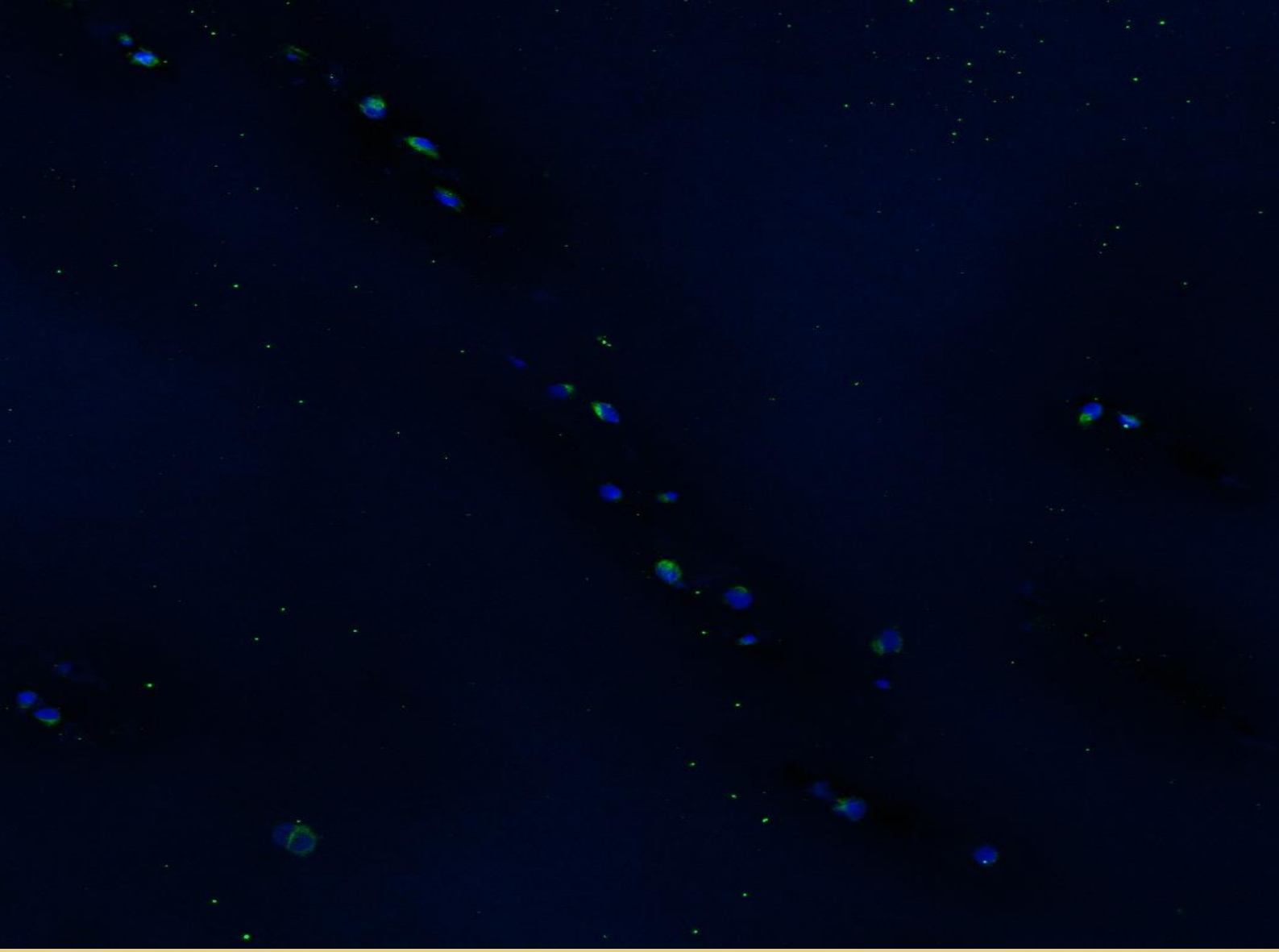

Inês Sofia dos Santos Rodrigues Ferreira

IDENTIFICATION OF NEUROPEPTIDE Y RECEPTORS IN HUMAN ARTICULAR CARTILAGE: INFLUENCE OF GENDER AND OSTEOARTHRITIS

Dissertação de Mestrado em Farmacologia Aplicada, orientada pela Professora Doutora Alexandrina Ferreira Mendes e pela Professora Doutora Cláudia Cavadas e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Julho 2017

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Universidade de Coimbra

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IDENTIFICATION OF NEUROPEPTIDE Y RECEPTORS IN HUMAN ARTICULAR CARTILAGE: INFLUENCE OF GENDER AND OSTEOARTHRITIS

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Farmacologia Aplicada

UNIVERSIDADE DE COIMBRA

2017

Capa: Imagem de microscopia confocal de ensaios de imunofluorescência em cartilagem articular humana ligeiramente degradada (G1), proveniente de um dador do sexo masculino. A verde, imunoreatividade do recetor Y2 e a azul, coloração dos núcleos dos condrócitos com DAPI.

Microscópio e objetiva utilizados: AxioObserver LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss, Germany) e Objective Plan – Apochromat 20x/0.8.

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"She who likes cherries soon learns to climb"

German Proverb

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Index

List of Abbreviations

ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin motifs
AGEs	Advanced glycation end products
ANOVA	Analysis of Variance
ARs	Adrenoceptors
Atg5	Autophagy-related protein 5
BCA	Bicinchoninic acid
BMSCs	Bone marrow stromal cells
BSA	Bovine serum albumin
CGRP	Calcitonin gene-related peptide
ChQ	Chloroquine
CHUC	University and Hospital Center of Coimbra
COX-2	Clyclooxygenase-2
CRLR	Calcitonin receptor-like receptor
DALYs	Disability-adjusted life years
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMM	Destabilization of the medial meniscus
DOC	Sodium deoxycholate
ECF	Enhanced chemifluorescence reagent
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
HA	Hyaluronan
HCl	Hydrogen chloride
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHH	Indian hedgehog
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
KL system	Kellgren and Lawrence system
LC3	Microtubule-associated protein 1A/1B-light chain 3
MMPs	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NE	Norepinephrine
NK1R	Neurokinin-1 receptor
NPY	Neuropeptide Y
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PGs	Proteoglycans
PP	Pancreatic polypeptide
PPARγ	Peroxisome proliferator-activated receptor gamma

PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RAMP-1	Receptor activity-modifying protein-1
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SOX-6	SRY (sex determining region Y)-box 6
SOX-9	SRY (sex determining region Y)-box 9
SP	Substance P
TBS	Tris-buffered saline
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor alfa
VIP	Vasoactive intestinal peptide
Y_1R	Neuropeptide Y receptor Y_1
Y_2R	Neuropeptide Y receptor Y_2
Y_4R	Neuropeptide Y receptor Y_4
Y_5R	Neuropeptide Y receptor Y_5
Y_6R	Neuropeptide Y receptor Y_6

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Abstract

Osteoarthritis (OA) is a degenerative synovial joint disease, primarily characterized by progressive degradation of the articular cartilage. It brings about joint stiffness and pain, ultimately leading to loss of joint mobility and considerably impaired quality of life. However, it is currently defined as a complex and multifactorial disease and its etiology remains incompletely perceived. Besides, there are presently no therapeutic interventions capable of regenerating or repairing the degraded articular cartilage or, at least, of delaying the development and progression of OA.

Sensory and sympathetic nerve fibers arising from the peripheral nervous system innervate several joint tissues. Seemingly, changes in this peripheral joint innervation contribute to the emergence of degenerative alterations that elicit OA progression. Various resident cell types of the musculoskeletal system express receptors for sensory and sympathetic neurotransmitters, thus enabling their response to peripheral neuronal stimuli, and some of these cells even synthesize the neurotransmitters themselves.

Neuropeptide Y (NPY) acts as a neurotransmitter and is widely distributed in the central and peripheral nervous systems. In the latter, it is detected in sympathetic nerve fibers. NPY is involved in the regulation of numerous physiological processes, namely metabolic and age-associated mechanisms, both of which are also involved in the pathophysiology of OA. Importantly, elevated levels of NPY were reported in osteoarthritic joints. Nevertheless, contrarily to other neurotransmitters and their receptors, no studies have depicted the presence of NPY receptors in articular cartilage, nor has the role of this neuropeptide in joint tissue functions been elucidated.

In this sense, this study firstly aimed at determining whether and which NPY receptor subtypes (Y_1, Y_2 and Y_5) are present in human chondrocytes and in human articular cartilage. The presence of each receptor subtype was investigated by immunofluorescence with primary antibodies against the NPY receptors, Y_1, Y_2 and Y_5, in the human chondrocytic cell line, C28/I2, and in healthy and osteoarthritic human cartilage sections. Human cartilage was obtained from multi-organ donors at the Bone and Tissue Bank, University and Hospital Center of Coimbra, with approval by the Ethics Committee. Immunoreactivity for Y_1, Y_2 and Y_5 NPY receptors was observed in the chondrocyte cell line. In human cartilage, only the Y_2 receptor was found in all samples and Y_5 receptor immunoreactivity was undetectable, regardless of disease state, gender and age of the donors. On the other hand, Y_1 receptor

immunoreactivity was observed in male and female OA cartilage samples, as well as in those from healthy females, but not in those from healthy males.

Hereafter, this study intended to assess whether the NPY receptor subtypes identified are functional. Notably, basal autophagic activity diminishes both with aging and OA and the protective role of autophagy modulation in age-associated joint diseases has been advocated. Furthermore, NPY was found to stimulate autophagy in rodent hypothalamus and to possibly have an aging-protective effect. Thus, this study aimed at determining whether NPY could induce this same stimulatory effect in human chondrocytes. To achieve this, the human chondrocytic cell line, C28/I2, was used and the ratio between the levels of LC3-II and I, a marker of autophagy activation, was compared by Western Blot analysis in the presence or absence of the lysosomal protein degradation inhibitor, chloroquine (ChQ).

To assure that NPY and ChQ did not have cytotoxic effects to human chondrocytes, cell viability assays were foremost carried out. The results obtained demonstrated that**,** under the experimental conditions used, none of the compounds affects cell viability in comparison with control untreated cells.

Western Blot analysis indicated that a 6-hour stimulation with NPY 50 nM or 100 nM apparently decreased LC3-II and LC3-I levels in comparison with untreated chondrocytes, suggesting either an increase of the autophagic flux in those conditions, and thus an increased synthesis and clearance of LC3-II, or a decreased synthesis of LC3. To elucidate this question, the levels of LC3-I and II were measured in the presence of ChQ, which blocks LC3-II degradation. In these conditions, an increase in LC3-II levels in cells treated with NPY and ChQ relative to those treated with ChQ alone indicates an increase in the autophagic delivery of LC3-II to the lysosome, thus reflecting autophagy activation by NPY. To clarify this increase, the net autophagic flux was measured, by subtracting LC3-II levels in the absence of ChQ from those obtained in its presence. The results show a mean increase of the net flux at 6 hours stimulation with 50 or 100 nM NPY, which did not reach statistical significance, likely due to the observed inter-assay variability. Treatment with NPY for 24h, especially at the 100 nM concentration, also seems to increase the net flux, even though no conclusions can be taken, as these are preliminary results that did not allow statistical analysis to be performed.

As a whole, this study shows that NPY receptors are present in human chondrocytic cells and in human chondrocytes *in situ* in the articular cartilage. Moreover, the expression of the NPY receptor subtypes seems to be determined by gender and, in males, also by the disease state, strongly suggesting that non-neuronal cells and tissues of the joints, namely chondrocytes, are relevant as NPY targets. Future studies are required to understand the role

of NPY and its receptors in modulating male and female chondrocyte autophagy and how it affects their homeostasis in health and disease conditions. These studies will provide the basis for further studies aimed at establishing the role of NPY and its receptors in cartilage and other joint tissues health and in OA development and progression. Such studies may ultimately lead to the development of novel comprehensive therapeutic strategies with captivating prospects for the treatment of this disabling disease.

Keywords: Aging, autophagy, gender, human cartilage, human chondrocytes, NPY, osteoarthritis, receptors.

Resumo

A osteoartrite (OA) é uma doença degenerativa que afeta as articulações sinoviais e que se caracteriza principalmente pela degradação progressiva da cartilagem articular. Gera rigidez e dor articulares e culmina na perda de mobilidade articular e na diminuição da qualidade de vida. No entanto, é definida com uma doença complexa e multifatorial e a sua etiologia não é ainda totalmente compreendida. Além disso, não há atualmente intervenções terapêuticas capazes de regenerar ou de reparar a cartilagem articular degradada ou de, pelo menos, adiar o desenvolvimento e a progressão da OA.

Fibras nervosas sensoriais e simpáticas com origem no sistema nervoso periférico inervam vários tecidos articulares. Aparentemente, modificações nesta inervação articular periférica contribuem para o surgimento de alterações degenerativas que induzem a progressão da OA. Vários tipos de células residentes do sistema músculo-esquelético expressam recetores para neurotransmissores sensoriais e simpáticos, permitindo desta forma a sua resposta a estímulos neuronais periféricos, e algumas destas células sintetizam também os próprios neurotransmissores.

O neuropeptídeo Y (NPY) atua como um neurotransmissor e está amplamente distribuído nos sistemas nervosos central e periférico. Neste último, é detetado em fibras nervosas simpáticas. O NPY está envolvido na regulação de vários processos fisiológicos, tais como os mecanismos metabólicos e os associados ao envelhecimento, sendo que ambos estão envolvidos na fisiopatologia da OA. Notavelmente, níveis elevados de NPY foram detetados em articulações osteoartríticas. Contudo, ao contrário do que sucede com outros neurotransmissores e respetivos recetores, não há estudos que descrevam a presença de recetores do NPY na cartilagem articular e o papel deste neuropeptídeo nas funções dos tecidos articulares ainda não foi elucidado.

Neste sentido, este estudo teve como primeiro objetivo determinar se e quais os subtipos de recetores do NPY (Y_1, Y_2 e Y_5) presentes em condrócitos humanos e em cartilagem articular humana. A presença de cada subtipo de recetor foi investigada por imunofluorescência com anticorpos primários contra os recetores do NPY, Y_1, Y_2 e Y_5, na linha celular condrocítica humana, C28/I2, e em cortes de cartilagem humana saudável e osteoartrítica. A cartilagem humana foi obtida através de dadores multi-órgãos no Banco de Tecidos do Centro Hospitalar e Universitário de Coimbra, com aprovação da Comissão de Ética. Na linha celular condrocítica, foi observada imunoreatividade para os recetores do NPY Y_1, Y_2 e Y_5. Em cartilagem articular humana, apenas o recetor Y_2 foi encontrado em todas as

amostras e o recetor Y_5 não foi detetado em nenhuma das amostras, independentemente do grau de OA, sexo e idade dos dadores. Por outro lado, a imunoreatividade para o recetor Y_1 foi observada em amostras de cartilagem de mulheres e de homens com OA, assim como em amostras de cartilagem de mulheres saudáveis, mas não nas de homens saudáveis.

De seguida, este estudo pretendeu avaliar se os subtipos de recetores do NPY identificados são funcionais. É importante notar que a atividade autofágica basal diminui tanto com o envelhecimento como com a OA e que o papel protetor da modulação da autofagia em doenças articulares associadas à idade tem sido defendido. Além disso, determinou-se que o NPY estimula a autofagia no hipotálamo de roedores e que pode ter um efeito protetor contra o envelhecimento. Desta forma, o objetivo seguinte deste estudo foi determinar se o NPY conseguia induzir este efeito estimulatório na autofagia em condrócitos humanos. Para isso, a linha celular condrocítica humana, C28/I2, foi utilizada e a razão entre os níveis de LC3-II e I, um marcador da ativação da autofagia, foi comparada através de análise de Western Blot na presença ou na ausência de um inibidor da degradação lisossomal de proteínas, a cloroquina (ChQ).

Para assegurar que o NPY e a ChQ não tinham efeitos citotóxicos nos condrócitos humanos, foram realizados *à priori* ensaios de viabilidade celular. Os resultados obtidos demonstraram que, sob as condições experimentais utilizadas, nenhum dos compostos afetou a viabilidade celular em comparação com as células não tratadas.

A análise de Western Blot indicou que uma estimulação de 6 horas com NPY 50 nM ou 100 nM pareceu diminuir os níveis de LC3-II e LC3-I em comparação com os condrócitos não tratados, sugerindo um aumento do fluxo autofágico nessas condições, e, dessa forma, uma síntese e *clearance* de LC3-II aumentadas, ou uma síntese diminuída de LC3. Para elucidar esta questão, os níveis de LC3-I e II foram medidos na presença de ChQ, que inibe a degradação de LC3-II. Nestas condições, um aumento dos níveis de LC3-II em células tratadas com NPY e ChQ, relativamente às células tratadas apenas com ChQ, indica um aumento da transferência autofágica de LC3-II para o lisossoma, refletindo assim a ativação da autofagia pelo NPY. Para clarificar este aumento, o fluxo autofágico foi medido, subtraindo os níveis de LC3-II obtidos na ausência de ChQ àqueles obtidos na sua presença. Os resultados mostram um aumento médio do fluxo autofágico após 6 horas de estimulação com NPY 50 nM ou 100 nM, que não alcançou significância estatística, provavelmente devido à variabilidade verificada entre ensaios. O tratamento com NPY durante 24 horas, especialmente na concentração de 100 nM, também parece aumentar o fluxo autofágico, apesar de não poderem ser retiradas

conclusões, pois estes são apenas resultados preliminares, que não permitiram que a análise estatística fosse realizada.

Em suma, este estudo mostra que recetores do NPY estão presentes em células condrocíticas humanas e em condrócitos humanos *in situ* na cartilagem articular. Além disso, a expressão dos subtipos de recetores do NPY parece ser determinada pelo sexo e, em homens, também pelo grau de OA, sugerindo fortemente que células não neuronais, nomeadamente os condrócitos, e tecidos das articulações são relevantes como alvos do NPY. São necessários estudos futuros para compreender o papel do NPY e dos seus recetores na modulação da autofagia em condrócitos, em mulheres e homens, e a forma como esse papel afeta a sua homeostasia em condições de saúde e de doença. Estes estudos fornecerão a base para mais estudos, que tenham como objetivo estabelecer o papel do NPY e dos seus recetores na homeostasia da cartilagem e de outros tecidos articulares, assim como no desenvolvimento e na progressão da OA. Esses estudos podem, por fim, levar ao desenvolvimento de novas e abrangentes estratégicas terapêuticas, com perspetivas atraentes para o tratamento desta doença incapacitante.

Palavras-chave: Autofagia, cartilagem humana, condrócitos humanos, envelhecimento, NPY, osteoartrite, recetores, sexo.

CHAPTER I

INTRODUCTION

1.1 Osteoarthritis

1.1.1 Epidemiology

Osteoarthritis (OA), the most common degenerative joint disease, is a principal health burden and is associated with high morbidity [Wei & Bai, 2016]. It affects an estimated 10% of men and 13% of women in the age of 60 or older [Zhang & Jordan, 2010] and its prevalence is likely to increase continuously over the next years, due to aging and to longer working lives in the global population [Rannou et al., 2014]. Notably, the overall burden of OA, expressed in disability-adjusted life years (DALYs), increased by 35% between 1990 and 2015 [Kassebaum N et al., 2017] and OA is foreseen to be the sole leading cause of disability in the general population by 2030 [Thomas et al., 2014].

1.1.2 Etiology and Risk Factors

In spite of being highly prevailing with a marked public health impact, OA etiology remains incompletely perceived. A plethora of risk factors has been linked to OA, including genetic predisposition, aging, obesity, previous joint injury, abnormal limb development and mechanical stress on the joint [Silverwood et al., 2015; Lane et al., 2011; Glyn-Jones et al., 2015; Brandt et al., 2009]. This disease, which was formerly seen as a consequence of "wear and tear" of the articular cartilage along with its incapacity to repair itself [Berenbaum & Meng, 2016], is now recognized as the result of a defective remodeling process of the entire joint [Loeser et al., 2012], involving not only the articular cartilage, but also the synovial tissue, tendons, ligaments and the subchondral bone [Bijlsma et al., 2011].

1.1.3 Pathogenesis

Articular cartilage injury is normally the most evident pathological feature of joint dysfunction [Wei & Bai, 2016]. This hyaline tissue coats the surface of bones in synovial joints and allows free and painless movement, as well as transmission of forces through the skeleton [Fellows et al., 2016]. These mechanical properties are a result of the structure of its extracellular matrix (ECM) [Jeffery et al., 1991]. The ECM is mainly composed of a notably organized collagen network (essentially type II) with high tensile strength that holds aggregating proteoglycans (PGs) and hyaluronan (HA) capable of conferring compressive stiffness and elasticity to this tissue [Poole et al., 2001]. The articular cartilage also has the ability to reduce the friction between oposed and pressurized cartilaginous surfaces by lubricating the joint

[Sophia Fox et al., 2009]. It is maintained by a unique cell type, the chondrocytes [Buckwalter et al., 2005] which are embedded in the abundant and self-synthesized ECM and are responsible for its constitution and integrity [Archer and Francis-West, 2003]. The articular cartilage is an avascular tissue and chondrocytes are adapted to operate at a low oxygen tension environment, relying on the diffusion of nutrients and metabolites from the articular surface [Poole, 1997].

The balance between anabolism and catabolism of the ECM relies on the capacity of chondrocytes to identify modifications in the matrix composition, such as the presence of damaged macromolecules, the placement of biomechanical forces on the articular surface or the biochemical stimuli of hormones, growth factors, cytokines, neurotransmitters and of other intercellular mediators [Ponchel et al., 2015; Sanchez-Adams et al., 2014] and to reply by producing appropriate types and quantities of new ECM components [Sherwood et al., 2014]. During aging and disease, a phenotypic shift towards catabolism is observed, as it exceeds the rate of deposition of promptly synthesized molecules [Goldring and Marcu, 2009]. Moreover, the upregulated catabolic mediators actively suppress the anabolic processes in the articular cartilage, thus further aggravating the imbalance between chondrocyte catabolism and anabolism [Houard et al., 2013]. Besides disruption of the articular cartilage homeostasis and progressive destruction of this tissue, the OA synovial joint may also display the following pathological features: hypertrophy and cloning of chondrocytes (local proliferation), vascularization of the subchondral bone and vascular penetration into the calcified matrix, presence of nerve endings in the emerging osteophytes, meniscus, posterior cruciate ligament and synovium; subchondral bone sclerosis, trabecular thinning and synovial inflammation [Loeser et al., 2012; Reichenbach et al., 2008; Felson, 2009; Konttinen et al., 2012].

Although OA was originally regarded as a non-inflammatory form of arthritis, it is now largely accepted that synovitis is closely linked to enhanced cartilage damage [Felson et al., 2016]. In established OA, proliferation of synoviocytes, infiltration of mononuclear cells and thickening of the sinovial lining layer are notable, with increased vascularity [Scanzello & Goldring, 2012]. Altered production of pro-inflammatory cytokines, predominantly interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) plays a crucial role in the loss of cartilage [Loeser, 2006], since these cytokines induce the expression of catabolic enzymes, namely matrix metalloproteinases (MMPs) and aggrecanases [Aigner et al., 2006] and of inflammatory mediators, such as clyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [Goldring 2000; Pelletier et al., 2001]. Activation of these injury-associated patterns is probably secondary to cartilage alterations during an initial insult to the

joint, after which synovial inflammation and joint degeneration are exacerbated in a positive feedback loop [Scanzello & Goldring, 2012]. A synergy between local tissue damage and the immune system is observed and ultimately leads to a recognised state of low-grade, chronic joint inflammation [Sokolove & Lepus, 2013]. Although this activation of the innate immune system has been regarded as a trigger of local inflammation in OA [Berenbaum, 2013], the local production of inflammatory mediators by joint tissues and cells induces synovial angiogenesis and further synthesis of pro-inflammatory cytokines and catabolic enzymes by synovial cells and by chondrocytes, thus playing a major role in the perpetuation of cartilage degradation in OA [Kapoor et al., 2011].

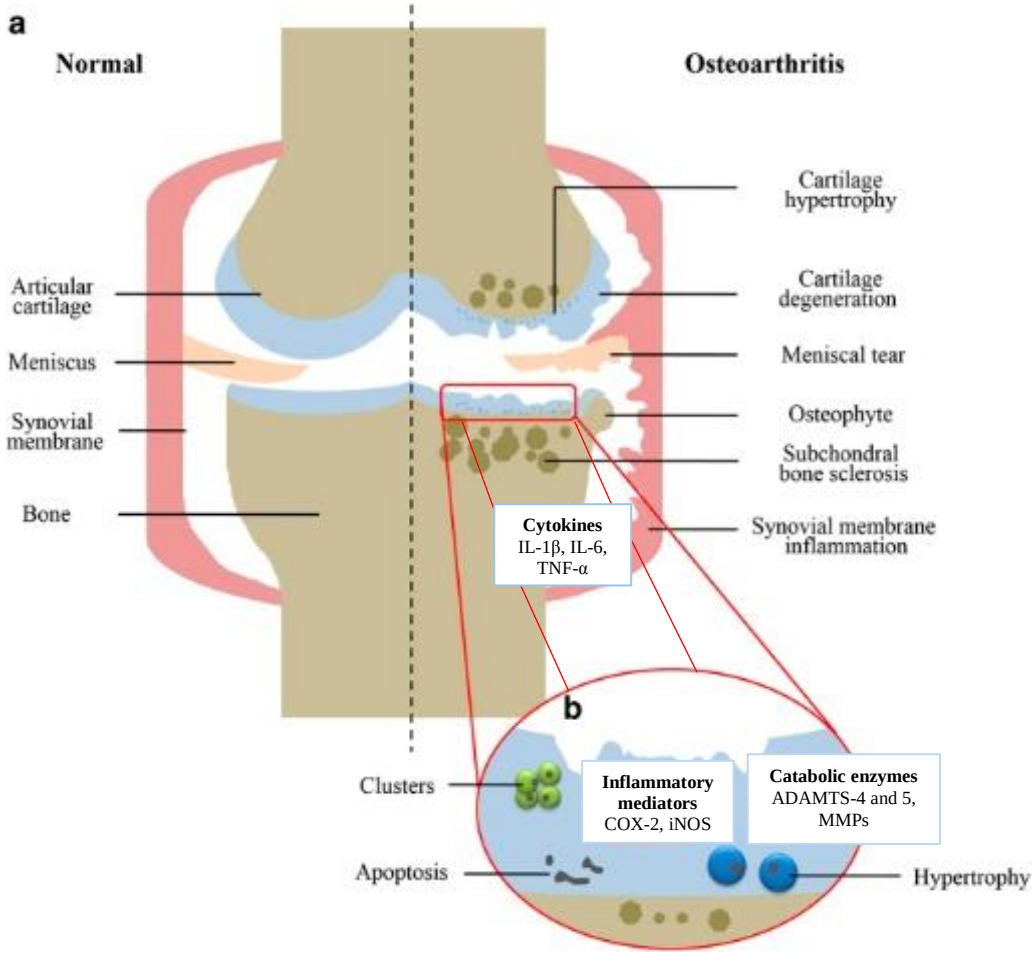


Figure 1: Schematic representation of a healthy and an osteoarthritic synovial joint. (a) The changes in the articular structure during OA development and progression. (b) Cellular responses in OA articular cartilage. Adapted with permission from Zhang *et al.*, 2016.

Macroautophagy (hereafter refered to as autophagy) is a mechanism of cellular homeostasis, highly preserved in eukaryotic organisms, whereby damaged and deleterious cytoplasmic components are delivered to the lysossome for degradation [Chen & Klionsky, 2011; Levine & Kroemer, 2008]. Is it remarkably known for its roles in protecting cells from pathogens [Mizushima et al., 2008] and from conditions of cellular stress, namely hypoxia, oxidative stress, endoplasmic reticulum stress and nutrient and growth factor scarcity, thus sustaining cellular function [Schneider & Cuervo, 2014]. The key features of the autophagic pathway are presented in figure 2.

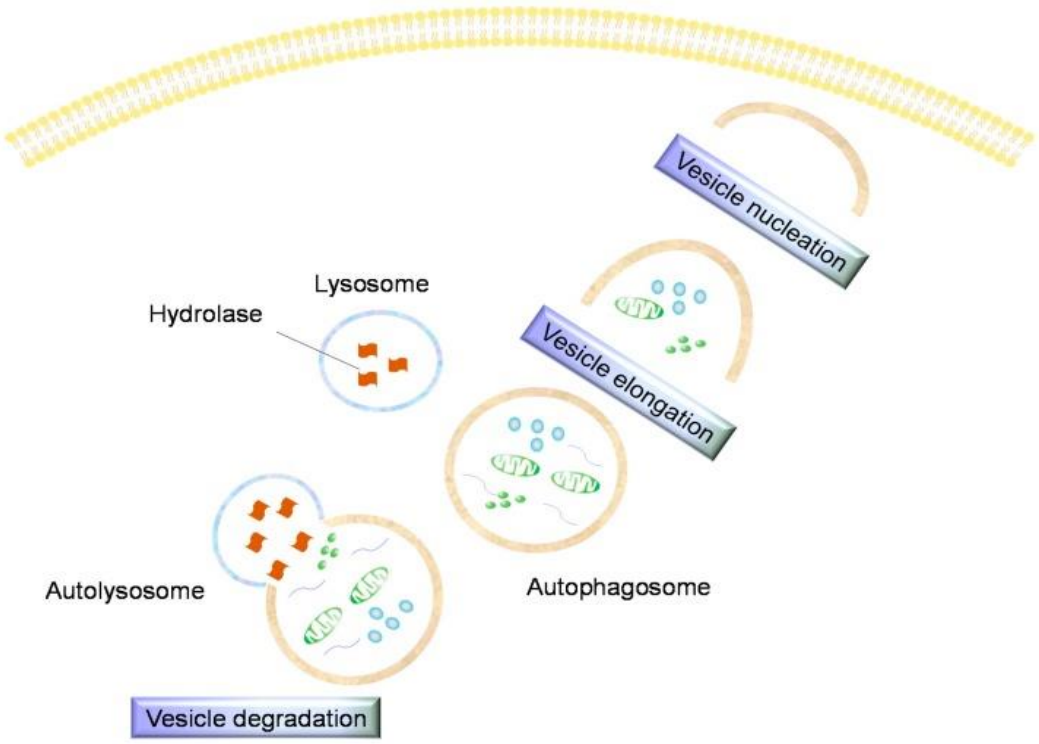


Figure 2: Basic steps of the autophagic pathway. This physiological process starts with the formation of a double membrane structure, known as phagophore or isolation membrane. The double membrane of a phagophore sequesters damaged cytoplasmic and intracellular organelles and forms a vesicular structure called autophagosome. By fusing with a lysosome, the autophagosome turns into an autolysosome. At this point, the inner membrane of the autophagosome and the cytoplasmic material are broken down by lysosomal hydrolases. The autophagic process allows recycling of the degraded products and of energy and, therefore, basal autophagy represents a reparative and life-sustaining process [Martinet et al., 2017]. Reprinted with permission from Musumeci *et al.,* 2015.

Autophagy impairment arises in a wide variety of disease states, particularly in age-related diseases [Bergamini, 2006], and contributes to their aggravation [Cuervo, 2008]. Thus, understanding the processes involved in its dysregulation may bear therapeutic significance.

An extensive association between autophagy and OA has been described [Li et al., 2016]. Autophagy not only regulates the final stages of the chondrocyte life cycle, but also the rate at which chondrocytes enroll the maturation process [Shapiro et al., 2014]. During the initial degenerative stage of OA, catabolic and nutritional stresses lead to a compensatory increase in the expression of key autophagy markers, such as Microtubule associated protein light chain 3 (LC3) and Beclin-1 [Klionsky et al., 2016], in OA chondrocytes [Sasaki et al., 2012]. However, the expression of these autophagy markers is reduced in late stages of OA, in parallel with increased chondrocyte apoptosis [Caramés et al., 2010]. Notably, aging of mouse knee joints is associated with reduced autophagy and cellularity, as well as with increased apoptotic cell death [Caramés et al., 2015]. The link between autophagy and OA was further elucidated when mice with cartilage-specific deletion of mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that acts as key supressor of autophagy, presented protection from OA induced by surgical destabilization of the medial meniscus (DMM) [Zhang et al., 2015]. In line with this, peroxisome proliferator-activated receptor gamma (PPARγ) KO mice subjected to the DMM model of OA presented, among other features, an increased expression of mTOR and an accelerated OA phenotype, which were reversed using PPARγ expression vector *in vitro* [Vashegani et al., 2015]. Moreover, in this study, PPARγ-mTOR double knock out mice exhibited significant protection from the OA phenotype, which shows that PPARγ is involved in the regulation of mTOR/autophagy signaling in the articular cartilage and also provides evidence on its chondroprotective role. Besides, in primary human chondrocytes, advanced glycation end products (AGEs) down-regulated PPARγ expression and increased the levels of inflammatory mediators and this effect was reversed by the PPARγ agonist, pioglitazone, thus suggesting a protective role of PPARγ in the pathophysiology of AGEs-induced OA [Ma et al., 2015]. Considering the known roles of PPARγ in homeostasis, inflammation and adipogenesis [Barish et al., 2006], its implication in mTOR signaling streghtens the crosstalk between cellular metabolism, autophagy and cell survival [Ahmadian et al., 2013]. Additionally, in a mouse model consisting of the green fluorescent protein (GFP) merged to LC3 on the C57BL/6J genetic background (GFP-LC3 transgenic mice), the highest levels of GFP-LC3 and of the key autophagy proteins [autophagy-related-gene 5 (Atg5) and LC3] were found in chondrocytes in the superficial and upper-mid zones of articular cartilage

in young mice, whereas the levels were much lower in deep zone articular chondrocytes and in old mice [Caramés et al., 2015].

These insights are consistent with the hypothesis that basal autophagic activity dimishes with age [Caramés et al., 2015; Cuervo & Dice, 2000] and that the pronounced inhibition of autophagy negatively affects chondrocyte survival and differentiation [Shapiro et al., 2014], thereby taking part in the build-up of damaged macromolecules and in the susceptibility to age-related OA [Cuervo & Dice, 2000]. Therefore, pharmacological activation of autophagy could be a suitable therapeutic approach for OA.

1.1.4 Current therapeutic approaches

OA clinical symptoms often comprise severe pain, stiffness and irreversible loss of joint mobility [Loeser, 2009]. In spite of being one of the leading causes of disability in adults, there are presently no therapeutic interventions to halt or, at least, delay disease progression and/or to promote regeneration or repair of the damaged tissue [Chen et al., 2017]. The signaling pathways involved in OA development and progression and the different phenotypes of this disease need to be further explored, as a means to generate specifically targeted therapies. Both phenotype-specific and comprehensive novel drugs targeting molecules or pathways relevant in several or all distinct OA phenotypes might provide captivating prospects for the treatment of this disease.

1.2 Peripheral nerve fibers and their neurotransmitters in bone and cartilage

Accumulating evidence has hinted that sensory and sympathetic nerves and their neurotransmitters are relevant effectors regulating bone and cartilage physiology and playing pivotal roles in musculoskeletal pathophysiology [Haberland et al., 2001; Pongratz & Straub, 2013]. Importantly, these nerve fibers innervate the synovium, the trabecular and subchondral bone, the bone marrow, the periosteum and the fracture callus [Hukkanen et al., 1992; Madsen et al., 1998] and partly regulate vascularization and matrix differentiation during endochondral bone ossification in embryonic and fetal development [Gajda et al., 2000], which strongly suggests a distinct role in modulating the processes of skeletal growth and limb generation.

Sensory nerve fibers generally hold two different nociceptive neuropeptide families: the tachykinins [Zhang et al., 2000] and calcitonin gene-related peptides (CGRPs). Tachykinins mediate their biological effects through three distinct neurokinin (NK1, 2, 3) receptors.

Among these, the tachykinin, substance P (SP), has the highest affinity to NK1 receptor (NK1R) [Harrison & Geppeti, 2001; Severini et al., 2002] and is known as a mediator of nociception and of inflammation [Hartung & Toyka, 1983]. CGRP has a variety of pro-inflammatory effects due to its influence on immune cells [Tang et al., 1998; Cuesta et al., 2002] and is thought to play a relevant role in the development of inflammatory pain [Bowler et al., 2013]. The vasoactive intestinal peptide (VIP) is a widely distributed sympathetic neuropeptide [Henning & Sawmiller, 2001], which belongs to a family of regulatory peptides including secretin, glucagon and growth hormone-releasing hormone and mediates its effects through G protein-coupled receptors (VPAC1 and VPAC2) [Dickson & Finlayson, 2009]. It displays a robust anti-inflammatory and immune-regulatory activity and modulation of its levels has been considered a potential player for the treatment of inflammatory and auto-immune diseases [Gutierrez-Cañas et al., 2006; Delgado et al., 2001]. Sympathetic nerve fibers also contain, among others, norepinephrine (NE) and express tyrosine hydroxylase (TH), the key enzyme for biosynthesis of cathecolamines [Bjurholm et al., 1988]. NE is a catecholaminergic neurotransmitter that mediates its actions by binding to α- and β- adrenoceptors (ARs) [Hein, 2006]. At high concentrations (> 10^{-7} M), NE acts through α- and β-ARs, while at low concentrations (≤ 10^{-7} M), its effects are mainly mediated through α-ARs [Miller et al., 2000].

Notably, unlike other musculoskeletal connective tissues, the articular catilage does not contain blood vessels and is not profoundly innervated by nerve fibers. Nevertheless, there is slight evidence that cartilage metabolism and regenerating capacity is influenced by neurotransmitters released either from nerve fibers present in neighbouring tissues or directly from chondrocytes [Grässel, 2014; Jenei-Lanzl et al., 2014]. Next, a brief summary of efferent functions and roles of sympathetic and sensory nerve fibers and their neurotransmitters in bone and cartilage physiology and pathophysiology is exhibited.

1.2.1 Sensory nerve fibers in joint physiology and in OA

CGRP- and SP-positive nerve fibers precede the development of cartilage canals which are organized during skeletal growth promptly after birth and were detected when penetrating the canals of growth cartilage in the epiphysis of young rats, thus coming close to chondrocytes [Edoff et al., 2000; Hara-Irie et al., 1996]. Sensory nerve fibers contained in these cartilage canals seem to regulate chondrogenic differentiation during limb growth in embryonic development [Grässel, 2014]. In line with this, it was reported that shortly after fracture, there is a strong induction of sensory nerve regeneration and growth into the site of injury

[Hukkanen et al., 1995], indicating that a restored nerve supply can be pivotal for normal fracture healing [Li et al., 2011].

The presence of NK1 receptors on bone cells was demonstrated [Goto et al., 1998] and studies on the role of SP in bone tissue revealed that it can induce osteogenesis [Shih and Bernard, 1997] and late stage osteoblastic bone formation [Goto et al., 2007]. This was recently corroborated by Niedermair and colleagues [2014], who demonstrated that the absence of SP impacts on bone strucuture by promptly increasing bone resorption and suggested that endogenous SP production by fracture callus chondrocytes acts as a crucial trophic and anabolic factor in bone physiology. On the other hand, in a study where murine costal chondrocytes were used as a model of adult human chondrocytes, it was shown that these cells express SP and NK1 receptors and that SP dose-dependently increases chondrocyte proliferation rate [Opolka et al., 2012], therefore suggesting that SP also modulates chondrocyte metabolism and has trophic effects on chondrocytes. Still, opposite effects for SP on bone formation have been described, depending on its concentration. While SP concentrations above 10^{-8} M promote osteoblast differentiation and bone matrix mineralization [Goto et al., 2007; Wang et al., 2009], SP concentrations below 10^{-8} M inhibit osteogenic differentiation of rat bone marrow stromal cells (BMSCs) but stimulate proliferation and protein synthesis [Adamus & Dabrowski, 2001] and SP absence in an adult murine model of endochondral ossification negatively affected bone architecture, by decreasing pain sensitivity and mechanical stability of the newly formed bone [Niedermair et al., 2014]. These findings suggest that this neuropeptide is required for normal endochondral ossification.

As yet, the production of CGRP and the presence of its receptor, consisting of the two elements CRLR/RAMP1, in articular cartilage has not been described [Grässel & Muschter, 2017]. Although the expression of CRLR in articular cartilage chondrocytes isolated from both OA and non-OA patients was demonstrated, primarily in middle and deep zones, similarly to the NK1R [Grässel et al., 2016], the role of CGRP in cartilage homeostasis remains to be determined. In bone metabolism, it is defined as an anabolic factor, as it stimulates osteoblast activity and thus bone formation [Elefteriou, 2005; Schinke et al., 2004]. On the other hand, it was shown that CGRP concentrations in the serum and synovial fluid of patients with primary knee OA were elevated, compared with healthy controls, and were associated with increasing Kellgren and Lawrence (KL) scores of cartilage damage [Dong et al., 2015]. In this study, CGRP was presented as a possible biomarker and predictor of knee OA severity and progression.

1.2.2 Sympathetic nerve fibers in joint physiology and in OA

Sympathetic nerve fibers and/or neurotransmitter-producing cells are abundantly detected in the subchondral bone marrow [Maestroni, 2000], in the periosteum and in the synovial membrane [Bjurholm et al., 1988; Lorenz et al., 2016]. Yet, there are no reports regarding its presence in the articular cartilage or in the avascular zone of the meniscus [Miller et al., 2000; Jenei-Lanzl et al, 2014]. Notwithstanding, β2- and α2-ARs have been identified on growth plate chondrocytes at different developmental phases [Lai & Mitchell, 2008; Mitchell et al., 2011] and in chondrogenic progenitor cells obtained from human OA cartilage explants [Lorenz et al., 2016; Jenei-Lanzl et al., 2014]. Expression of β2- and α1/α2- ARs was also verified in newborn murine costal chondrocytes [Opolka et al., 2012]. Given that specific ARs receptors are located on chondrocytes, it might be presumed that the neurotransmitters released into the synovial fluid can influence cartilage tissue and chondrocyte metabolic activity [Grässel & Muschter, 2017]. It was proposed that signaling through β2-ARs inhibits collagen II, collagen X and indian hedgehog (IHH) expression partly through suppression of SOX-6 and SOX-9, thereby interfering with chondrogenic differentiation [Lai & Mitchell, 2008; Mitchell et al., 2011; Takarada et al., 2009]. This was corroborated by Jenei-Lanzl and colleagues [2014], who demonstrated that NE inhibited chondrogenic differentiation of BMSCs and OA-cartilage-derived progenitor cells. At high concentrations (10^{-6}), NE acted through β2-AR signaling and repressed collagen II and glycosaminoglycan (GAG) deposition, while inducing the expression of hyperthrophic markers, such as collagen X and MMP-13. At lower concentrations (10^{-7} and 10^{-9}), NE, acting primarily through α-ARs, exerted no effects. Given the fact that inflammation is accepted as a relevant OA hallmark, Lorenz and colleagues [2016] looked at the effects of NE on chondrocyte function in the presence of the inflammatory trigger, IL-1β. At high concentrations, NE reversed IL-1β-induced decrease of collagen II and GAG synthesis and decreased chondrocyte metabolism and proliferation, presumptively through β-AR signaling. Thus, in contrast to previous findings, it appeared that β-AR signaling stimulated an anti-inflammatory, non-proliferative and metabolically stable articular chondrocyte phenotype, which may impede OA onset and progression. Contrarily, α-AR signaling appeared to promote apoptosis and/or proliferation of articular chondrocytes, which may contribute to OA progression.

The precise role of VIP in the pathogenesis of OA remains equivocal [Sutton et al., 2009]. Until now, the presence of VIP receptors on chondrocytes has not been reported [Grässel & Muschter, 2017]. VIP has been proposed to play a protective role towards progression of OA, just like in rheumatoid arthritis (RA), since VIP levels in the synovial fluid and in the articular

cartilage of OA patients are negatively associated with progressive joint injury and disease severity [Jiang et al., 2012]. Interestingly, in a study by McDougall and colleagues [2006], intra-articular injection of VIP generated a prompt and transient algesic effect, by sensitizing aferent nerves and increasing the nerve fire rate. Moreover, administration of the VIP receptor antagonist, $VIP_{6-28,}$ into the knee joint of rats with monoiodacetate-induced arthritis decreased pain-related behaviour, thus elucidating the potential role of VIP in the emergence of knee joint allodynia and secondary hyperalgesia. Together, these studies suggest that VIP may act through different mechanisms in the synovial joint and, thus, may exert distinct and partly contradictory effects on disease progression and pain.

Collectively, these reports provide different and partly conflicting data, which do not allow a clear understanding of the role of sympathetic neurotransmitters in the regulation of joint tissue homeostasis and OA related-pathways. It may though be affirmed that sensory and sympathetic nerve fibers and their neurotransmitters remarkably influence subchondral bone, articular cartilage and other joint tissue function and homeostasis, as well as joint tissue pathophysiology in OA.

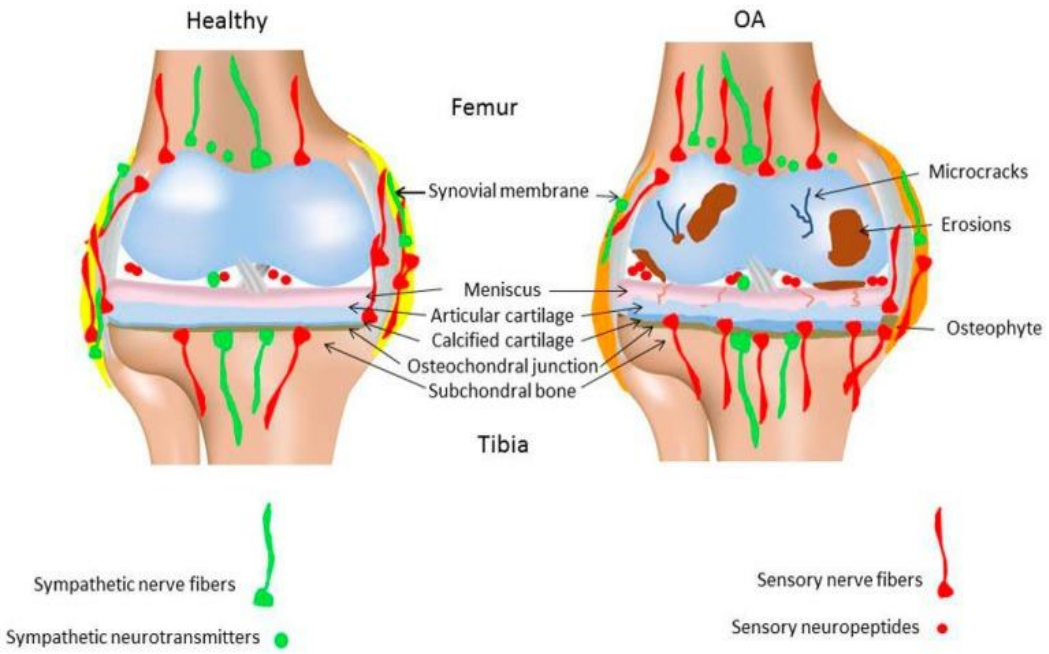


Figure 3: Proposed alteration of sensory and sympathetic joint innervation in OA. It is suggested that, in OA, there is an increase of sensory innervation in the subchondral bone, which reaches into the calcified cartilage zone or which comes into close contact to the articular cartilage. Contrarily, synovial sensory innervation appears to decrease in OA. Sympathetic innervation is presumably not profoundly changed during OA development. Reprinted with permission from Grässel & Muschter, 2017.

1.3 Neuropeptide Y (NPY)

1.3.1 The NPY system

Neuropeptide Y (NPY) is a 36 amino-acid peptide abundantly expressed in the central and peripheral nervous systems [Driessler & Baldock, 2010; Zengin et al., 2010]. Within the central nervous system, it displays particular high concentrations in the hypothalamus, cerebral cortex and brainstem [Baraban, 1998; Lin et al., 2005]. In the hypothalamus, NPY is involved in the modulation of different physiological functions, such as regulation of food consumption, body temperature, circadian rhythms, anxiety, memory processing, and cognition [Beck & Pourié, 2013; Decressac & Barker, 2012; Nguyen et al., 2011; Wettstein et al., 1995; Wiater et al., 2011]. Recently, it was reported that NPY is able to interfere with various age-related mechanisms and has a role in aging and lifespan determination [Botelho & Cavadas, 2015]. Within the peripheral nervous system, NPY is detected in the sympathetic nervous system, where it is co-stored and co-delivered with NE during nerve stimulation [Ekblad et al., 1984]. Also in peripheral organs, NPY has been shown to contribute to a broad range of physiological processes, including vascular and immune function, pain, stress coping, namely in anxiety, post-traumatic stress disorder, and depressive-like behavior, adipogenesis and angiogenesis [Brothers & Wahlestedt, 2010; Hirsch & Zukowska, 2012; Pedrazzini et al., 2003; Kuo et al., 2007; Ekstrand et al., 2003; Wu et al., 2011].

NPY mediates its actions through the activation of, at least, five G-protein-coupled receptors subtypes termed NPY Y_1, Y_2, Y_4, Y_5 and y_6 [Michel et al., 1998; Lin et al., 2004]. To date, the Y_3 receptor subtype has not been cloned [Pedragosa-Badia et al., 2013]. Besides, the Y_4 receptor subtype is preferentially activated by the pancreatic polypeptide (PP), one of the three polypeptides of the NPY family, and the y_6 receptor subtype is not functional in rats and humans [Balasubramaniam, 1997]. As a result, Y_1, Y_2 and Y_5 receptors are seemingly the three major subtypes of NPY receptors that mediate NPY biological functions in both rats and humans [Masliukov et al., 2016].

1.3.2 The role of NPY in cartilage and bone

Existing data reveal that bone is innervated by NPY-positive fibers from the sympathetic nervous system [Hill & Elde, 1991; Bjurholm et al., 1988]. Furthermore, NPY is expressed by non-neuronal cells found in the bone microenvironment, including osteoblasts, osteocytes, bone marrow cells and endothelial cells [Silva et al., 2005; Nunes et al., 2010]. Among the five NPY receptors, Y_1R and Y_2R have been reported as major contributors to bone homeostasis

[Shi & Baldock, 2012]. While Y_2R seemingly mediates hypothalamic control of bone mass, Y_1R has been demonstrated to be expressed in bone, namely in osteoblasts and bone marrow cells, and is, therefore, more likely to contribute to the peripheral pathway [Igwe et al., 2009; Lundberg et al., 2007; Baldock et al., 2002]. Notably, these observations support a functional role for the NPY system in bone regulation.

In synovial joints, NPY-positive sympathetic nerve fibers were identified at the basis of tibial osteophytes, the subchondral bone marrow and within vascular channels of articular cartilage in OA [Suri et al., 2007]. In a recent study, Wang and colleagues [2014] revealed that the synovial fluid concentrations of NPY were substantially higher in the knees of OA patients, compared with healthy controls, and also that middle and advanced stage OA patients exhibited higher NPY synovial fluid concentrations compared to early-stage OA patients. Besides, increasing synovial fluid concentrations of NPY positively correlated with increasing pain scores. NPY is recognized as a potent neuromodulator [Hua et al., 1991], being stored in the terminal branches of Aδ and C fibers and, when delivered into the joint, it reduces the activation threshold of nociceptive nerve endings, which possibly contributes to chronic, sensitized pain responses [Woolf & Salter, 2000]. In line with these reports, the authors suggested a role for NPY as a putative regulator of pain transmission and perception in knee OA pain and also hypothesized that concentrations of NPY may reflect the pathological progression and severity of OA. This was partly corroborated by Adães and colleagues [2015], who evaluated whether neuronal injury could be related to the development of OA-associated nociception. For this purpose, they used the collagenase-induced rat OA model described by van der Kraan et al., [1989], where they found that nociceptive behaviours were associated with movement and loading of the OA joint, mimicking the main symptoms reported by patients. Those researchers also observed that the expression of neuronal injury markers, including NPY, was upregulated in primary afferent neurons innervating OA joints after collagenase injection, both at an early inflammatory phase and at a later phase, when articular degradation was extensive.

Together, these studies heighten the hypothesis that injury of NPY-positive peripheral nerve fibers may be a fundamental feature of OA-associated pain. Moreover, they point towards a potential role for NPY in joint tissue function and homeostasis, as reported for other neurotransmitters and for the peripheral nerve fibers in which they are localized. Nervetheless, the precise mechanims through which NPY may be involved in the pathogenesis of OA require further investigation.

1.3.3 The role of NPY in autophagy

The role of NPY in autophagy has not been extensively described. However, it is well established that basal autophagy decreases with age [Mariño & López-Otín, 2008] and that autophagy impairment contributes to distinct features of the aging phenotype [Cuervo, 2008]. Interestingly, a few studies have demonstrated that aging dimishes NPY levels in rodent hypothalamus [Gruenewald et al., 1994; Higuchi et al., 1988; Vela et al., 2003]. In line with this, Aveleira and colleagues [2015] hypothesized that NPY can be involved in autophagy modulation in the hypothalamus. Using both in vitro and in vivo models, they observed that NPY stimulates autophagy in rodent hypothalamus. Because both NPY levels and hypothalamic autophagy decrease with aging [Kaushik et al., 2012] and NPY regulates autophagy in the hypothalamus, this group suggested that modulation of NPY levels may afford protective effects against age-associated hypothalamic alterations.

Importantly, several of the hallmarks of aging have been suggested to contribute to OA development and progression [Loeser et al., 2016]. Besides, a reduction in autophagic activity and key regulators is observed in OA chondrocytes [Shapiro et al., 2014] and a chondroprotective role of autophagy activation has been suggested [Caramés et al., 2010; Ribeiro et al., 2016a, 2016b]. However, whether NPY stimulates autophagy in synovial joint tissues, remains to be determined.

1.4 Aims

The etiology of OA remains incompletely understood and the distinct phenotypes of the disease need to be further investigated. Aging is apparently the most prominent risk factor triggering OA development and progression, possibly due to the poor self-healing capacity of the articular cartilage [Hunziker, 2002]. The presently available therapies for this disease merely provide symptomatic relief and do not have the capacity to restore the injured articular cartilage or to postpone the development of OA. Therefore, it is fundamental to clarify the molecular and signaling mechanisms eliciting OA progression, as a means to generate novel drugs and agents potentially capable of reversing or, at least, halting this disease.

Evidence has indicated that sensory and sympathetic nerves fibers arising from the peripheral nervous system, as well as their neurotransmitters, regulate bone and cartilage physiology. Importantly, under musculoskeletal pathological conditions, the innervation pattern of these fibers is generally altered in the subchondral bone, in the synovial membrane and in other joint tissues. Although this strongly suggests their involvement in the modulation

of the function and homeostasis of both bone and cartilage, these data remain unclear and partly controversial.

Therefore, the main purpose of this study was to contribute to understand the role of NPY in synovial joint homeostasis. Firstly, we aimed at determining whether any NPY receptor subtypes are present in human chondrocytes and in human articular cartilage and whether their presence is influenced by gender or disease state. To achieve this, we used both the human chondrocyte cell line, C28/I2, and healthy and osteoarthritic human articular cartilage. Then, we sought to determine whether the NPY receptors present in human articular cartilage are functional. For this and considering that autophagy activation seems to protect against age-associated joint diseases [Lotz and Caramés, 2011] and further considering the stimulatory effect of NPY on autophagy in rodent hypothalamus, we investigated whether NPY might induce this same stimulatory effect in human chondrocytes. For this purpose, we used the human chondrocyte cell line, C28/I2, to investigate the role of NPY in inducing the autophagic flux. This study also provides the basis for further studies aimed at elucidating the role of NPY in joint physiology and in the patophysiology of articular diseases, namely OA.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell culture

The immortalized C28/I2 human chondrocyte cell line (a kind gift from Prof. Mary Goldring and Harvard University) was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) and Nutrient Mixture F-12 Ham (Sigma-Aldrich®), supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 1% antibiotic mixture (Penicillin and Streptomycin), at 37°C, in an atmosphere of 5% CO_2. The media was changed every 2-3 days. Cells were passaged at 70 to 80% confluence using 0.05% trypsin – 0.02% EDTA solution.

2.2 Immunocytochemistry

Chondrocytic cells were seeded on 8-well µ-slides (IbiTreat, Ibidi) at a density of 2.5 x 10^5 cells/mL and cultured for 16 h. The chondrocyte monolayers were subsequently fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0,1% Tween 20 in PBS for 10 minutes at room temperature (RT). Thereafter, the cells were blocked for 1h in 5% Goat Serum in 0,5% BSA in PBS and incubated either with Anti-NPY Y_1 Receptor (1:50; Immunostar), Anti-NPY Y_2 Receptor (1:500; Alomone Labs) or Anti-NPY Y_5 Receptor (1:250; Alomone Labs) primary antibodies overnight at 4°C. Negative controls for each antibody were set up by omitting the primary antibodies. The slides were then incubated for 1h at RT in the dark with an Alexa 488-conjugated secondary antibody (Anti-Rabbit IgG (H+L); 1:400; Sigma-Aldrich®). Nuclei were stained with DAPI (0.2 µg/µL; Invitrogen). Lastly, the slides were mounted with Ibidi Mounting Medium (Ibidi) and visualized by fluorescence microscopy in an Axio Observer Z1 microscope (Carl Zeiss, Germany). Each antibody was tested in, at least, three different cell slides ($n \geq 3$).

2.3 Articular cartilage samples

Non-osteoarthritic and osteoarthritic human knee cartilage samples were collected from the distal femoral condyles of 4 multi-organ donors, of which 2 were men (mean age: 69.5 ± 0.5 years) and 2 were women (mean age: 50.5 ± 17.5 years) and, with informed consent, of 3 patients undergoing total knee arthroplasty at the Orthopaedic Department and Bone Bank of the University and Hospital Center of Coimbra (CHUC). Two of those patients were men (mean age: 65 ± 10 years) and 1 was a 65 year old woman. The cartilage samples were macroscopically evaluated and classified as to the degree of degradation using the Outerbridge classification (1961). Briefly, cartilage samples were classified as normal or undamaged if presenting no macroscopic signs of degradation (Grade 0), mildly damaged if presenting only

a softened surface (Grade 1), damaged if presenting a fibrillated or fissured surface without evident signs of surface erosion (Grades 2-3) and severely damaged if presenting areas of full thickness erosion that exposed the subchondral bone, corresponding to advanced OA (Grade 4). All procedures were approved by the Ethics Committee of CHUC (protocol approval numbers 8654/DC and HUC-13-05).

Table 1: List of the human knee cartilage samples used in the immunohistochemistry assays.

Origin	Gender / age (years)	Outerbridge classification
Multi-organ donors	Male / 69	G1
	Male / 70	G1
	Female / 33	G0
	Female / 68	G0
Patients	Male / 75	G4
	Male / 55	G4
	Female / 65	G4

2.4 Immunohistochemistry

The cartilage explants were first embedded in the optimum cutting temperature (OCT) formulation, Shandon Cryomatrix (Thermo Scientific), and serial 10 µm sections were obtained in a MEV cryostat (SLEE medical GmbH, Germany). For immunofluorescence reactions, the sections were fixed in cold acetone for 10 minutes and rehydrated in PBS, pH=7.4, for 5 minutes at RT. Then, the sections were permeabilised in PBS with 0,25% Triton X-100 for 30 minutes and blocked in 10% Goat Serum in PBS with 1% BSA for another 30 minutes at RT. The sections were then incubated overnight at 4°C with one of the following primary antibodies: anti-NPY Y_1 Receptor (1:100; Immunostar), anti-NPY Y_2 Receptor (1:2000; Alomone Labs) or anti-NPY Y_5 Receptor (1:2000; Alomone Labs). Negative controls for each sample were set up by omitting each primary antibody. After thorough washing with PBS pH=7.4, the sections were incubated with Alexa488-conjugated secondary antibody (1:400, Anti-Rabbit IgG (H+L), Sigma-Aldrich®) for 1h at RT in the dark. Nuclei were stained with DAPI (0.2 µg/µL; Invitrogen) and the sections were mounted with Ibidi Mounting Medium

(Ibidi) and coverslipped**.** Immunostaining images were acquired on an AxioObserver LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss, Germany).

2.5 Assessment of cell viability

The viability of C28/I2 chondrocytic cells in the presence of NPY and/or chloroquine, a lysosomal protein degradation inhibitor, was assessed by the Resazurin reduction assay [Ansar Ahmed et al., 1994]. The chondrocytes were cultured in 24-well plates at a density of 0.5 x 10^6 cells/mL for 16h. Briefly, the cells were pretreated with or without chloroquine (ChQ) (50 µM; Sigma-Aldrich) for 30 minutes, followed by stimulation with or without 50 nM or 100 nM NPY (Phoenix Pharmaceuticals) for 6 h. After that, 100 µL of rezasurin (0,125 mg/mL) were added to each well and incubated at 37°C for 2h. The absorbance was measured using the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc. USA) set at a test wavelength of 570 nm and a reference wavelength of 620 nm. Each condition was performed in duplicate in, at least, three independent experiments**.**

2.6 Evaluation of autophagy

C28/I2 chondrocytic cells were cultured in 6-well plates at a density of 0.5 x 10^6 cells/mL for 16 h. To estimate the effect of NPY on autophagy, chondrocytes were cultured with or without ChQ (50 µM; Sigma-Aldrich), a lysosome inhibitor, for 30 minutes, followed by the addition of distinct concentrations (0nM, 50 nM or 100 nM) of NPY (Phoenix Pharmaceuticals) for 6 h or 24 h. Total cell extracts were then prepared for assessment of LC3 I and II levels by western blot and calculation of the net autophagic flux.

2.7 Cell lysis and protein extraction

At the end of the treatment periods, chondrocyte cultures were washed twice with cold PBS pH = 7.4 and the proteins extracted with ice-cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 1% Triton X100, 0.5% DOC, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails (Complete Mini and PhosStop, Roche) according to the manufacturer's instructions. Lysates were kept on ice for 30 minutes and were then centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatants were collected and kept at -20°C until further use.

2.8 Measurement of protein concentration

The protein concentration in cell lysates was determined with the bicinchoninic acid (BCA) / copper (II) sulfate assay (Sigma-Aldrich®), according to the manufacturer's instructions. Absorbance values were measured at a wavelenght of 570 nm using the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc. USA). The protein concentration in each cell lysate was determined through interpolation in the linear regression built from previously prepared standard solutions with known concentrations of BSA (Merck, EMD Millipore Corporation, USA).

2.9 Western Blot

Whole cell extracts were prepared as previously described [Rosa et al., 2009]. Briefly, the proteins in the extracts (25 μg) and molecular weight markers (All blue, Precision Plus molecular weight markers, Bio-Rad Laboratories Inc., Hercules, CA) were resolved on 12% SDS – polyacrylamide gels and then transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk in 0.1% TBS-Tween 20 for 2 h at RT and were further probed with the primary antibody against human LC3 (1:1000; Cell Signaling Technology) overnight at 4°C. After extensive washings, the membranes were incubated with an alkaline phosphatase-linked secondary antibody (Anti-Rabbit IgG, GE Healthcare, UK) for 1 h at RT. Immune complexes were detected using the Enhanced ChemiFluorescence reagent (GE Healthcare, UK) in a Typhoon™ FLA 9000 scanner (GE Healthcare Life Sciences, Uppsala, Sweden). β-tubulin was used as a loading control. For this purpose, the membranes were repeatedly washed with 0,1% TBS-Tween 20, newly blocked in 5% non-fat dry milk in 0,1% TBS-Tween 20 for 30 minutes and probed with the primary antibody against β-tubulin (1:20000**,** Sigma-Aldrich®) for 1 h at RT. Additional washings with 0,1% TBS-Tween 20 were performed every 5 minutes for 30 minutes, followed by incubation with the corresponding secondary antibody (Anti-Mouse IgG + IgM, alkaline phosphatase-linked; 1:20000; GE Healthcare, UK) for 1 h at RT. The intensity of the bands was estimated using the CLIQS software (TotalLab Ltd., England). For each sample, the ratio between the intensities of the bands corresponding to LC3-II and LC3-I was determined.

2.10 Statistical Analysis

Data analysis was carried out using GraphPad Prism 5 (GraphPad Software, www.graphpad.com). For each experimental condition, the results are presented as the mean ± SEM. Evaluation of the statistical significance between experimental conditions and the control was performed by the Analysis of Variance (ANOVA) test, followed by the Dunnet's post hoc test for multiple comparisons. Values of $p<0.05$ were considered statistically significant.

CHAPTER III

RESULTS

3. 1 Immunoreactivity of NPY receptors in the human chondrocyte cell line, C28/I2

The presence of NPY receptors in the C28/I2 chondrocyte cell line was investigated as a preliminary indicator of their potential presence in human primary chondrocytes and to establish their usefulness as a model to study the role of NPY and its receptors in chondrocytes. As shown in Fig. 4, immunoreactivity for all three NPY receptor subtypes was observed in the chondrocyte cell line, C28/I2.

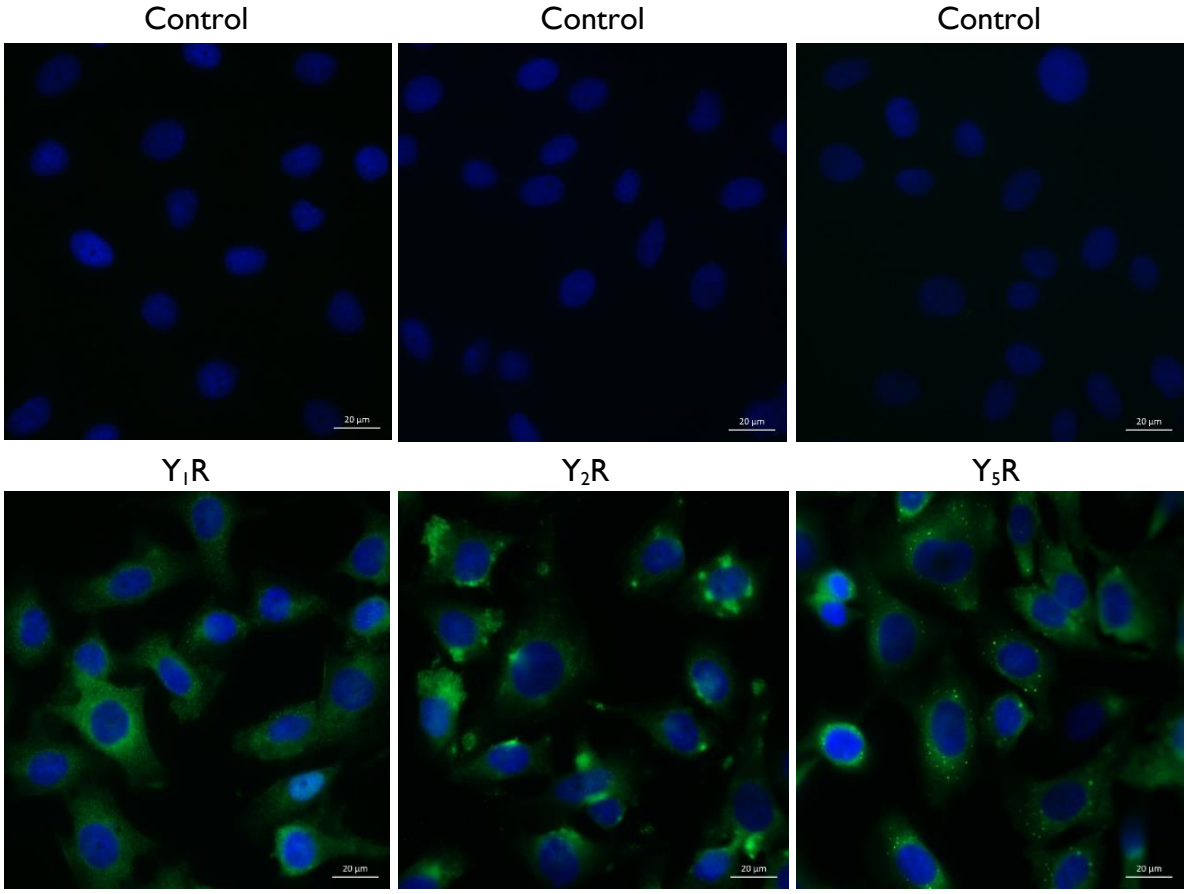


Figure 4. Immunoreactivity for Y_1, Y_2 and Y_5 NPY receptors in the chondrocyte cell line, C28/I2. Cells were immunolabelled for Y_1, Y_2 or Y_5 NPY receptors (green). Nuclei were stained with DAPI (blue). Negative controls were set up by omitting the primary antibodies. The images shown are representative of, at least, three independent experiments. Scale bar: 20 µM.

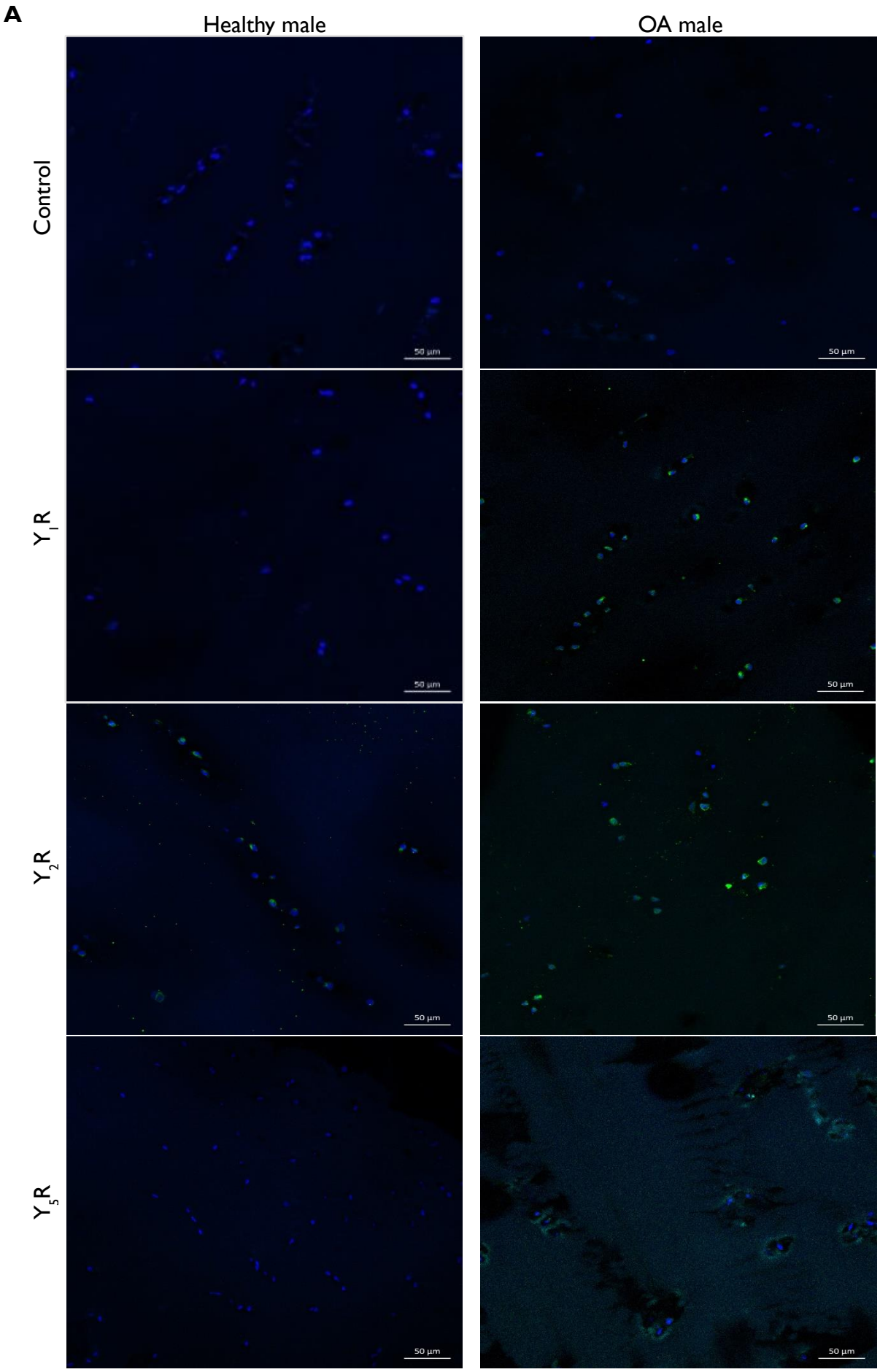
3.2 Immunoreactivity of NPY receptors in human articular cartilage

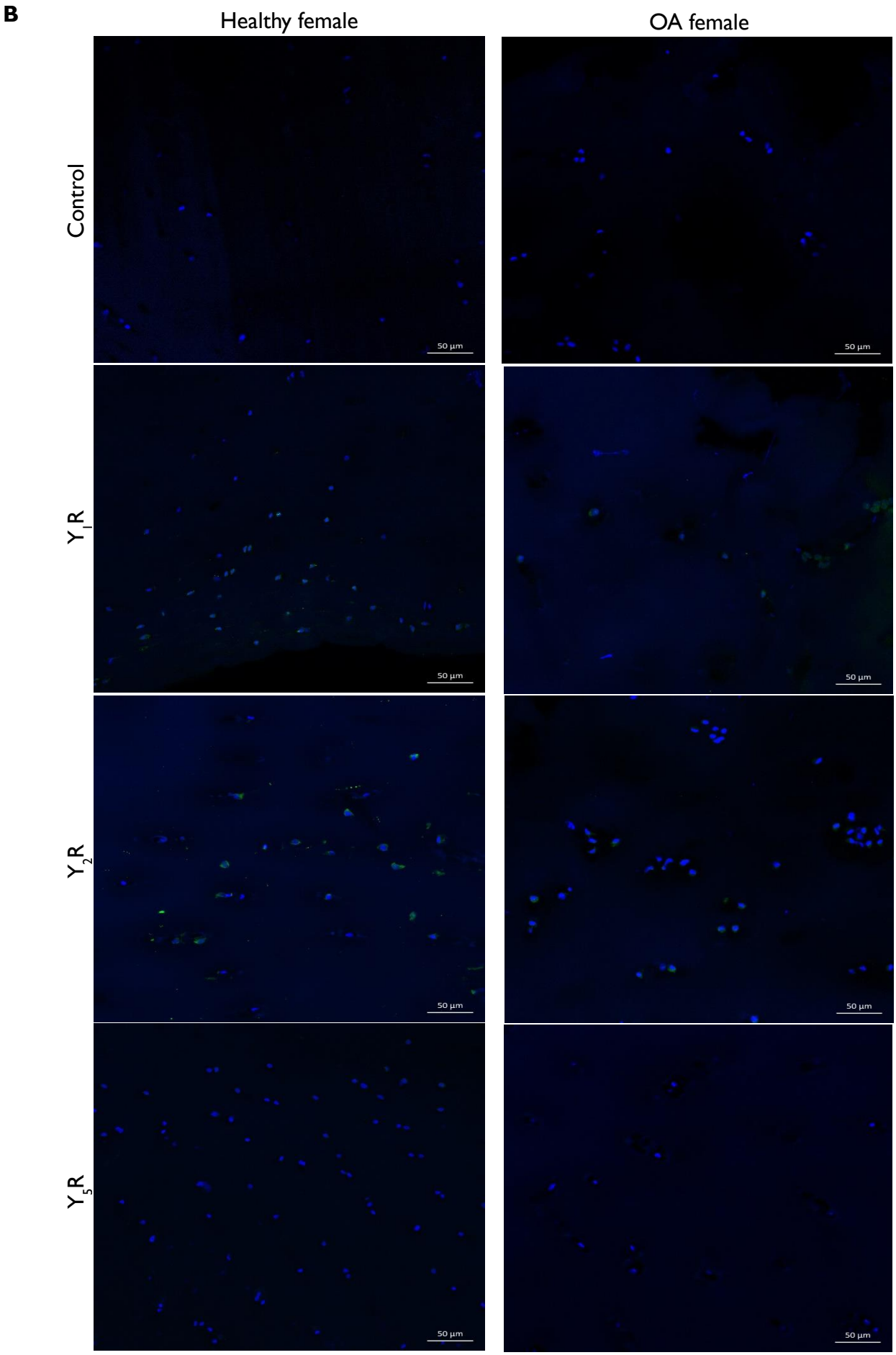
Immunohistochemical staining of non-OA and OA human knee cartilage samples was evaluated using the same antibodies as in the immunocytochemistry assays. To investigate the presence of the NPY receptors, Y_1, Y_2 and Y_5, in the cartilage samples, the age, gender (male vs. female) and the joint disease state (healthy vs. osteoarthritic) of the donors were taken into consideration. Also, grade 0 (G0) and grade 1(G1) cartilage samples were considered "healthy", while grade 4 (G4) samples are exhibited as "osteoarthritic".

As shown in Fig. 5, only the Y_2 receptor was found in all samples, independently of the gender and the disease state of the donors, while Y_5 receptor immunoreactivity was undetectable in all samples. On the other hand, Y_1 receptor immunoreactivity was observed in male and female OA cartilage samples, as well as in those from healthy females, but not in those from healthy males.

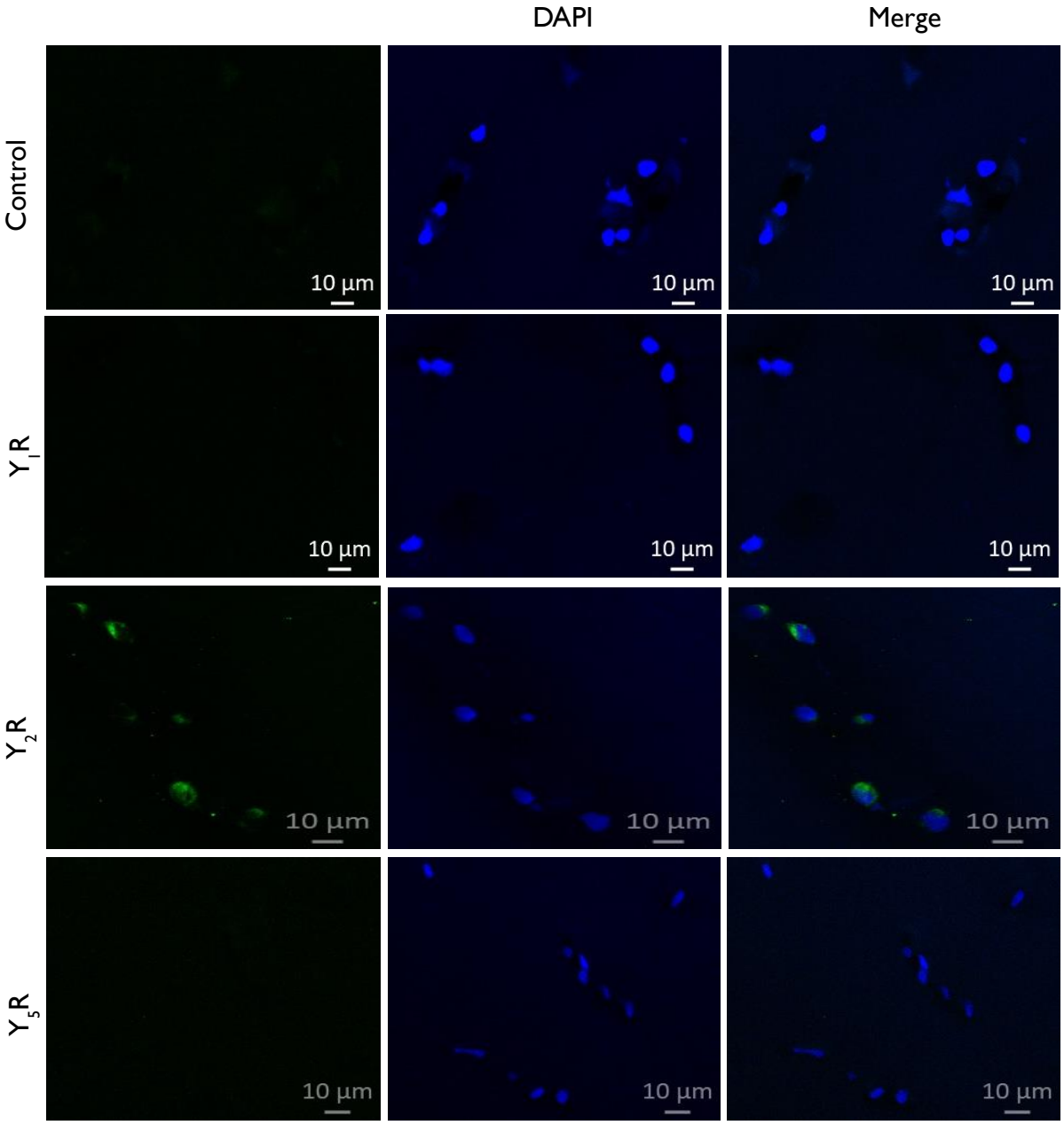
Furthermore, age doesn't seem to affect the presence of NPY receptors since the age range of healthy and OA cartilage donors, both in males and females and between genders, doesn't seem substantially different (table 1). Indeed, no differences in any of the NPY receptor subtypes were observed between a 33-year old female (G0) and the other two females aged 65 (G4) and 68 (G0) years. In men, the presence of the Y_1 receptor was associated with the disease state, but such difference doesn't seem associated with age since the age range of the donors (60-70 versus 55-75) was similar in minimally damaged (G1) and OA (G4) cartilage, respectively. Nonetheless, further studies including a larger number of samples from individuals of a wider age range will help confirm these observations.

Figures 5 C-F show details of the immunoreactivity for each NPY receptor subtype in each experimental group.

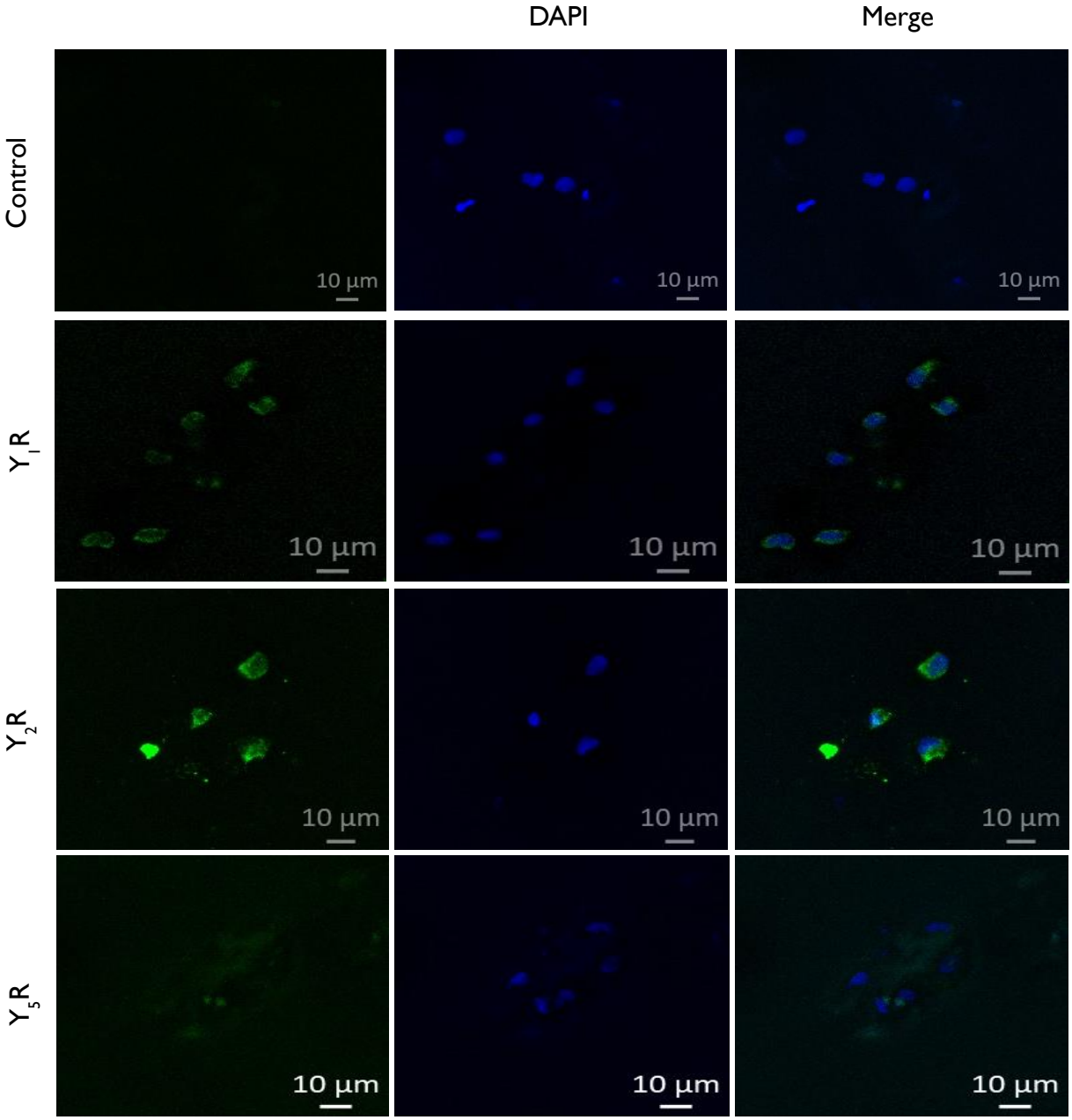

A
Healthy male
OA male
Control
Y1R
Y2R
Y5R
50 µm


B
Healthy female
OA female
Control
Y1R
Y2R
Y5R
50 µm

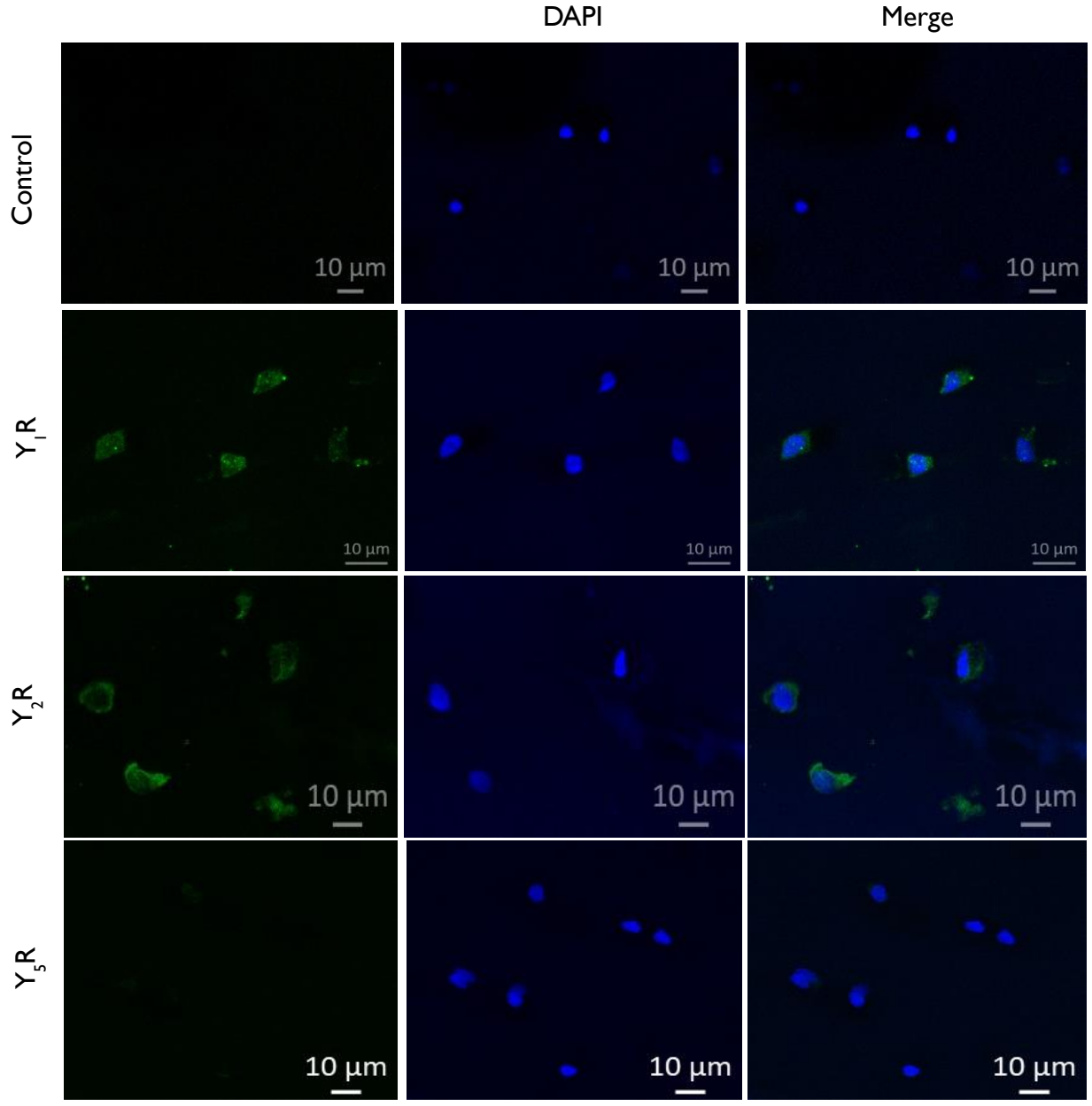
C: Healthy male



D: OA male



E: Healthy female



F: OA female

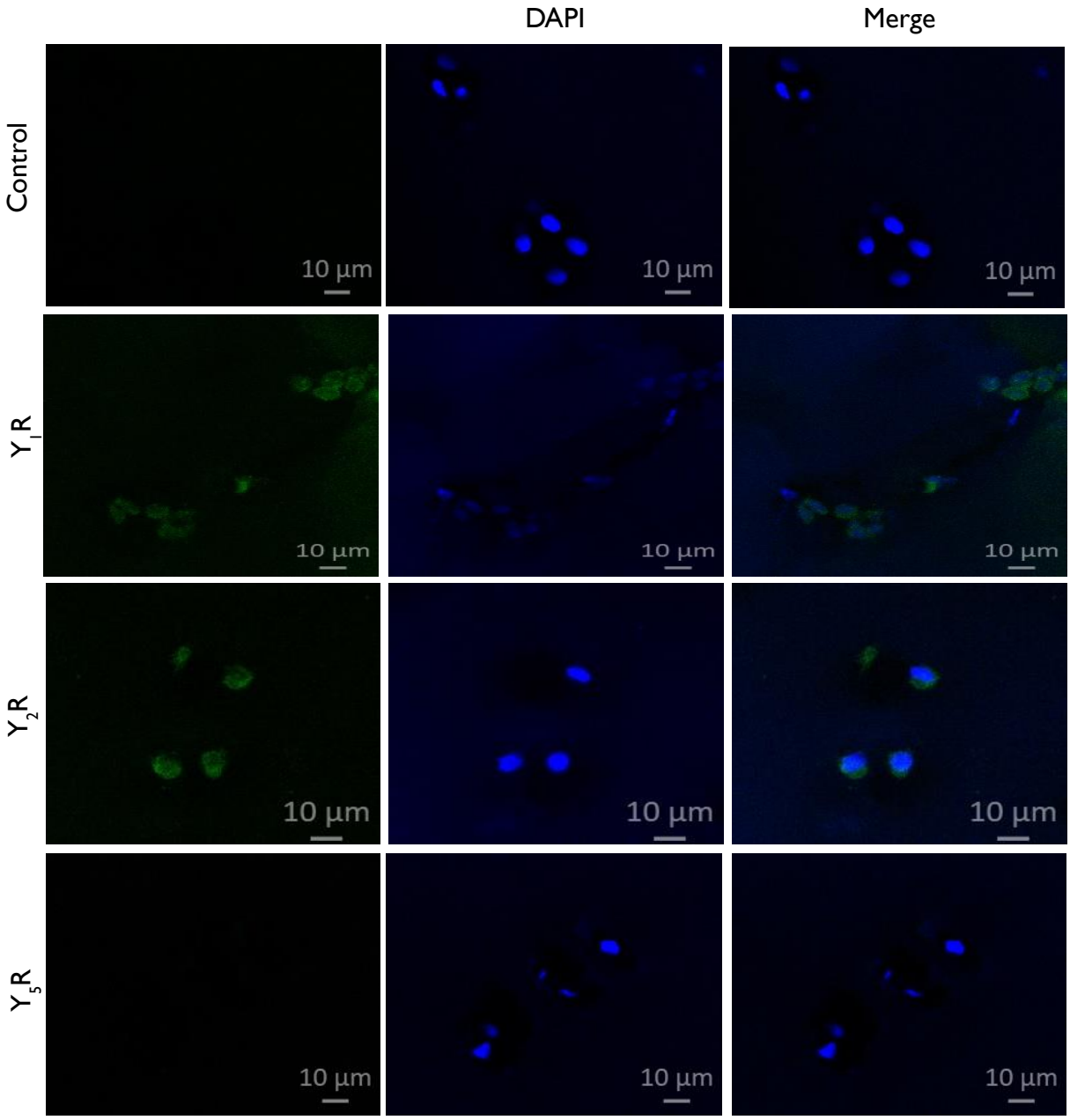


Figure 5. Immunoreactivity for NPY receptors, Y_1, Y_2 and Y_5, in healthy and OA human cartilage is determined by gender and by disease state in men. Cartilage sections were immunolabelled for Y_1, Y_2 or Y_5 NPY receptors (green). Nuclei were stained with DAPI (blue). Negative control assays were performed in the absence of the primary antibodies. A-B: Representative images of the immunoreactivity for each NPY receptor subtype and nuclei staining in each experimental group. Scale bar: 50 µm. C-F: Details of the immunoreactivity for each NPY receptor subtype in each experimental group. Scale bar: 10 µm.

3.3 Effect of NPY and ChQ on C28/I2 cell viability

To determine whether NPY and /or ChQ induced toxic effects in C28/I2 chondrocytic cells, cell viability under distinct concentrations of both compounds was assessed by the Resazurin reduction assay. The results presented in Fig. 6 show that, under the experimental conditions used, none of the test compounds affected cell viability in comparison with control untreated cells.

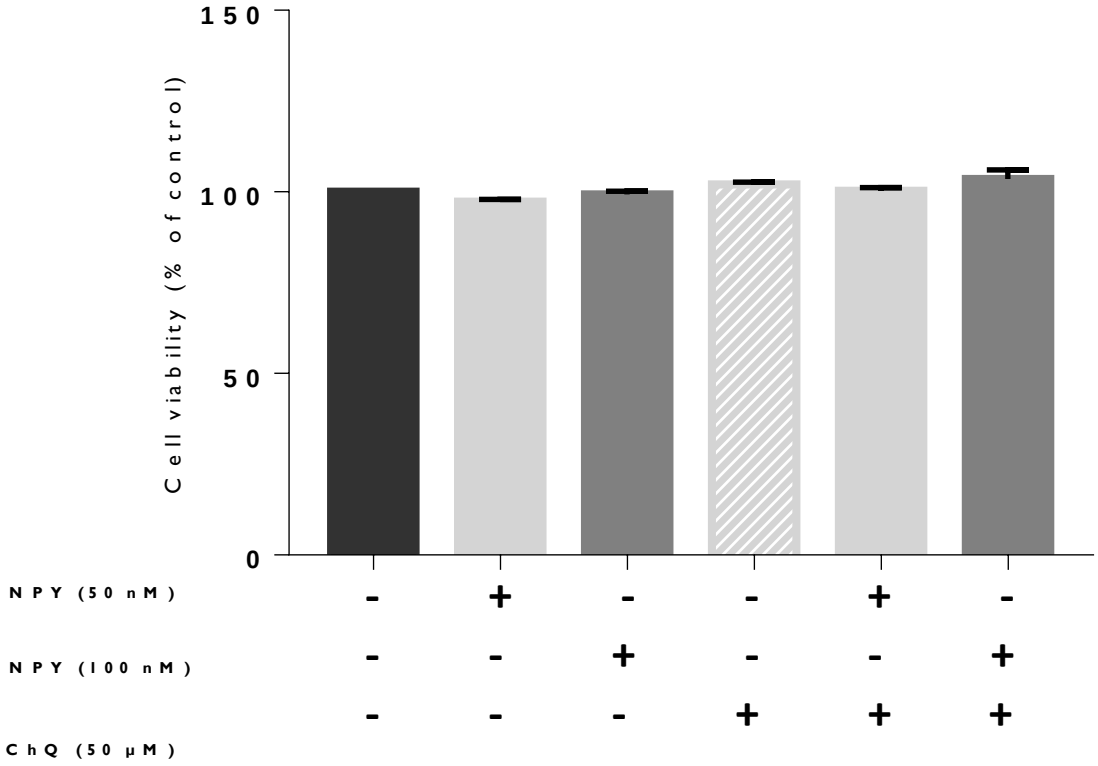
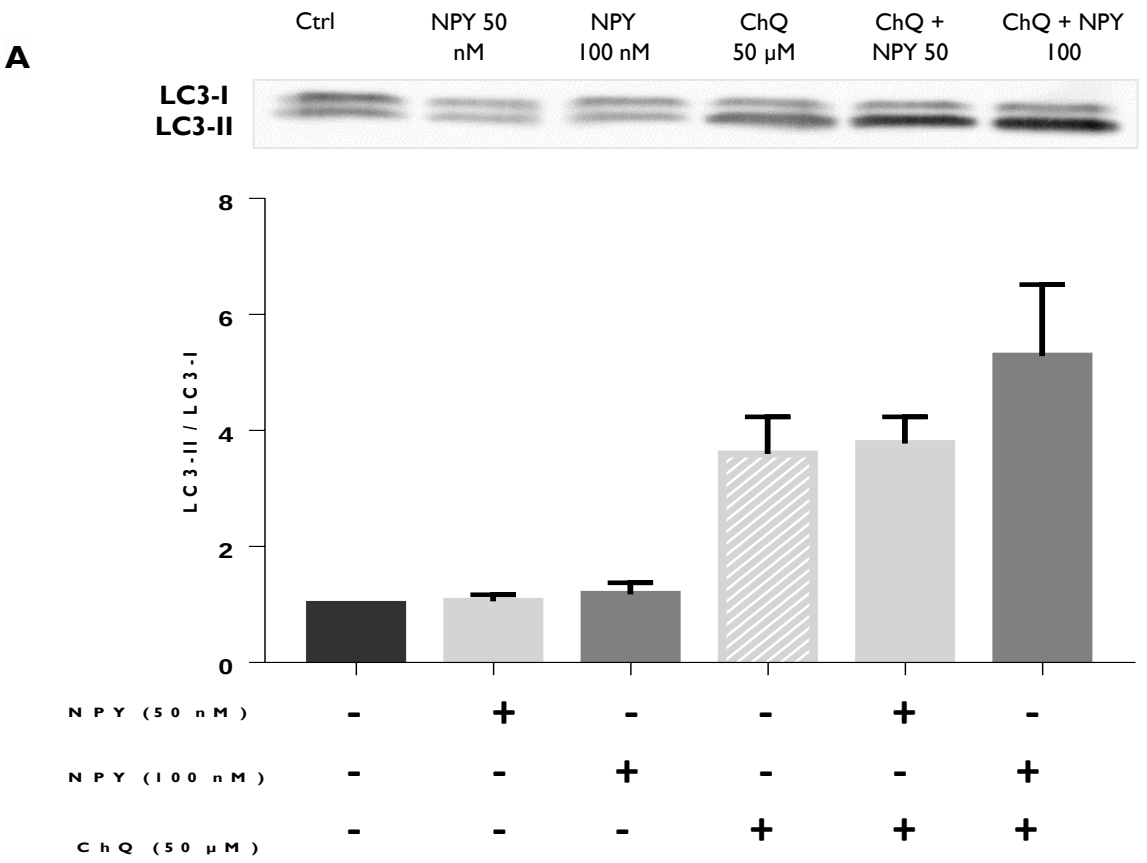
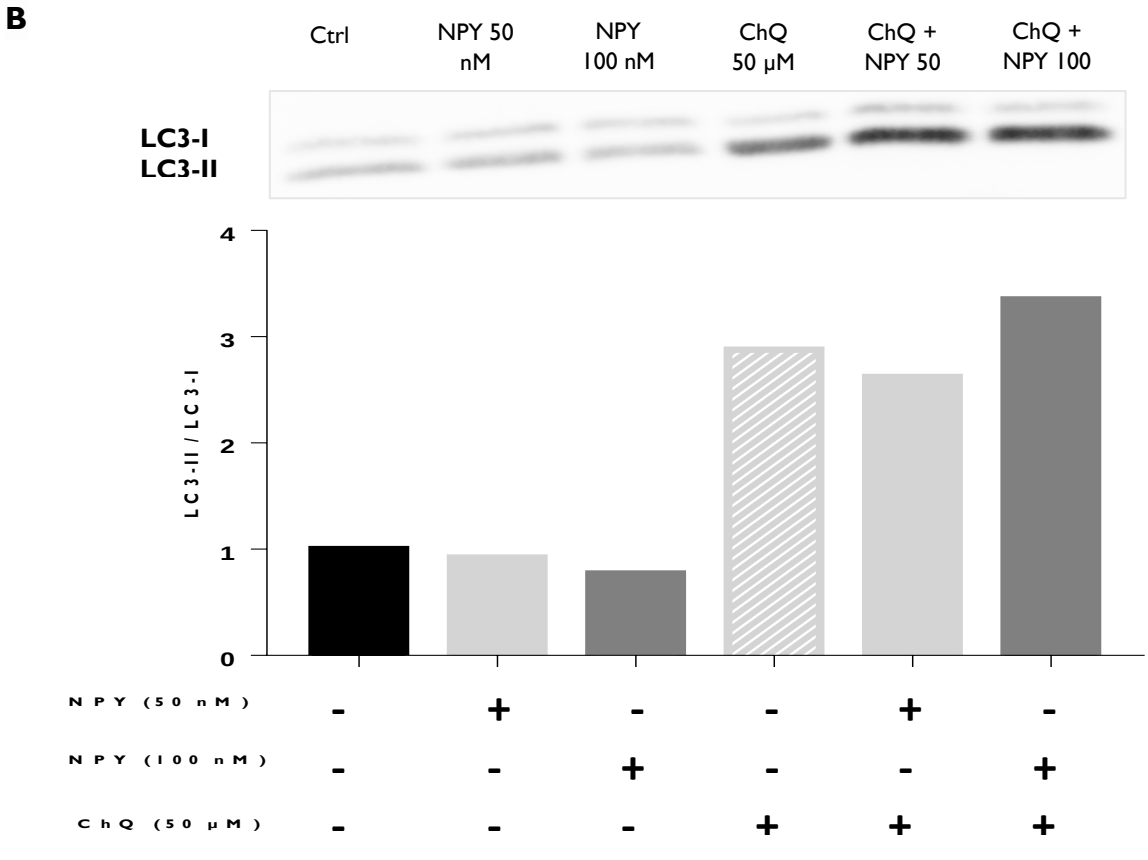


Figure 6. Effect of NPY and ChQ on cell viability. C28/I2 chondrocytic cells were pretreated with or without ChQ (50 μM, 30 minutes), followed by stimulation with the indicated concentrations of NPY for 6h. Cell viability was assessed by the Resazurin reduction assay, as described in "Materials and Methods", and the results are expressed in percentage relative to untreated cells (control). Each column represents the mean ± SEM of, at least, 3 experiments.

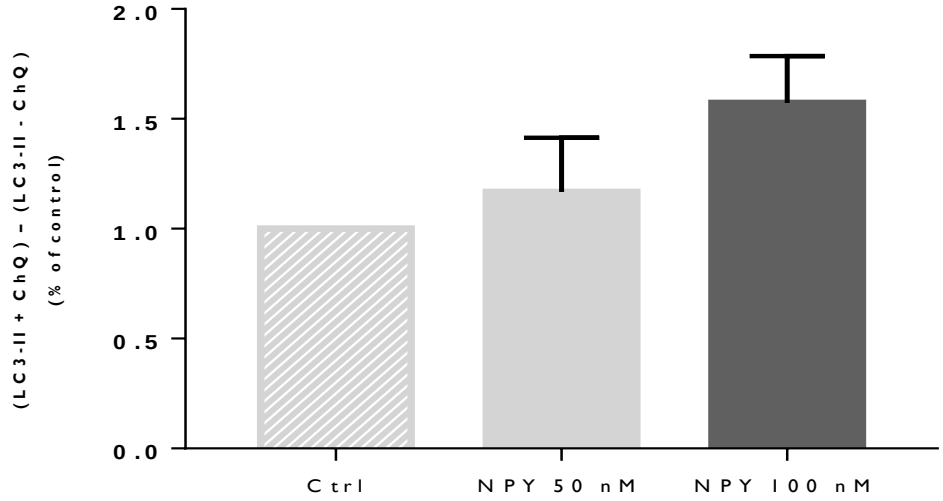
3.4 Role of NPY in modulating autophagy in C28/I2 chondrocytic cells

Since we found NPY receptors in human chondrocytes, we hypothesized that those receptors are likely functional and allow NPY to stimulate autophagy in chondrocytes. To assess autophagy, we evaluated the protein levels of LC3-I and II, as upon autophagy activation, LC3-I is converted into LC3-II through conjugation with phosphatidylethanolamine and adheres to the membrane of the phagophore to start the elongation process that originates the autophagossome. Thus, conversion of LC3-I into LC3-II, assessed as the ratio between the levels of LC3-II and I, is regarded as a marker of autophagy activation [Mizushima et al., 2010; Klionsky et al., 2016]. Western blot analysis indicated that a 6-hour stimulation with NPY 50 nM or 100 nM apparently decreased LC3-II and LC3-I protein levels in comparison with untreated chondrocytes (Fig. 7 A), suggesting either an increase of the autophagic flux in those conditions and thus an increased synthesis and clearance of LC3-II or a decreased synthesis of LC3. To elucidate this question, we measured LC3-I and II levels in cells treated with NPY in presence of the lysosomal protein degradation inhibitor, ChQ, which blocks LC3-II degradation. In these conditions, an increase in LC3-II levels relative to cells treated with ChQ alone indicates an increase in the autophagic delivery of LC3-II to the lysosome thus reflecting autophagy activation. Nonetheless, such increase can be better appreciated by calculating the autophagic flux. For this, LC3-II levels in the absence of ChQ are subtracted from those obtained in its presence. Figures 7 C and D show the results of the net autophagic flux in C28/I2 cells treated with the indicated concentrations of NPY in the presence or absence of ChQ for 6h and 24h, respectively. The results obtained show a mean increase of the net flux at 6 hour stimulation with 50 or 100 nM NPY which did not reach statistical significance, although the increase was evident in every experiment performed, as can be observed in the representative image shown in figures 7 A and C. However, inter-assay variability as to the relative increase was substantial (SEM=0,2469 and 0,2133 for 50 and 100 nM NPY, respectively) and likely precluded statistically significant differences to be disclosed. Moreover, treatment with NPY for 24h, especially at the 100 nM concentration, also seems to increase the autophagic flux, even though no conclusions can be taken as these are preliminary results (n=1) that did not allow statistical analysis to be performed (Figures 7 B and D).


A
Ctrl
NPY 50 nM
NPY 100 nM
ChQ 50 µM
ChQ + NPY 50
ChQ + NPY 100
LC3-I
LC3-II
LC3-II / LC3-I
0
2
4
6
8
NPY (50 nM)
NPY (100 nM)
ChQ (50 µM)


B
Ctrl
NPY 50 nM
NPY 100 nM
ChQ 50 µM
ChQ + NPY 50
ChQ + NPY 100
LC3-I
LC3-II
LC3-II / LC3-I
0
1
2
3
4
NPY (50 nM)
NPY (100 nM)
ChQ (50 µM)

C



D

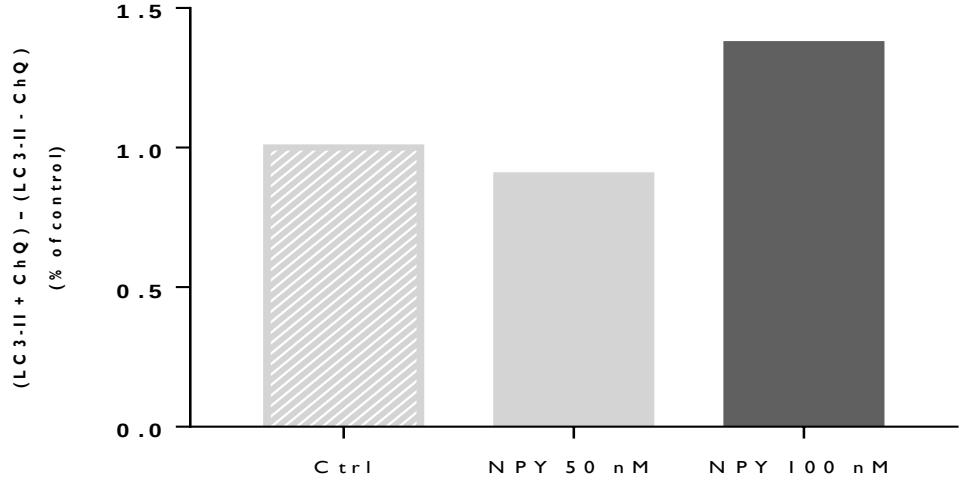


Figure 7. Modulation of autophagy by NPY in C28/I2 chondrocytic cells. C28/I2 chondrocytic cells were treated with the indicated concentrations of NPY for 6 h (A) or 24 h (B) in the absence or presence of 50 μM ChQ. LC3 expression was measured by Western Blot (A and B). The net LC3-II flux after 6 (C) and 24 h (D) treatments was determined by subtracting the volume of the LC3-II band in samples not treated with ChQ (LC3-II – ChQ) from the volume of the corresponding samples treated with ChQ (LC3-II + ChQ). The control was set up by subtracting the volume of LC3-II in untreated cells (LC3-II - ChQ) from that obtained in cells treated with ChQ (LC3-II + ChQ). In A and C, each column represents the mean ± SEM of 3 independent experiments; panels B and D show the results of a single experiment.

CHAPTER IV

DISCUSSION

4.1 Discussion

OA is the most common muskuloskeletal condition that affects the world population, resulting in a substantially impaired quality of life in affected individuals and raising sizable costs to health care systems [Grässel & Muschter, 2017]. Since OA progression has been linked to aging [Johnson & Hunter, 2014] and human longevity is currently increasing, it is foreseeable that the social and economic burden of OA will grow significantly in the next decades [Rannou et al., 2014]. There is presently no curative therapy for OA, which emphasizes the need to further understand the mechanisms underlying the development of this synovial joint disease.

As previously mentioned under "Aims", the main goal of this study was to contribute to understand the role of NPY in synovial joints and, particularly, in articular cartilage homeostasis. In this sense, we first aimed at determining whether and which NPY receptor subtypes are present in human chondrocytes. We observed that all the three major NPY receptor subtypes [Y_1, Y_2 and Y_5] were present in the human chondrocyte cell line, C28/I2. This initial identification of the immunoreactivity of each NPY receptor subtype in a cell line was essential, not only to predict their potential immunoreactivity in native human articular cartilage samples, but also to determine the conditions in which this cell line can be used in future studies regarding the role of NPY and its receptors in chondrocyte health and disease.

Afterwards, we investigated the immunoreactivity of these NPY receptor subtypes in articular cartilage samples from male and female healthy donors and osteoarthritic patients. As shown in Figure 5, we observed that receptor immunoreactivity was determined by the disease state and the gender of the donors, but not by their age. To our knowledge, this is the first study identifying NPY receptors in articular cartilage, human or from other species. Importantly, NPY had already been detected in the synovial fluid of knee OA patients and healthy controls and its levels were found to be positively correlated with increasing pain scores in knee OA patients [Wang et al., 2014]. Besides, it was observed that the expression of neuronal injury markers, including NPY, was upregulated in primary afferent neurons innervating joints in a collagenase-induced OA model in rats [Adães et al., 2015]. Together, these studies suggest a role for NPY as a regulator of nociception in OA, but, so far, no evidence existed as to other non-neuronal sources and targets of NPY in joints. The results presented in this study, although requiring further confirmation in a larger cohort, show the presence of NPY receptors in human chondrocytes and their differential expression as a function of gender and disease state, strongly suggesting that non-neuronal cells and tissues of the joints, namely chondrocytes, are relevant as NPY targets.

Nonetheless, these results per se do not provide evidence as to the functional status of those receptors or their role in modulating chondrocyte functions in health and disease. In this regard, the increased concentrations of NPY in the synovial fluid of OA patients [Wang et al., 2014] may not only be associated with pain, but can also reflect either an attempt of joint tissues to counteract OA progression or another mechanism that contributes to joint tissue damage and OA progression. Thus and considering previous studies that link decreased basal autophagic ativity with aging [Mariño & López-Otín, 2008] and OA [Shapiro et al., 2014] as well as the stimulatory effect of NPY on autophagy in rodent hypothalamus with its potential anti-aging effect [Aveleira et al., 2015], we hypothesized that NPY may modulate autophagy in chondrocytes. The results obtained, although requiring further confirmation in primary human chondrocytes, suggest that NPY increases the autophagic flux [Figure 7] in conditions that do not affect cell viability [Fig. 6]. These results indicate that NPY receptors are functional in C28/I2 chondrocytic cells and that NPY may have a protective role in chondrocytes by activating autophagy. It remains to be determined whether this effect is present in primary human chondrocytes and the receptor subtype(s) involved. Given the gender- and disease state-associated expression of the NPY receptor subtypes observed in human cartilage samples, it is conceivable that the ability of NPY to induce autophagy in the human chondrocytic cells, C28/I2, is differentially maintained in male and female, healthy and OA chondrocytes. Thus, future work is required to understand the role of NPY and its receptors in modulating male and female chondrocyte autophagy and how it affects their homeostasis in health and disease conditions.

4.2 Concluding remarks and future perspectives

In summary, this study shows that NPY receptors are present in human chondrocytic cells and in human chondrocytes *in situ* in the articular cartilage. Moreover, the expression of the NPY receptor subtypes seems to be determined by gender and, in males, also by the disease state. Those receptors seem to be functional in chondrocytic cells and whether and which are functional in primary human chondrocytes remains to be elucidated. Future work will address this issue and, using pharmacological and molecular tools, namely selective antagonists and siRNAs, will also elucidate the role of each NPY receptor subtype in modulating autophagy in male and female, healthy and OA human chondrocytes. These studies will provide the basis for further studies aimed at establishing the role of NPY and its receptors in cartilage and other joint tissues health and in OA development and progression. Such studies may ultimately

lead to the development of novel comprehensive therapeutic strategies with captivating prospects for the treatment of this disabling disease.

REFERENCES

- ADÃES S *et al.* (2015). Injury of primary afferent neurons may contribute to osteoarthritis induced pain: an experimental study using the collagenase model in rats. **Osteoarthritis and Cartilage**, 23 (6), 914–924.
- ADAMUS M A & DABROWSKI Z J (2001). Effect of the neuropeptide substance P on the rat bone marrow-derived osteogenic cells in vitro. **Journal of Cellular Biochemistry**, 81 (3), 499–506.
- AHMADIAN M et al. (2013). PPARγ signaling and metabolism: the good, the bad and the future. **Nature Medicine**, 99(5), 557–566.
- AIGNER T *et al.* (2006). Osteoarthritis: Pathobiology - targets and ways for therapeutic intervention. **Advanced Drug Delivery Reviews**, 58 (2), 128–149.
- AKKIRAJU H & NOHE A (2015). Role of Chondrocytes in Cartilage Formation, Progression of Osteoarthritis and Cartilage Regeneration. **Journal of Developmental Biology**, 3 (4), 177–192.
- ANSAR AHMED S, GOGAL R M, WALSH J E. (1994). A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay. **Journal of Immunological Methods**. 170 (2), 211–224.
- ARCHER C W, & FRANCIS-WEST P. (2003). The chondrocyte. **The International Journal of Biochemistry & Cell Biology**, 35 (4), 401-404.
- AVELEIRA C A *et al.* (2015). Neuropeptide Y stimulates autophagy in hypothalamic neurons. **Proceedings of the National Academy of Sciences**. 112 (13), E1642–E1651.
- BALASUBRAMANIAM A. (1997). Neuropeptide Y Family of Hormones: Receptor Subtypes and Antagonists. **Peptides.** 18 (3), 445–457.
- BALDOCK P A *et al.* (2002). Hypothalamic Y2 receptors regulate bone formation. **Journal of Clinical Investigation**, 109 (7), 915–921.
- BARABAN S C (1998). Neuropeptide Y and limbic seizures. **Rev. Neurosci**., 9, pp. 117–128.
- BARISH G D, NARKAR V A & EVANS R M (2006). PPARδ: a dagger in the heart of the metabolic syndrome. **J Clin Invest.** 116:590–597.
- BECK B & POURIÉ G (2013). Ghrelin, neuropeptide Y, and other feeding-regulatory peptides active in the hippocampus: role in learning and memory. **Nutr Rev** 71 (8): 541–561.
- BERENBAUM F (2013). Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). **Osteoarthritis and Cartilage**, 21(1), 16–21.

- BERENBAUM F, & MENG Q J (2016). The brain-joint axis in osteoarthritis: nerves, circadian clocks and beyond. **Nat Rev Rheumatol**, 12 (9), 508-516.
- BERGAMINI E. (2006). Autophagy: A cell repair mechanism that retards ageing and age-associated diseases and can be intensified pharmacologically. **Molecular Aspects of Medicine**, *27* (5–6), 403–410.
- BIJLSMA J, BERENBAUM F, & Lafeber F (2011). Osteoarthritis: an update with relevance for clinical practice. **The Lancet**, 377 (9783), 2115-2126.
- BJURHOLM A *et al.* (1988). Neuropeptide Y-, tyrosine hydroxylaseand vasoactive intestinal polypeptide-immunoreactive nerves in bone and surrounding tissues. **J Auton Nerv Syst.** 25:11-125.
- BJURHOLM A *et al.* (2017). Neuropeptide Y-, tyrosine hydroxylase- and vasoactive intestinal polypeptide-immunoreactive nerves in bone and surrounding tissues. **Journal of the Autonomic Nervous System**, 25 (2), 119–125.
- BOTELHO M & CAVADAS C (2015). Neuropeptide Y: An Anti-Aging Player? **Trends in Neurosciences**, 38 (11), 701–711.
- BOWLER K E *et al. (*2013). Evidence for anti-inflammatory and putative analgesic effects of a monoclonal antibody to calcitonin gene-related peptide. **Neuroscience**, 228, 271–282.
- BRANDT K D, DIEPPE P, & RADIN E. (2009). Etiopathogenesis of Osteoarthritis. **Medical Clinics of North America**, 93 (1), 1-24.
- BRIGHTON C T, HEPPENSTALL R B. (1971). Oxygen tension in zones of the epiphyseal plate, the metaphysis and diaphysis. An in vitro and in vivo study in rats and rabbits. **J Bone Joint Surg Am**; 53:719-28.
- BROTHERS S P & WAHLESTEDT C (2010). Therapeutic potential of neuropeptide Y (NPY) receptor ligands. **EMBO Molecular Medicine**, 2 (11), 429–439.
- BUCKWALTER J, MANKIN H J, & GRODZINSKY A J. (2005). Articular cartilage and osteoarthritis. **Instructional Course Lectures**. 54:465-80.
- CARAMÉS B *et al.* (2010). Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. **Arthritis and Rheumatism**, 62 (3), 791–801.
- CARAMÉS B *et al*. (2015). The relationship of autophagy defects to cartilage damage during joint aging in a mouse model. **Arthritis & Rheumatology**. 67(6), 1568–76.
- CHEN D *et al.* (2017). Osteoarthritis: toward a comprehensive understanding of pathological mechanism. **Bone Research**, 5, 16044–.

- CHEN Y & KLIONSKY D J. (2011). The regulation of autophagy - unanswered questions. **Journal of Cell Science**, 124 (2), 161–170.
- CUERVO A M & DICE J F (2000). Age-related Decline in Chaperone-mediated Autophagy. **Journal of Biological Chemistry**, 275 (40), 31505–31513.
- CUERVO A M. (2008). Autophagy and aging: keeping that old broom working. **Trends in Genetics**, 24 (12), 604–612.
- CUESTA M C *et al.* (2002). Substance P and calcitonin gene-related peptide increase IL-1β, IL-6 and TNFα secretion from human peripheral blood mononuclear cells. **Neurochemistry International**, 40 (4), 301–306.
- DECRESSAC M & BARKER R A (2012). Neuropeptide Y and its role in CNS disease and repair. **Exp Neurol** 238 (2):265–272.
- DELGADO M *et al.* (2001). Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. **Nat Med**, 7 (5), 563–568.
- DICKSON L & FINLAYSON K (2009). VPAC and PAC receptors: From ligands to function. **Pharmacology & Therapeutics**, 121 (3), 294–316.
- DONG T *et al.* (2015). Calcitonin gene-related peptide can be selected as a predictive biomarker on progression and prognosis of knee osteoarthritis. **International Orthopaedics**, 39 (6), 1237–1243.
- DRIESSLER F & BALDOCK P A (2010). Hypothalamic regulation of bone. **Journal of Molecular Endocrinology**, 45 (4), 175–181.
- EDOFF K, GRENEGARD M & HILDEBRAND C (2000). Retrograde tracing and neuropeptide immunohistochemistry of sensory neurones projecting to the cartilaginous distal femoral epiphysis of young rats. **Cell Tissue Res**., 299, 193–200.
- EKBLAD E *et al.* (1984). Neuropeptide Y co-exists and co-operates with noradrenaline in perivascular nerve fibers. **Regulatory Peptides**, 8(3), 225–235.
- EKSTRAND A J *et al.* (2003). Deletion of neuropeptide Y (NPY) 2 receptor in mice results in blockage of NPY-induced angiogenesis and delayed wound healing. **Proceedings of the National Academy of Sciences**, 100 (10), 6033–6038.
- ELEFTERIOU F (2005). Neuronal signaling and the regulation of bone remodeling. **Cell. Mol. Life Sci**. 62, 2339–2349.
- FELLOWS C *et al.* (2016). Adipose, Bone Marrow and Synovial Joint-Derived Mesenchymal Stem Cells for Cartilage Repair, **Front. Genet**, 7:213 1-20.

- FELSON D T (2009). Developments in the clinical understanding of osteoarthritis. **Arthritis Research & Therapy**, 11 (1), 203.
- FELSON D T *et al.* (2016). Synovitis and the risk of knee osteoarthritis: the MOST Study. **Osteoarthritis and Cartilage**, 24 (3), 458-464.
- GAJDA M, ADRIAENSEN D, CICHOCKI T. (2000). Development of the innervation of long bones: expression of the growth-associated protein 43. **Folia Histochem Cytobiol** 38: 103–10.
- GLYN-JONES S *et al. (*2015). Osteoarthritis. **The Lancet**, 386 (9991), 376-387.
- GOLDRING M B (2000). The role of the chondrocyte in osteoarthritis. **Arthritis and Rheumatism**, 43: 1916–1926.
- GOLDRING M B, & MARCU K B (2009). Cartilage homeostasis in health and rheumatic diseases. **Arthritis Research & Therapy,** 11 (3), 224.
- GOTO T (1998). Light- and electron-microscopic study of the distribution of axons containing substance P and the localization of neurokinin-1 receptor in bone. **Cell and Tissue Research**, 293 (1), 87–93.
- GOTO T *et al.* (2007). Substance P stimulates late-stage rat osteoblastic bone formation through neurokinin-1 receptors. **Neuropeptides**, 41 (1), 25–31.
- GRÄSSEL S & MUSCHTER D (2017). Peripheral Nerve Fibers and Their Neurotransmitters in Osteoarthritis Pathology. **International Journal of Molecular Sciences**, 18 (5), 931.
- GRÄSSEL S G (2014). The role of peripheral nerve fibers and their neurotransmitters in cartilage and bone physiology and pathophysiology. **Arthritis Research & Therapy**, 16 (6), 485.
- GRÄSSEL S, STRAUB R H & JENEI-LANZL Z (2016). The sensory and sympathetic nervous system in cartilage physiology and pathophysiology. **In Cartilage Vol. 2, Pathophysiology**; Grässel, S., Aszódi, A., Eds.; Springer: New York, NY, USA.
- GRUENEWALD D A *et al.* (1994) Age-related decrease in neuropeptide-Y gene expression in the arcuate nucleus of the male rat brain is independent of testicular feedback. **Endocrinology** 134 (6):2383–2389.
- GUTIÉRREZ-CAÑAS I *et al.* (2006). VIP down-regulates TLR4 expression and TLR4-mediated chemokine production in human rheumatoid synovial fibroblasts. **Rheumatology**, 45 (5), 527–532.
- HABERLAND M *et al.* (2001). Brain and bone: central regulation of bone mass. A new paradigm in skeletal biology. **J Bone Joint Surg Am**; 83-A: 1871–6.

- HARA-IRIE F, AMIZUKA N & OZAWA H (1996). Immunohistochemical and ultrastructural localization of CGRP-positive nerve fibers at the epiphyseal trabecules facing the growth plate of rat femurs. **Bone**, 18 (1), 29–39.
- HARRISON S & GEPPETTI P (2001). Substance P. **The International Journal of Biochemistry & Cell Biology**, *33* (6), 555–576.
- HARTUNG H P & TOYKA K V. (1983). Activation of macrophages by substance P: induction of oxidative burst and thromboxane release. **Eur J Pharmacol.** May 6;89 (3-4):301-5.
- HEIN L. (2006). Adrenoceptors and signal transduction in neurons. **Cell and Tissue Research.** 326 (2), 541–551.
- HENNING R J & SAWMILLER D R (2001) Vasoactive intestinal peptide: cardiovascular effects. **Cardiovasc Res.**; 49(1):27–37.
- HIGUCHI H, YANG H Y, COSTA E (1988) Age-related bidirectional changes in neuropeptide Y peptides in rat adrenal glands,brain, and blood. **J Neurochem**. 50 (6):1879–1886.
- HILL E L & ELDE R (1991). Distribution of CGRP-, VIP-, D beta H-, SP-, and NPY-immunoreactive nerves in the periosteum of the rat. **Cell and Tissue Research**, 264 (3), 469–80.
- HIRSCH D & ZUKOWSKA Z (2012). NPY and Stress 30 Years Later: The Peripheral View. **Cellular and Molecular Neurobiology**, 32 (5), 645–659.
- HOUARD X, GOLDRING M B & BERENBAUM F (2013). Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. **Current Rheumatology Reports**, 15 (11), 375.
- HUA X Y *et al.* (1991). The antinociceptive effects of spinally administered neuropeptide Y in the rat: systematic studies on structure-activity relationship. **The Journal of Pharmacology and Experimental Therapeutics**, 258 (1), 243–8.
- HUKKANEN M *et al.* (1992). Distribution of nerve endings and sensory neuropeptides in rat synovium, meniscus and bone. **Int J Tissue React**.;14 (1):1-10.
- HUKKANEN M *et al.* (1995). Effect of sciatic nerve section on neural in- growth into the rat tibial fracture callus. **Clin Orthop**, 311: 247-57.

- HUNZIKER E B. (2002). Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. **Osteoarthritis and Cartilage.** 10 (6), 432–463.
- IGWE J C *et al.* (2009). Neuropeptide Y is expressed by osteocytes and can inhibit osteoblastic activity. **Journal of Cellular Biochemistry**, 108 (3), 621–630.
- INOUE H *et al.* (2001). Production of neuropeptide substance P by synovial fibroblasts from patients with rheumatoid arthritis and osteoarthritis. **Neurosci. Lett.**, 303, 149–152.
- JEFFERY AK *et al.* (1991). Three-dimensional collagen architecture in bovine articular cartilage. **J Bone Joint Surg Br.** 73-B:795-801.
- JENEI-LANZL Z *et al.* (2014). Norepinephrine Inhibition of Mesenchymal Stem Cell and Chondrogenic Progenitor Cell Chondrogenesis and Acceleration of Chondrogenic Hypertrophy. **Arthritis & Rheumatology**, 66 (9), 2472–2481.
- JIANG W *et al.* (2012). Expression of synovial fluid and articular cartilage VIP in human osteoarthritic knee: A new indicator of disease severity? **Clinical Biochemistry**, 45 (18), 1607–1612.
- JOHNSON V L & HUNTER D J (2014). The epidemiology of osteoarthritis. **Best Practice & Research Clinical Rheumatology**, 28 (1), 5–15.
- KABEYA Y *et al.* (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. **The EMBO Journal.** 19 (21), 5720–8.
- KAPOOR M *et al.* (2011). Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. **Nature Reviews Rheumatology**, 7 (1), 33–42.
- KASSEBAUM N et al. (2017). Global, regional, and national disability-adjusted life-years (DALYs) for 315 diseases and injuries and healthy life expectancy (HALE), 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. **The Lancet**, 388 (10053), 1603–1658.
- KAUSHIK S *et al.* (2012) Loss of autophagy in hypothalamic POMC neurons impairs lipolysis. **EMBO Rep**. 13 (3):258–265.
- KELLGREN J H & LAWRENCE J S. (1957). Radiological assessment of osteo-arthrosis. **Annals of the Rheumatic Diseases.** 16 (4), 494–502.
- KLIONSKY D J *et al.* (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). **Autophagy.** 12:1-222.
- KONTTINEN Y T *et al.* (2012). Editorial: Osteoarthritis as an autoinflammatory disease caused by chondrocyte-mediated inflammatory responses. **Arthritis & Rheumatism**, 64 (3), 613-616.

- KUO L E *et al.* (2007). Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. **Nature Medicine**, 13 (7), 803–811.
- LAI L P & MITCHELL J (2008). β2-adrenergic receptors expressed on murine chondrocytes stimulate cellular growth and inhibit the expression of Indian hedgehog and collagen type X. **Journal of Cellular Biochemistry**, 104 (2), 545–553.
- LANE N E, *et al*. (2011). OARSI-FDA initiative: Defining the disease state of osteoarthritis. **Osteoarthritis and Cartilage**, *19* (5), 478-482.
- LEVINE B & KROEMER G. (2008). Autophagy in the Pathogenesis of Disease. **Cell**, 132 (1), 27–42.
- LI R *et al.* (2011). Endothelial Progenitor Cells for Fracture Healing: A Microcomputed Tomography and Biomechanical Analysis. **Journal of Orthopaedic Trauma**, 25 (8), 467–471.
- LI Y S, *et al*. Autophagy in osteoarthritis. ***Joint Bone Spine***. 2016;83(2):143–148.
- LIN S *et al.* (2005). Compensatory changes in [125I]-PYY binding in Y receptor knockout mice suggest the potential existence of further Y receptor(s). **Neuropeptides**, 39 (1), 21–28.
- LIN S, BOEY D & HERZOG H (2004). NPY and Y receptors: lessons from transgenic and knockout models. **Neuropeptides**, 38 (4), 189–200.
- LOESER R F (2006). Molecular Mechanisms of Cartilage Destruction: Mechanics, Inflammatory Mediators, and Aging Collide. **Arthritis and Rheumatism**, 54 (5), 1357-1360.
- LOESER R F *et al.* (2012). Osteoarthritis: A disease of the joint as an organ. **Arthritis & Rheumatism**, 64 (6), 1697-1707.
- LOESER R F, COLLINS J A & DIEKMAN B O (2016). Ageing and the pathogenesis of osteoarthritis. **Nature Reviews Rheumatology**, 12 (7), 412–420.
- LOESER R F. (2009). Aging and Osteoarthritis: The Role of Chondrocyte Senescence and Aging Changes in the Cartilage Matrix. **Osteoarthritis and Cartilage / OARS, Osteoarthritis Research Society**. 17(8), 971-979.
- LORENZ J *et al.* (2016). Norepinephrine modulates osteoarthritic chondrocyte metabolism and inflammatory responses. **Osteoarthritis and Cartilage**, 24 (2), 325–334.
- LOTZ M K & CARAMÉS B. (2011). Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. **Nature Reviews Rheumatology**. 7 (10), 579–87.

- LUNDBERG P *et al.* (2007). Greater bone formation of Y2 knockout mice is associated with increased osteoprogenitor numbers and altered Y1 receptor expression. **The Journal of Biological Chemistry**, 282 (26), 19082–91.
- MA C *et al.* (2015). The Role of PPARγ in Advanced Glycation End Products-Induced Inflammatory Response in Human Chondrocytes. **PLOS ONE**, 10 (5), e0125776.
- MADSEN J E et al. (1998). Fracture healing and callus innervation after peripheral nerve resection in rats. **Clin Orthop Relat Res.** Jun; (351):230-40.
- MAESTRONI G J (2000). Neurohormones and catecholamines as functional components of the bone marrow microenvironment. **Annals of the New York Academy of Science**s, 917, 29–37.
- MARIÑO G & LÓPEZ-OTÍN C. (2008). Autophagy and aging: New lessons from progeroid mice. **Autophagy**. 4 (6), 807–809.
- MARTINET W, ROTH L & DE MEYER G R Y (2017). Standard Immunohistochemical Assays to Assess Autophagy in Mammalian Tissue. **Cells**, 6 (3), 17.
- MASLIUKOV P M, *et al.* (2016). Development of neuropeptide Y-mediated heart innervation in rats. **Neuropeptides**. 55, 47–54.
- MCDOUGALL J J, WATKINS L & LI Z (2006). Vasoactive intestinal peptide (VIP) is a modulator of joint pain in a rat model of osteoarthritis. **Pain**, 123 (1), 98–105.
- MICHEL M C *et al.* (1998). International Union of Pharmacology XVI. Recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. ***Pharmacol Rev* 50:** 143–150.
- MILLER L E *et al.* (2000). The loss of sympathetic nerve fibers in the synovial tissue of patients with rheumatoid arthritis is accompanied by increased norepinephrine release from synovial macrophages. **The FASEB Journal**, 14 (13), 2097–2107.
- MITCHELL J *et al.* (2011). β2-Adrenergic receptors inhibit the expression of collagen type II in growth plate chondrocytes by stimulating the AP-1 factor Jun-B. **AJP: Endocrinology and Metabolism**, 300 (4), E633–E639.
- MIZUSHIMA N *et al.* (2008). Autophagy fights disease through cellular self-digestion. **Nature**, 451 (7182), 1069–1075.
- MIZUSHIMA N, YOSHIMORI T & LEVINE B. (2010). Methods in Mammalian Autophagy Research. **Cell.** 140 (3), 313–326.
- MUSUMECI G *et al.* (2015). Biomarkers of Chondrocyte Apoptosis and Autophagy in Osteoarthritis. **International Journal of Molecular Sciences**, 16 (9), 20560–20575.

- NGUYEN A D, HERZOG H & SAINSBURY A (2011). Neuropeptide Y and peptide YY: important regulators of energy metabolism. **Curr Opin Endocrinol Diabetes Obes** 18 (1):56–60.
- NIEDERMAIR T *et al.* (2014). Absence of substance P and the sympathetic nervous system impact on bone structure and chondrocyte differentiation in an adult model of endochondral ossification. **Matrix Biology**, 38, 22–35.
- NUNES A F *et al.* (2010). Neuropeptide Y expression and function during osteoblast differentiation - insights from transthyretin knockout mice. **FEBS Journal**, 277 (1), 263–275.
- OPOLKA A *et al.* (2012). Substance P and norepinephrine modulate murine chondrocyte proliferation and apoptosis. **Arthritis & Rheumatism**, 64 (3), 729–739.
- PEDRAGOSA-BADIA X, STICHEL J, & BECK-SICKINGER A G. (2013). Neuropeptide Y receptors: how to get subtype selectivity. **Frontiers in Endocrinology.** 4, 5.
- PEDRAZZINI T, PRALONG, F & GROUZMANN E (2003). Neuropeptide Y: the universal soldier. **Cellular and Molecular Life Sciences CMLS**, 60 (2), 350–377.
- PELLETIER J P, MARTEL-PELLETIER J, ABRAMSON S B (2001). Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. **Arthritis Rheum** 44: 1237–1247.
- PONCHEL F *et al.* (2015). Changes in peripheral blood immune cell composition in osteoarthritis. **Osteoarthritis and Cartilage**, *23* (11), 1870-1878.
- PONGRATZ G & STRAUB R H (2013). Role of peripheral nerve fibres in acute and chronic inflammation in arthritis. **Nat Rev Rheumatol**, 9(2), 117–126.
- POOLE A R *et al.* (2001). Composition and Structure of Articular Cartilage: A Template for Tissue Repair. **Clinical Orthopaedics and Related Research**, 391.
- POOLE C A (1997). Articular cartilage chondrons: form, function and failure. **Journal of Anatomy**, 191 (Pt 1), 1–13.
- RANNOU F, *et al.* (2014). The burden of osteoarthritis: development and validation of a new assessment tool (BONe'S). **Current Medical Research and Opinion**, *30* (4), 741-751.
- REICHENBACH S *et al.* (2008). Prevalence of bone attrition on knee radiographs and MRI in a community-based cohort. **Osteoarthritis and Cartilage**, 16 (9), 1005-1010.
- RIBEIRO M *et al.* (2016a). Insulin decreases autophagy and leads to cartilage degradation. **Osteoarthritis and Cartilage**, 24(4), 731–739.

- RIBEIRO M *et al.* (2016b). Diabetes-accelerated experimental osteoarthritis is prevented by autophagy activation. **Osteoarthritis and Cartilage**, 24(12), 2116–2125.
- ROSA S C *et al.* (2009). Impaired glucose transporter-1 degradation and increased glucose transport and oxidative stress in response to high glucose in chondrocytes from osteoarthritic versus normal human cartilage. **Arthritis Research & Therapy**, 11 (3), R80.
- SANCHEZ-ADAMS J *et al.* (2014). The mechanobiology of articular cartilage: bearing the burden of osteoarthritis. **Current Rheumatology Reports**, 16 (10), 451.
- SASAKI H *et al.* (2012). Autophagy modulates osteoarthritis-related gene expression in human chondrocytes. **Arthritis & Rheumatism**, 64 (6), 1920–1928.
- SCANZELLO C R & GOLDRING S R (2012). The role of synovitis in osteoarthritis pathogenesis. **Bone**, 51 (2), 249-257.
- SCHINKE T *et al.* (2004). Decreased Bone Formation and Osteopenia in Mice Lacking α-Calcitonin Gene-Related Peptide. **Journal of Bone and Mineral Research**, 19 (12), 2049–2056.
- SCHNEIDER J L, & CUERVO A M. (2014). Autophagy and human disease: emerging themes. **Current Opinion in Genetics & Development**, 26, 16 – 23.
- SEVERINI C *et al.* (2002). The Tachykinin Peptide Family. **Pharmacological Reviews**, 54 (2), 285 LP-322.
- SHAPIRO I M *et al.* (2014). Boning up on autophagy. **Autophagy,** 10 (1), 7–19.
- SHERWOOD J C *et al.* (2014). Cellular and molecular mechanisms of cartilage damage and repair. **Drug Discovery Today**, 19 (8), 1172-1177.
- SHI Y C & BALDOCK P A (2012). Central and peripheral mechanisms of the NPY system in the regulation of bone and adipose tissue. **Bone**, 50 (2), 430–436.
- SHIH C & BERNARD G W (1997). Neurogenic substance P stimulates osteogenesis in vitro. **Peptides**, 18 (2), 323–6.
- SILVA A, *et al.* (2005). The Putative Neuroprotective Role of Neuropeptide Y in the Central Nervous System. **Current Drug Target -CNS & Neurological Disorders**, 4 (4), 331–347.
- SILVERWOOD V, *et al.* (2015). Current evidence on risk factors for knee osteoarthritis in older adults: A systematic review and meta-analysis. **Osteoarthritis and Cartilage**, 23 (4), 507-515.
- SOKOLOVE J & LEPUS C M (2013). Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. **Therapeutic Advances in Musculoskeletal Disease**, 5 (2), 77–94.

- SOPHIA FOX A J, BEDI A & RODEO S A (2009). The basic science of articular cartilage: structure, composition, and function. **Sports Health**, 1 (6), 461–8.
- SURI S *et al.* (2007). Neurovascular invasion at the osteochondral junction and in osteophytes in osteoarthritis. **Annals of the Rheumatic Diseases**. 66 (11), 1423–8.
- SUTTON S *et al.* (2009). The contribution of the synovium, synovial derived inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis. **The Veterinary Journal**, 179 (1), 10–24.
- TAKARADA T *et al.* (2009). Interference by adrenaline with chondrogenic differentiation through suppression of gene transactivation mediated by Sox9 family members. **Bone**, 45 (3), 568–578.
- TANG Y, FENG Y & WANG X. (1998). Calcitonin gene-related peptide potentiates LPS-induced IL-6 release from mouse peritoneal macrophages. **Journal of Neuroimmunology**, *84* (2), 207–212.
- THOMAS E, PEAT G, & CROFT P. (2014). Defining and mapping the person with osteoarthritis for population studies and public health. **Rheumatology (United Kingdom)**, 53 (2), 338-345.
- VAN DER KRAAN P M *et al.* (1989) Development of osteoarthritic lesions in mice by 'metabolic' and 'mechanical' alterations in the knee joint. **Am. J. Pathol.** 135, 1001-1014.
- VASHEGHANI F *et al.* (2015). PPARγ deficiency results in severe, accelerated osteoarthritis associated with aberrant mTOR signalling in the articular cartilage. **Annals of the Rheumatic Diseases**, 74 (3), 569–578.
- VELA J *et al.* (2003) Rat hippocampal GABAergic molecular markers are differentially affected by ageing. **J Neurochem.** 85 (2):368–377.
- WANG H *et al.* (2015). Increasing expression of substance P and calcitonin gene-related peptide in synovial tissue and fluid contribute to the progress of arthritis in developmental dysplasia of the hip. **Arthritis Research & Therapy**, 17(1), 4.
- WANG L *et al.* (2009). Substance P stimulates bone marrow stromal cell osteogenic activity, osteoclast differentiation, and resorption activity in vitro. **Bone**, 45 (2), 309–320.
- WANG L *et al.* (2014). Levels of neuropeptide Y in synovial fluid relate to pain in patients with knee osteoarthritis. **BMC Musculoskeletal Disorders**, 15, 319.
- WEI Y, BAI L. (2016). Recent advances in the understanding of molecular mechanisms of cartilage degeneration, synovitis and subchondral bone changes in osteoarthritis. **Connect Tissue Res.** 57(4):1-17.

- WETTSTEIN J G, EARLEY B & JUNIEN J L (1995). Central nervous system pharmacology of neuropeptide Y. **Pharmacol Ther** 65 (3):397–414.
- WIATER M F *et al.* (2011). Circadian integration of sleep-wake and feeding requires NPY receptor-expressing neurons in the mediobasal hypothalamus. **AJP: Regulatory, Integrative and Comparative Physiology**, 301 (5).
- WOOLF C J & SALTER M W (2000). Neuronal plasticity: increasing the gain in pain. **Science (New York, N.Y.)**, 288 (5472), 1765–9.
- WU G *et al.* (2011). Central functions of neuropeptide Y in mood and anxiety disorders. **Expert Opin. Ther. Targets** 15, 1317–1331.
- ZENGIN A *et al.* (2010). Neuropeptide Y and sex hormone interactions in humoral and neuronal regulation of bone and fat. **Trends in Endocrinology & Metabolism**, 21 (7), 411–418.
- ZHANG W *et al.* (2016). Current research on pharmacologic and regenerative therapies for osteoarthritis. **Bone Research**, 4, 15040.
- ZHANG Y *et al.* (2000). Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. **Nat Immunol**, 1 (5), 392–397.
- ZHANG Y *et al.* (2015). Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis. **Annals of the Rheumatic Diseases**, 74 (7), 1432–1440.
- ZHANG Y, JORDAN JM. (2010). Epidemiology of Osteoarthritis. **Clinics in geriatric medicine.** 26(3):355-369.