL-1β, and further incubated for 24 h. Each column represents the mean ± SEM of 3 independent experiments, except for the combination of Y1 and Y2 agonists which represents two independent experiments (no statistical analysis was performed for this condition). **p< 0.01.*

3.4. Role of NPY and its receptors in the regulation of iNOS and nitric oxide levels induced by IL-1β

To further investigate the anti-inflammatory role of NPY, its effect on IL-1β-induced iNOS levels was investigated because it is up regulated by IL-1β and is responsible for the production of nitric oxide, an important proinflammatory and destructive mediator in OA. NPY was also able to decrease iNOS levels induced by IL-1β (Figure 8A). In addition, NPY even in the presence of IL-1β, significantly reducing NO production (Figure 8B).

To assess which receptor(s) would be responsible for promoting the effect of NPY on decreasing both iNOS and NO levels, chondrocytes were treated with NPY or agonists for 3 h and then with IL-1β and further incubated for 24 h. As shown in figure 8A and B, none of the agonists decreased iNOS levels and NO production. These results are similar to those found for pro-IL-1β (Figure 5) and show that NPY may have anti-inflammatory effects in chondrocytes via reduction of iNOS and pro-IL-1β expression and suggest that more than one receptor subtype may be required for these effects.

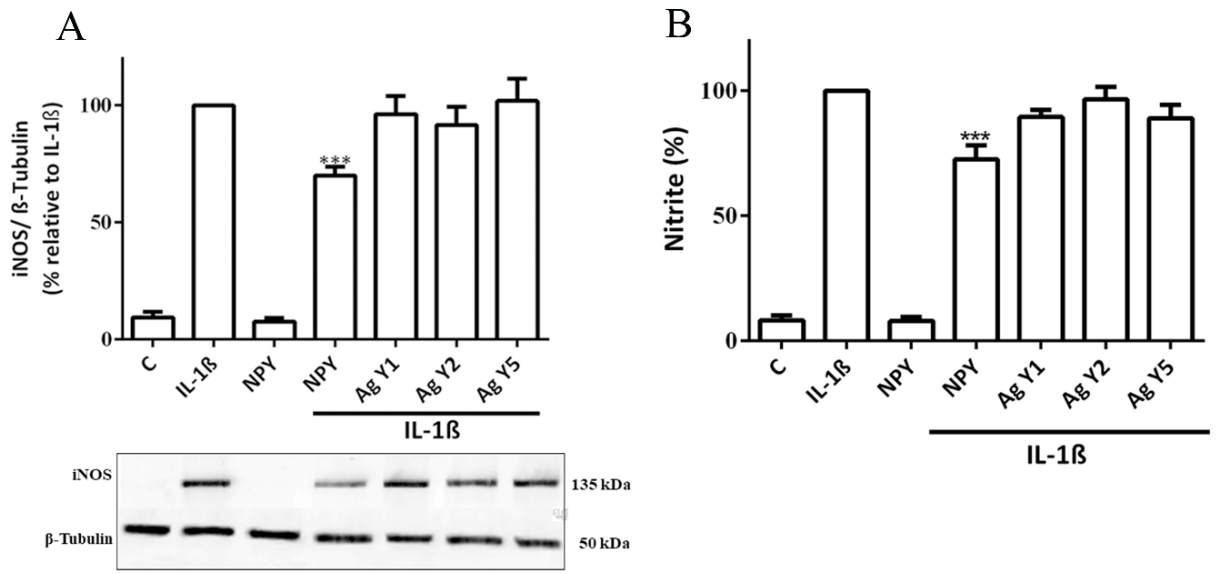

Figure 8. Role of NPY and its receptors in reducing iNOS and NO production. *A)Protein levels of iNOS and B) NO production. Human chondrocytes were treated with NPY (50 nM) or Y1, Y2 and Y5 agonists (100 nM) for 3 h before addition of 10 ng/ml IL-1β and then further incubated for 24 h. iNOS expression was measured by Western Blot and NO levels were measured by the Griess reaction. Each column represents the mean ± SEM of 9 independent experiments, ***p< 0.001 relative to IL-1β.*

3.5. Evaluation of the NPY receptor subtypes responsible for the effects of NPY on the regulation of iNOS expression and activity

The above result show that possibly more than one NPY receptor can be required to interact with NPY to promote the reduction of iNOS and NO levels. To assess which receptors might be involved in the NPY effect, human chondrocytes were pre-treated with selective antagonists for Y1, Y2 and Y5 receptors for 30 min, followed by addition of NPY and incubation for another 3 h and then addition of IL-1β and incubation for 24 h, at the same concentrations used for the study of pro-IL-1β levels. Figure 9 shows that only treatment with the Y5 antagonist was able to counteract the inhibitory effect elicited by NPY on iNOS levels, while the other two antagonists did not alter the NPY effect, although the mean value obtained with the Y2 antagonist is smaller than that obtained in NPY+IL-1β-treated cells (Figure 9 A). These results suggest that the Y5 receptor has a greater importance in the effect of NPY, however, this effect is incomplete, suggesting a cooperative role between receptor subtypes. Nonetheless, NO production in the presence of Y5 antagonist did not significantly differ from that of cells treated with either IL-1β alone or NPY+IL-1β. This may be due to the fact that nitrite, the product of the spontaneous oxidation of NO, accumulates in the culture supernatants and so, small reductions of the enzyme protein levels may not be accompanied by significant reductions of nitrite concentrations. However, the Y2 antagonist decreased nitrite levels slightly more than NPY (Figure 9B). This suggests that blockade of the Y2 receptor enhances the effect of NPY to promote the decrease of iNOS and consequently decreased NO levels.

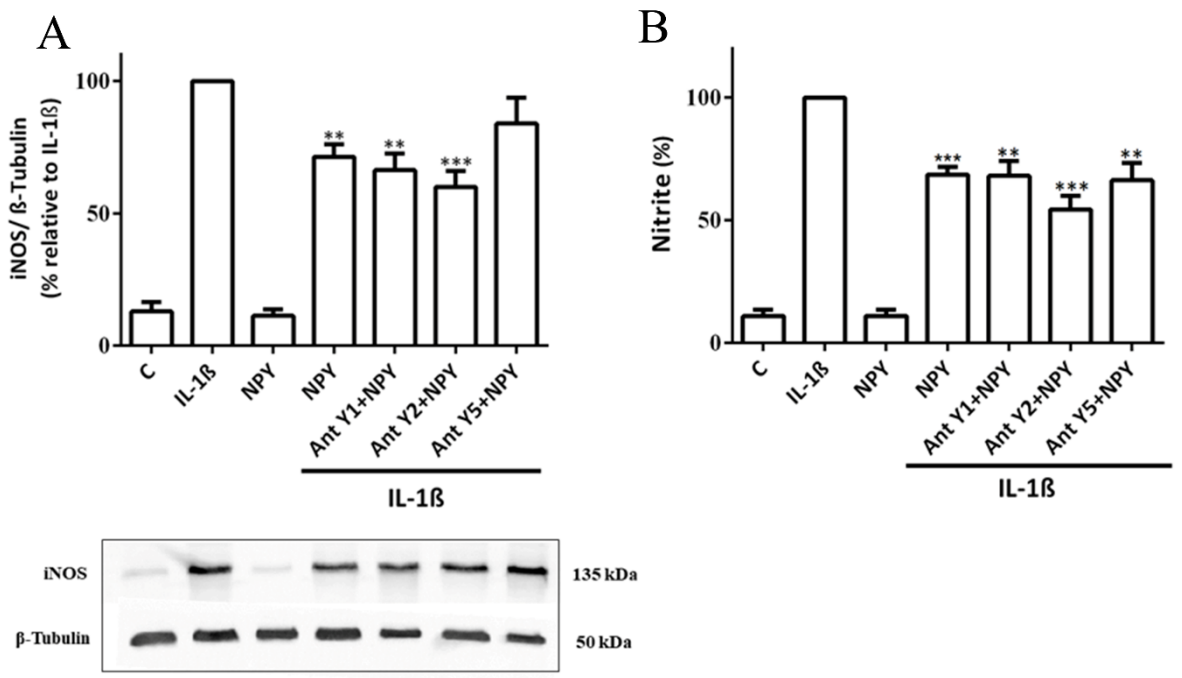

Figure 9. Evaluation of role of NPY and its receptors in reducing levels of iNOS and NO, after blockade with selective antagonists*. A) Protein levels of iNOS and B) NO production. Human chondrocytes were treated with NPY (50 nM) for 3h and Y1, Y2, Y5 antagonists (1 μM) for 3 h 30 min and IL-1β for 24 h (10 ng/mL). iNOS expression was measured by Western Blot (A). NO levels measured by the Griess reaction. Each column represents the mean ± SEM of 9 (A) and 6 (B) independent experiments, **p< 0.01 and p***< 0.001 relative to IL-1β.*

3.6. Evaluation of cooperative action between the Y1, Y2 and Y5 receptors in the interaction with NPY, promoting the regulation of iNOS

NPY significantly decreased iNOS levels induced by IL-1β. To understand which receptors were important for this purpose, chondrocytes were incubated with combinations of agonists in the presence of IL-1β. As shown in figure 10, agonists Y1, Y2 and Y5 in combination did not reduce iNOS levels compared to IL-1β. Interestingly, combinations of the Y1 and Y2 agonists and even the Y1 and Y5 combination also did not decrease iNOS levels (Figure 10). Nonetheless, further studies are required to understand the effect of NPY on iNOS reduction, such as a larger number of samples and increasing agonists concentration may be a possibility to understand if the effect of NPY on the reduction of iNOS and NO is mediated by Y1 and Y5 receptors

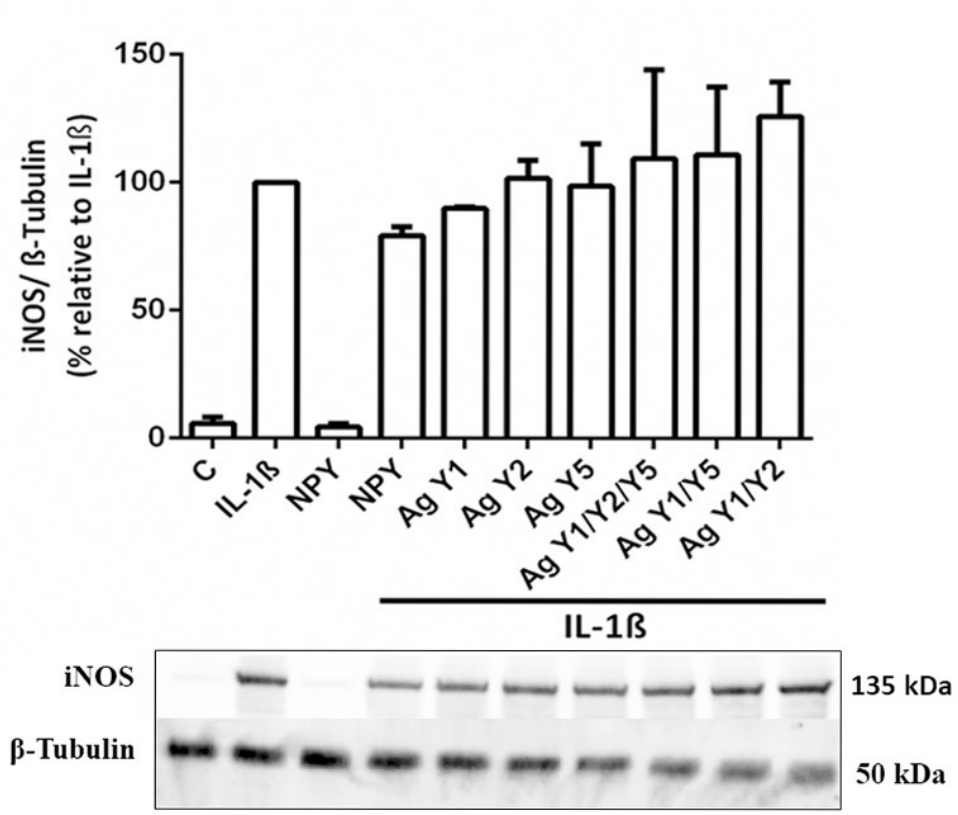

Figure 10. . Evaluation of cooperative action between NPY receptors, promoting the regulation of iNOS. *Protein levels of iNOS were measured by Western Blot. Human chondrocytes were treated with 50 nM NPY or 100 nM Y1, Y2 or Y5 agonists, alone or in various combinations, for 3 h and then further treated with 10 ng/ml IL-1β for 24 h. Each column represents the mean ± SEM of 3 independent experiments, except for the combination of Y1 and Y2 agonists which represents two independent experiments (no statistical analysis was performed for this condition).*

3.7. Role of NPY and its receptors in modulating the NFκB pathway

NF-κB plays a central role in the production of inflammatory mediators in inflammation and inflammation-related diseases, including OA. Given the ability of NPY to decrease IL-1β-induced IL-1β and iNOS levels, we hypothesised that it may inhibit IL-1β-induced NF-κB activation. NF-κB activation in response to inflammatory stimuli, like IL-1β, leads to IκB-α degradation in the cytoplasm and release of p65-containing dimers that migrate to the nucleus to activate inflammatory and catabolic gene expression. In order to determine whether the inhibitory effect of NPY on the protein levels of IL-1β and iNOS results from inhibition of NF-κB activation, human chondrocytes were treated with NPY or agonists for 3 h and IL-1β for 45 min. The cytoplasmic levels of IκB-α were evaluated by western blot. Figure 11A shows that neither NPY nor any of the NPY receptor agonists were able to prevent IL-1β-induced IκB-α degradation. Surprisingly, NPY alone significant decreased of IκB-α levels. Thus, the mechanism underlying inhibition of IL-1β and iNOS expression observed with NPY does not

seem to involve inhibition of NF-κB activation. Thus, we hypothesised that NPY may inhibit NPY-induced IL-1β and iNOS levels by mechanisms regulating its transcriptional activity, rather than its nuclear translocation. Acetylation of p65 on lysine 310 is required for the full transcriptional activity of NF-κB and thus for induction of inflammatory gene expression (170). Deacetylation of that Lys residue inhibits NF-κB transcriptional activity (171). Thus, we hypothesised that the ability of NPY to inhibit the expression of IL-1β and iNOS may result from inhibition of its acetylation and/or induction of its deacetylation without interfering with IκB-α degradation and p65 nuclear translocation. To elucidate this question, we evaluated the levels of Lys310-acetylated p65 (Acp65). NPY shows a tendency to decrease Acp65 levels relative to IL-1β, but the difference did not reach statistical significance. The Y1 and Y2 agonists significantly reduced Acp65 levels (Figure 11B), without affecting nuclear levels of total p65. These results indicate that NPY may be acting via these receptors to promote the decrease of Acp65. A larger number of samples needs to be evaluated, along with assessment of the levels of phosphorylated p65, to fully elucidate the participation of NPY in this pathway.

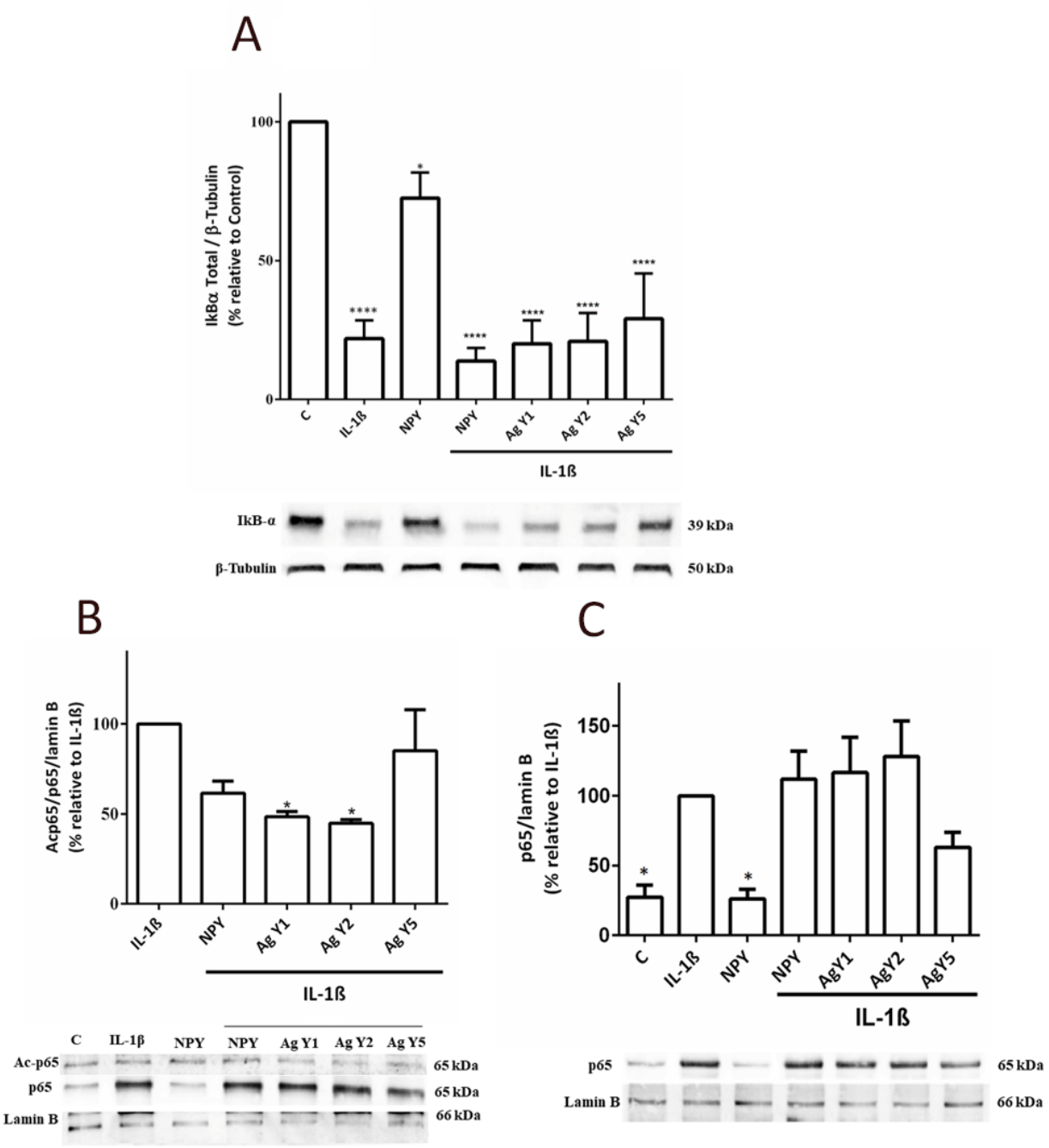

Figure 11. Role of NPY and its receptors in NF-κB activation. *Protein levels of IkB-α (A), acetylated (Ac)-p65 (B), and nuclear p65 (C). Human chondrocytes were treated with NPY (50 nM) or Y1, Y2, Y5 agonists (100 nM) for 3 h and IL-1β for 45 min (10 ng/mL). Each column represents the mean ± SEM of 3 independent experiments, *p< 0.05 and ****p< 0.0001, relative to control (A) and p*< 0.05 relative to IL-1β (B and C).*

3.8. Effect of NPY on the regulation of MMP-13

In OA, MMP-13 is an important enzyme in promoting the degradation of cartilage for its role in cleaving collagen 2 (172). Moreover, MMP-13 was found to be overexpressed in the joints of patients with OA (173). Studies show that MMP-13 can be directly up-regulated via NF-κB, as well as MAP kinases (174). Furthermore, studies showed that IKKα does not regulate

MMP-13 gene expression, however, it decreased TIMP levels, leading to an indirect increase in MMP-13 activity (175). Since we have found the effect of NPY on the reduction of inflammatory proteins, we raised the hypothesis that NPY can also reduce catabolic proteins in human chondrocytes. To elucidate this, human chondrocytes were treated with NPY for 3 h and with IL-1β for 24 h. The culture supernatants were used to analyze the protein levels of MMP-13 released to extracellular medium and total RNA extracted from cells was used to evaluate mRNA expression by real time RT-PCR. ELISA analysis indicated that NPY was not able to decrease MMP-13 levels when compared to IL-1β (Figure 12A). However, NPY alone significantly decreased MMP-13 mRNA expression and appears to decrease IL-1β-induced MMP-13 mRNA expression (Figure 12B). These results suggest that NPY can play an anti-catabolic role in human chondrocytes. Furthermore, due to differences in results between the techniques used, increasing the number of samples is imperative to understand whether NPY has anti-catabolic effects.

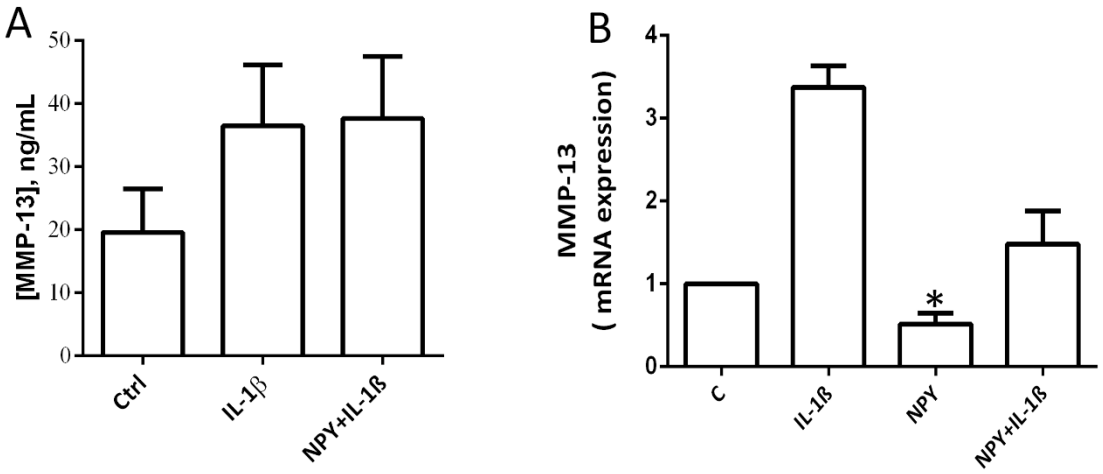

Figure 12. Effect of NPY on MMP-13 expression*. A) MMP-13 protein levels. B) MMP-13 mRNA levels. MMP-13 protein levels in culture supernatants were measured by Western Blot (A) and MMP-13 mRNA levels were evaluated by RT-PCR (B). Human chondrocytes were treated with NPY (50 nM) for 3 h and IL-1β for 24 h (10 ng/mL). Each column represents the mean ± SEM of 3 independent experiments. B) p*< 0.05 relative to IL-1β*

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3.9. NPY regulates collagen type 2 expression in human chondrocytes

Inflammation in OA may be one of the triggers for altered human chondrocyte activity. Human OA chondrocytes alter their synthesis activity and modify the proteins of the cartilage matrix. Reduction of colagen type 2 synthesis in OA causes mechanical instability of the ECM by reducing the capacity of the cartilaginous tissue to store elastic energy. We hypothesized that NPY could increase collagen 2 levels, since it acts on inflammation and has a tendency to decrease mRNA expression of MMP-13. To elucidate this question, treatment with NPY for

3 h and IL-1β for 24 h was performed and collagen 2 protein levels were evaluated with an ELISA kit and its mRNA levels by RT-PCR. The results show that NPY increased collagen 2 levels significantly compared to control (Figure 13A). Futhermore, we observed that NPY increased collagen 2 protein levels even in the presence of IL-1β (Figure 13B). Real time RT-PCR shows a tendency of NPY to increase collagen 2 mRNA levels and to reverse the inhibitory effect of IL-1β (Figure 13C), although the differences did not reach statistical significance due to the small number of samples. These results show that NPY likely plays a pro-anabolic role in human chondrocytes, increasing levels of collagen 2 even in the presence of IL-1β.

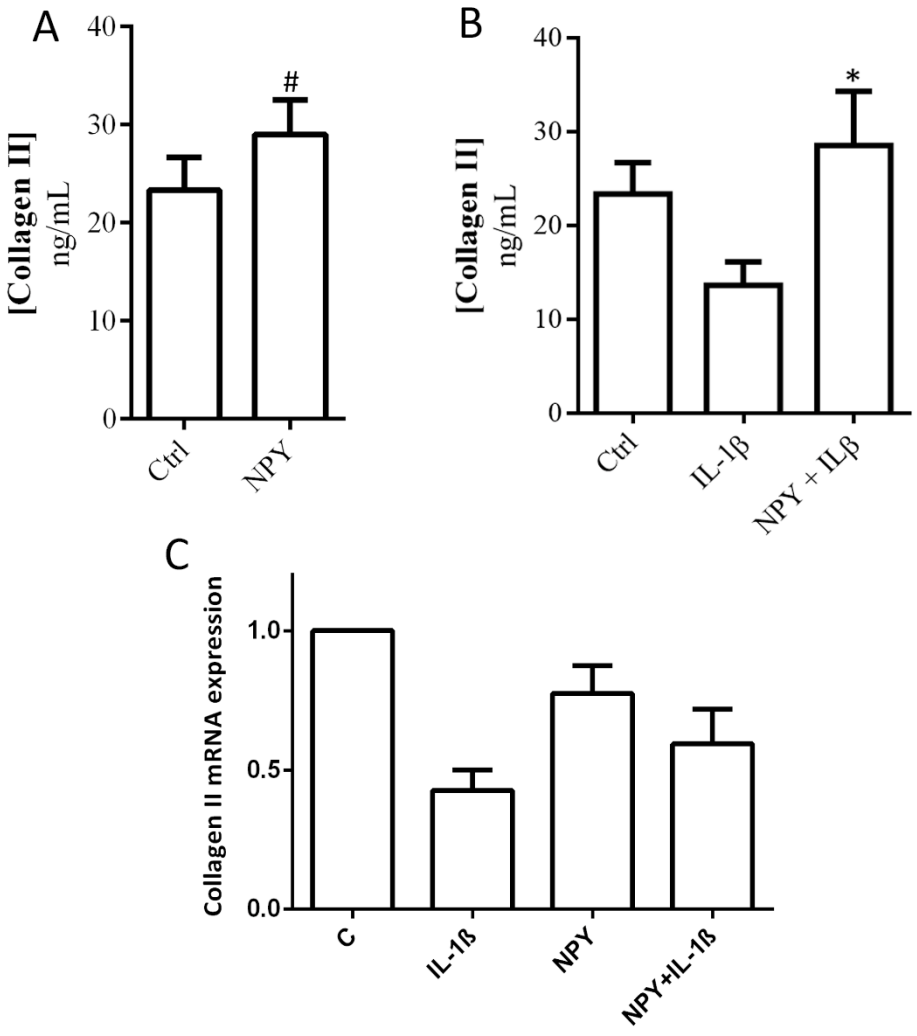

Figure 13. NPY has pro anabolic effects by increasing collagen type 2 expression. *Collagen type 2 protein levels were measured by ELISA (A and B) its mRNA levels by real time RT-PCR (C). Human chondrocytes were treated with NPY (50 nM) for 3 h and IL-1β for 24 h (10 ng/mL). Each column represents the mean ± SEM of 5 (A and B) or 3 (C) independent experiments, # $p < 0.05$ relative to Control (A) and * $p < 0.05$ relative to IL-1β (B)*

Chapter 4: Discussion

NPY appears to have a great therapeutic potential for aging-related diseases and several studies have shown its potential in maintaining cellular homeostasis and its anti-inflammatory potential (59). Evidence has shown that NPY is present in the joint microenvironment in osteoarthritis disease, being found in fibers of sympathetic nerves and in the synovial fluid of OA patients (80) (90). The therapeutic potential of NPY in OA is still poorly understood, nevertheless, Grassel et al (94), pointed out that NPY may be a potential biomarker since it is present in high concentration in the synovial fluid of OA patients (90). Synovial inflammation has been implicated in triggering changes in chondrocytes promoting inflammation and production of catabolic proteins in the articular microenvironment (176). As a result of inflammation, the cartilaginous tissues in OA have a reduced capacity to withstand compressive forces which leads to the cartilage fibrillation and fissure (131). Loeser et al (177), showed that articular chondrocytes display an age-related decrease in proliferative and synthetic capacity, maintaining the ability to produce pro-inflammatory mediators and matrix-degrading enzymes (177). Aging of chondrocytes may result in a microenvironment that is less able to support homeostasis when exposed to inflammatory mediators (178). All changes in the articular chondrocytes cause progressive loss of cartilaginous tissue, which can lead to biomechanical failure and loss of mobility in patients with OA (130). Until now, there is no specific treatment that prevents the progression of OA. Therefore, further understanding the role of inflammation in articular chondrocytes is indispensable for advances in future therapies.

As mentioned above, NPY is present in OA at elevated levels, so this study aimed to understand the role of NPY in modulating inflammatory responses in human chondrocytes. Previous studies in our laboratory had already identified that NPY modulates the responses of human chondrocytes to inflammatory stimuli by decreasing the levels of IL-1β and less prominently of iNOS. In this study, we confirmed the anti-inflammatory role of NPY in promoting the reduction of IL-1β and iNOS in inflammatory conditions (figures 5, 6, 7, 8 and 9) and we investigated which receptors mediate these NPY effects. The results in these figures show that treatment with Y1, Y2 and Y5 agonists, alone or in combination, did not decrease IL-1β and iNOS levels, suggesting that cooperation between receptor subtypes and/or simultaneous interaction of NPY with

various receptor subtypes is required for the inhibitory effects to occur. In addition, figures 6 and 9 show that the Y5 receptor antagonist was the only antagonist tested that could partially reverse the inhibitory effect of NPY on IL-1β and iNOS expression, thus likely being more relevant in mediating these NPY effect. Nonetheless, these results require careful interpretation as blockade of one receptor subtype may leave more agonist available to interact with the other receptor subtypes, thus masking the relevance of individual receptors.

Many studies support the anti-inflammatory effect of NPY (15) (43). For instance, in dendritic cells, antagonism of Y1, Y2 and Y5 receptors caused a significant increase of IL-6 and TNF-α (15). The results presented show that NPY also controls inflammation in human chondrocytes. Thus, the increased levels of NPY in the synovial fluid of OA patients can represent an attempt to control the inflammatory response.

Afterwards, we investigated whether NPY acts directly on the NF-κB signaling pathway. The results presented show that NPY does not appear to act directly on the NF-κB activation pathway, but more likely interferes with acetylation of p65 on Lys310 (Figure 11 B), thereby inhibiting its transcriptional activity. Whether the effect of NPY results from inhibition of acetyltransferase enzymes or activation of deacetylases deserved further investigation

Activation of catabolic pathways that culminate in degradation of ECM in cartilage plays a major role in OA development and progression (179). MMP-13 is an important enzyme that causes cartilage degradation. MMP-13 transgenic animals display joint pathology that greatly resembles OA (172). Our data shows of NPY alone decreasing the mRNA expression of MMP-13, as shown in figure 12, although further studies should be performed to confirm if NPY may play an anti-catabolic role in human chondrocytes.

Collagen type 2 is a critical component of the cartilage matrix. In OA, collagen 2 decreases exponentially, being replaced by non-native collagens (180). Therefore, we investigated whether NPY can affect the production of collagen type 2. As shown in figure 13, NPY was able to significantly increase the production of type collagen 2 compared to control, and reversed the inhibitory effect of IL-1β, increasing the protein levels of collagen 2. The mechanism underlying this pro-anabolic effect of NPY needs elucidation and considering the relevance of the transcription factor, SOX-9, for the expression of collagen type 2 and other matrix components (aggrecan, TIMPs), we hypothesise that NPY leads to SOX-9 activation and consequent transcription of its target genes. Furthermore, the possibility that NPY indirectly increases the levels of collagen type 2 has to be considered, since, as shown in this study, NPY has anti-inflammatory

effects in chondrocytes. Studies have shown that inflammatory mediators decrease SOX-9 expression in healthy and OA chondrocytes (181). In our study, NPY was effective in preventing the inhibitory effect of IL-1β on collagen type 2 expression, further supporting this mechanism. Thus, future studies are required to fully understand the mechanisms of NPY as an anti-inflammatory and pro-anabolic mediator in human cartilage.

Chapter 5: Conclusions and future perspectives

In summary, this study showed that NPY has an anti-inflammatory effect on human chondrocytes, decreasing levels of IL-1β and iNOS. Receptors responsible for the effect of NPY appear to act cooperatively, although Y5 appears to be of greater importance for the reduction of IL-1β and iNOS. NPY did not interfere directly in canonical NF-kB signaling, this might indicate that it promotes the reduction of pro-inflammatory proteins downstream through other proteins of this signaling pathway. In chondrocytes, NPY presents a decreased expression of MMP-13 indicating the possibility of an anti-catabolic role, however further studies are needed to confirm this effect.

To our knowledge, this is the first study to show that NPY plays a pro-anabolic role in human chondrocytes. Role of NPY as anti-inflammation and pro-anabolic in human chondrocytes create new opportunities regarding the use of NPY as a therapeutic target in OA. For future work, it will be necessary to analyse the role of NPY in the proteins that can activate or deactivate via NF-kB. It would be interesting to test other concentrations of agonists and antagonists to elucidate which receptors are important for the anti-inflammatory effect of NPY, other techniques such as siRNAs will also elucidate the role of each NPY receptor. In conclusion, these studies increase knowledge of NPY role in human cartilage and provide the basis for the effects of NPY on human chondrocytes and may lead to the development of a breakthrough in therapeutic strategies for the treatment of osteoarthritis.

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