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NEUROPEPTIDE Y
AT THE CELLULAR LEVEL

Studies on PreproNPY L7P Polymorphism and the Mitochondrial Form of NPY

by

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To my family

ABSTRACT

Katja Kaipio

Neuropeptide Y at the cellular level – Studies on PreproNPY L7P Polymorphism and the Mitochondrial Form of NPY

Department Pharmacology, Drug Development and Therapeutics and
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Neuropeptide Y (NPY) is a widely expressed neurotransmitter in the central and peripheral nervous systems. Thymidine 1128 to cytocine substitution in the signal sequence of the preproNPY results in a single amino acid change where leucine is changed to proline. This L7P change leads to a conformational change of the signal sequence which can have an effect on the intracellular processing of NPY. The L7P polymorphism was originally associated with higher total and LDL cholesterol levels in obese subjects. It has also been associated with several other physiological and pathophysiological responses such as atherosclerosis and T2 diabetes. However, the changes on the cellular level due to the preproNPY signal sequence L7P polymorphism were not known.

The aims of the current thesis were to study the effects of the [p.L7]+[p.L7] and the [p.L7]+[p.P7] genotypes in primary cultured and genotyped human umbilical vein endothelial cells (HUVEC), in neuroblastoma (SK-N-BE(2)) cells and in fibroblast (CHO-K1) cells. Also, the putative effects of the L7P polymorphism on proliferation, apoptosis and LDL and nitric oxide metabolism were investigated. In the course of the studies a fragment of NPY targeted to mitochondria was found. With the putative mitochondrial NPY fragment the aim was to study the translational preferences and the mobility of the protein.

The intracellular distribution of NPY between the [p.L7]+[p.L7] and the [p.L7]+[p.P7] genotypes was found to be different. NPY immunoreactivity was prominent in the [p.L7]+[p.P7] cells while the proNPY immunoreactivity was prominent in the [p.L7]+[p.L7] genotype cells. In the proliferation experiments there was a difference in the [p.L7]+[p.L7] genotype cells between early and late passage (aged) cells; the proliferation was raised in the aged cells. NPY increased the growth of the cells with the [p.L7]+[p.P7] genotype. Apoptosis did not seem to differ between the genotypes, but in the aged cells with the [p.L7]+[p.L7] genotype, LDL uptake was found to be elevated. Furthermore, the genotype seemed to have a strong effect on the nitric oxide metabolism. The results indicated that the mobility of NPY protein inside the cells was increased within the P7 containing constructs. The existence of the mitochondria targeted NPY fragment was verified, and translational preferences were proved to be due to the origin of the cells. Cell of neuronal origin preferred the translation of mature NPY ($NPY_{1\text{-}36}$) when compared to the non neuronal cells that translated both, NPY and the mitochondrial fragment of NPY. The mobility of the mitochondrial fragment was found to be minimal. The functionality of the mitochondrial NPY fragment remains to be investigated.

L7P polymorphism in the preproNPY causes a series of intracellular changes. These changes may contribute to the state of cellular senescence, vascular tone and lead to endothelial dysfunction and even to increased susceptibility to diseases, like atherosclerosis and T2 diabetes.

Key words: NPY, L7P polymorphism, mitochondrial protein, endothelial cell, cell of neuronal origin, non neuronal cell, nitric oxide, cellular senescence, atherosclerosis

TIIVISTELMÄ

Katja Kaipio

Neuropeptidi Y solutasolla – Tutkimus preproNPY:n L7P polymorfiaan ja NPY:n mitokondriaaliseen muotoon liittyen
Farmakologia, lääkekehitys ja lääkehoito ja Lääkekehityksen tutkijakoulu
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Väitöstutkimuksessa selvitettiin keskus- ja ääreishermoston yleisessä välittäjäaineessa (neuropeptidi Y eli NPY) tapahtuvan aminohappomuutoksen eli NPY:n signaalipeptidin leusiini 7 proliini 7 (L7P) polymorfian vaikutuksia solutasolla. Tutkimus osoitti, että NPY:n signaalipeptidin L7P polymorfia saattaa aiheuttaa solun sisällä sellaisia muutoksia, jotka saattavat johtaa pahimmillaan valtimonkovettumistautiin tai erilaisiin aineenvaihdunnan sairauksiin. Ihmisillä tehdyissä tutkimuksissa NPY:n signaalipeptidin L7P polymorfian on havaittu liittyvän mm. veren kohonneisiin kolesterolipitoisuuksiin lihavilla henkilöillä, valtimonkovettumistautiin ja tyypin 2 diabetekseen. Vaikka kyseisen polymorfian tiedetään aiheuttavan välittäjäaineessa sellaisen proteiinin rakenteen muutoksen, joka saattaa vaikuttaa NPY:n solun sisäiseen toimintaan, solutason muutoksia ei kuitenkaan tarkkaan tunneta.

Väitöskirjatutkimuksessa haluttiin selvittää NPY:n signaalipeptidin L7P polymorfian solutason vaikutuksia verisuonen sisäseinämän soluissa (HUVEC), hermostollista alkuperää olevissa soluissa (SK-N-BE(2)) sekä ei-hermostollista alkuperää olevissa soluissa (CHO-K1). Muutoksen vaikutusta tutkittiin verisuonen sisäseinämän solujen uudiskasvuun ja ohjelmoituun solukuolemaan eli solujen määrän luonnolliseen tasapainoon. Lisäksi tutkimuksen kohteena olivat vaikutukset typpioksidivälitteiseen verisuonten toiminnan säätelyyn sekä solujen kykyyn ottaa sisään LDL-kolesterolia eli "pahaa" kolesterolia. Polymorfiaa tutkittaessa löydettiin myös uusi proteiini, joka nimettiin NPY:n mitokondriaaliseksi muodoksi. Kyseinen proteiini kulkeutui solun sisällä poikkeuksellisesti mitokondrioon, toisin kuin NPY, joka normaalisti eritetään solusta ulos. Väitöskirjatutkimuksessa selvitettiin myös mitokondriaalisen NPY-muodon luentaketjua ja mitokondrioon kulkeutuvan proteiinin liikkuvuutta.

Tutkimuksissa verrattiin tietyn genotyypin soluja ([p.L7]+[p.P7]) verrokkisoluihin ([p.L7]+[p.L7]) ja solutason muutoksia havaittiin niin välittäjäaineen solunsisäisessä jakautumisessa kuin liikkuvuudessakin. NPY:n signaalipeptidin L7P polymorfian huomattiin vaikuttavan mm. solujen NPY-proteiini määrään, verisuonen sisäseinämän solujen uudiskasvuun ja kolesterolin sisäänottoon sekä typpioksidituotantoon ja typpioksidivälitteiseen verisuonten toiminnan säätelyyn. Tutkittaessa välittäjäaineen liikkuvuutta solun sisällä eroja löytyi esimerkiksi hermostollista alkuperää ja ei-hermostollista alkuperää olevien solujen kesken. NPY:n mitokondriaalisen muodon esiintyvyyden todettiin vaihtelevan eri alkuperää olevien solujen valillä. NPY:n mitokondriaalisen muodon merkitystä ei kuitenkaan toistaiseksi tiedetä.

Tutkimuksen tulosten mukaan NPY:n signaalipeptidin L7P polymorfia aiheuttaa useita solunsisäisten toimintojen muutoksia. Nämä muutokset vaikuttavat mitä todennäköisimmin solun vanhenemiseen, verisuonten joustavuuteen ja saattavat liittyä verisuonen sisäseinämän toimintahäiriöön. Tämän taas tiedetään altistavan valtimonkovettumistaudille ja tyypin 2 diabetekselle.

Avainsanat: NPY, NPY:n signaalipeptidin L7P polymorfia, NPY:n mitokondriaalinen muoto, verisuonen sisäseinämän solu, hermostollista alkuperää oleva solu, ei-hermostollista alkuperää oleva solu, typpioksidi, solujen vanheneminen, valtimonkovettumistauti

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ABBREVIATIONS

aa	amino acid
AgRP	agouti-related peptide
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMI	body mass index
bp	base pair
BSA	bovine serum albumin
CaM	calmodulin
CHD	coronary heart disease
CHO-K1	Chinese hamster ovary cell line
CNS	central nervous system
CRH	corticotrophin releasing hormone
C-PON	C-ponderance, C-terminal end, C-terminal peptide
CVD	cardiovascular disease
DAF-2DA	4,5-diaminofluorescein
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
DPP	dipeptidyl peptidase
EC	endothelial cell
ECGS	endothelial cell growth supplement
EDTA	ethylenediaminetetracetic acid
EEC	endocardial endothelial cell
EPC	endothelial progenitor cell
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FFA	free fatty acid
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
GHRH	growth hormone releasing hormone
GnRH	gonadotropin releasing hormone
GPCR	G-protein coupled receptor
HAC	hepatic arterial conductance
HUVEC	human umbilical vein endothelial cell
HRP	horseradish peroxidase
IMT	intima media thickness
KO	knockout
KRH	Krebs-Ringer-Hepes buffer
LDL	low density lipoprotein
LH	luteinizing hormone
L22M	leucine 22 to methionine 22 change
L7P	leucine 7 to proline 7 change
LPL	lipoprotein lipase
M199	Medium 199
MEM	Minimal essential medium
mRNA	messenger RNA
NA	noradrenaline

NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial NOS
iNOS	inducible NOS
mtNOS	mitochondrial NOS
nNOS	neuronal NOS
NP	alcohol non preferring rats
NPY-ir	neuropeptide Y immunoreactivity
OE	overexpressing
P	alcohol preferring rats
PAG	periaqueductal grey
PBS	phosphate buffered saline
PC	prohormone convertase
PP	pancreatic polypeptide
PTP	prohormone thiol protease
PYY	peptide YY
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROS	reactive oxygen species
SK-N-BE(2)	human neuroblastoma cell line
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
SOD	superoxide dismutase
T1D	type 1 diabetes
T2D	type 2 diabetes
TG	triglyceride
TGN	trans golgi network
TRH	thyrotropin releasing hormone
TRITC	tetramethyl rhodamine isothiocyanate
VED	vascular endothelial dysfunction
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
WAT	white adipose tissue

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original studies, which will be referred to with the Roman numeral I-V:

I Kallio J, Pesonen U, Kaipio K, Karvonen MK, Jaakkola U, Heinonen OJ, Uusitupa MIJ, Koulu M (2001): Altered intracellular processing of Neuropeptide Y due to leucine 7 to proline 7 polymorphism in the signal peptide of preproneuropeptide Y in humans. *FASEB J.* 15(7): 1242-1244.

II Kaipio K, Kallio J, Pesonen U (2005): Mitochondrial targeting signal in human neuropeptide Y gene. *Biochem Biophys Res Commun.* 337(2): 633-640.

III Kaipio K, Kallio J, Pesonen U (2009): The effect of endogenous preproneuropeptide Y Leucine 7 to Proline 7 polymorphism on growth and apoptosis in primary cultured HUVECs. *Biol Chem.* In press.

IV Kaipio K, Vahlberg T, Suominen M, Pesonen U (2009): The role of non-synonymous *NPY* gene polymorphism in the nitric oxide production in HUVECs. *Biochem Biophys Res Commun.* 381(4): 587-591.

V Kaipio K, Pesonen U (2009): The intracellular mobility of NPY and a putative mitochondrial form of NPY in neuronal cells. *Neurosci Lett.* 450(2): 181-185.

In addition some unpublished data is presented.

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1 INTRODUCTION

The procedure where a protein is formed according to the instructions encoded by DNA, is a rather complex process. DNA is transcribed to RNA and then translated to a protein. The protein adapts a tertiary structure that acts as an intracellular message. When DNA encoded messenger RNA (mRNA) is translated, ribosomes bind to the mRNA and initiate translation. A special sequence and codon are required for the translation to begin. A kozak sequence is a short recognition sequence which includes a translational start codon: AUG, coding for methionine. Multiple start codons can be included in one gene.

The protein is translated according to the instructions encoded by DNA. A precursor protein is an inactive protein or peptide that can be turned into an active form by posttranslational modifications, as is the case with prepronuropeptide Y (preproNPY). PreproNPY is a 97 amino acid (aa) precursor peptide for neuropeptide Y (NPY). PreproNPY contains two fragments that are cleaved off during the maturation process. The fragments are called the signal peptide and the C-terminal peptide (C-PON). The signal peptide directs the peptide in the cell, for example to the endoplasmic reticulum (ER) and further to the secretory pathway. A rather common polymorphism was found to exist in the signal sequence part of the preproNPY. The 7th aa, leucine, of the 28 aa long signal peptide is changed to proline. This leucine 7 to proline 7 (L7P) polymorphism has been associated with multiple pathophysiological conditions, such as higher total and low density lipoprotein (LDL) cholesterol levels in obese subjects (Karvonen *et al.* 1998), with higher serum triglyceride levels in preschool aged children (Karvonen *et al.* 2000; Pesonen 2006) and with accelerated atherosclerosis in middle aged men (Karvonen *et al.* 2001) as well as in patients with type 2 diabetes (T2D) (Karvonen *et al.* 2001; Niskanen *et al.* 2000a).

A 36 aa mature NPY is a widely expressed peptide hormone in the central and peripheral nervous systems. It is an evolutionally conserved peptide that has multiple functions such as regulation of energy balance and blood pressure, reproduction, hormonal balance, ethanol consumption and psychiatric disorders such as depression. In the periphery, NPY is located in multiple organs and tissues, such as adipose tissue, smooth muscle tissue, bone, kidney, liver and heart. NPY has also been localized in the endothelium, which lines all blood vessels. Endothelium contains a single cell layer and maintains physiological homeostasis (Bouis *et al.* 2001; Brandes *et al.* 2005). In vessels, except capillaries, endothelium is surrounded by a vascular smooth muscle cell (VSMC) layer. These two layers are in close relationship in maintaining the correct functions of the blood vessels, such as blood pressure. The aging of the endothelium and the VSMC layer are the key factors in the development of cardiovascular diseases (CVDs) for instance atherosclerosis and coronary heart disease. In the present study, the translation of the preproNPY was investigated and the effects of L7P polymorphism were studied in endothelial cells (ECs), neuroblastoma cells and fibroblast cells to approach the mechanisms at the cellular level resulting in the pathophysiological conditions caused by the NPY signal sequence L7P polymorphism.

2 REVIEW OF THE LITERATURE

2.1 BIOLOGY OF NEUROPEPTIDE Y

2.1.1 Introduction to NPY

Neuropeptides are generally synthesized as inactive precursors, prepronEuropeptides, which are first processed to proneuropeptides and then to mature neuropeptides (Kuiper & Martens 2000). The biologically active and evolutionally conserved mature NPY is derived from a 97 aa precursor peptide, preproNPY, which contains two fragments that are cleaved during the post-translational maturation processes (Mentlein *et al.* 1993). The cleaved fragments are commonly called a signal peptide and a C-PON. The signal peptide of NPY is a 28 aa's long peptide that directs the NPY peptide to the ER. The importance of the signal sequence is to enable the proper folding and packaging of the NPY peptide.

NPY was originally isolated from porcine brain in 1982 (Tatemoto 1982). Other members of the pancreatic polypeptide family are peptide YY (PYY) and pancreatic polypeptide (PP), which are gut endocrine peptides (Larhammar 1996). NPY, PYY and PP are all 36 aa's long peptides and they have the key residues required to adopt the so-called PP fold (Fuhlendorff *et al.* 1990b) (Figure 2.1). The PP fold consists of a polyproline helix and an alpha helix (Wood *et al.* 1977). The two helices are joined by a turn and are held in the folded configuration through hydrophobic interactions between side chains (Fuhlendorff *et al.* 1990b).

The mature NPY (NPY_{1-36}) is much more conserved in evolution than the signal peptide or the C-PON (Larhammar 1996). A low degree of identity is found for the signal peptide sequence (32-75%) and for the C-PON of NPY (43-73%) (van Riel *et al.* 1993). Recent findings of the sequence differences of NPY between species have given a lot of knowledge about the evolution of NPY. NPY has remained almost identical during evolution (Larhammar 1996). For instance, the NPY sequences of rat, rabbit, guinea pig and alligator are exactly the same as in humans and the NPY sequence in *Torpedo marmorata* (marbled electric ray) is identical to mammalian NPY in 33 out of 36 positions. Twenty-two positions are identical in all NPY sequences known (Larhammar 1996; Pedrazzini *et al.* 2003). The strong conservation of NPY is partly due to constraints forced to allow adoption of the PP fold. Among the fourteen variable positions of NPY, several have highly conservative replacements and only one position seems to tolerate more than two alternative aa's (position three) (Pedrazzini *et al.* 2003). The structural similarity between NPY and PYY in mammals is 70% and PP shows a 50% identity to NPY (Soderberg *et al.* 2000; Tatemoto 1982). These peptides have functional overlapping that has to do with the common ancestral origin (Larhammar 1996). NPY and PYY arose from a common ancestral gene in two genome duplications (tetraploidizations) in early vertebrate evolution and PP is a result of a more recent, separate duplication (Figure 2.2) (Larhammar 1996; Soderberg *et al.* 2000; Sundström *et al.* 2008).

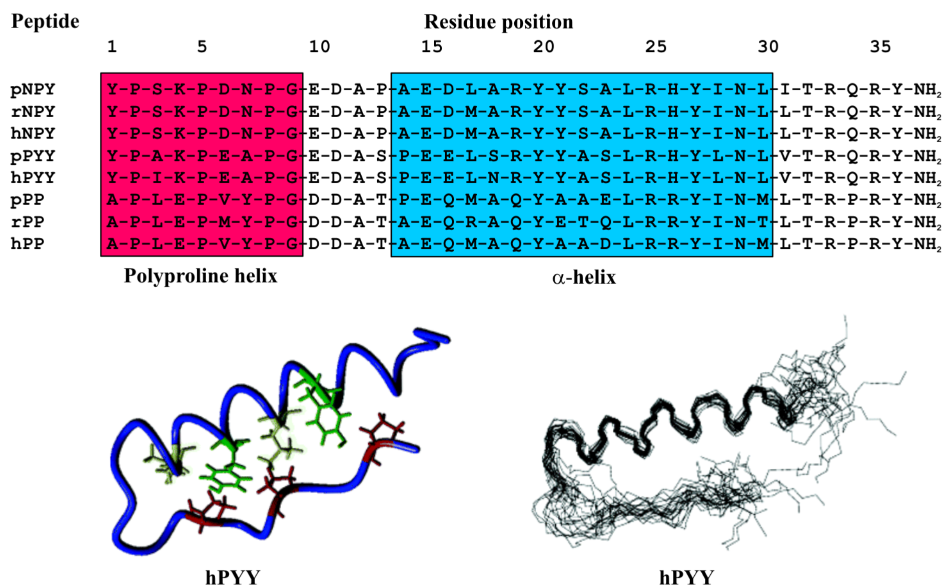

Figure 2.1. Top; aa sequences of NPY, PYY and PP. Residues 1-9 form the polyproline helix and residues 14-30 form the α-helix. The polyproline helix and α-helix are connected by type II β-turn. Bottom; Two molecular models of human PYY (Nygaard *et al.* 2006). Aa's are represented by their one-letter symbols. The small letters in front of the peptide symbols represent the species; p = porcine, r = rat, h = human. Modified from (Dumont *et al.* 1992; Nygaard *et al.* 2006).

NPY (MIM: 162640) exists as a single copy gene (Baker *et al.* 1995). Human *NPY* gene is located adjacent to *HOX1* (=*HOXA*) gene cluster on chromosome 7p15.1 in the human genome (Baker *et al.* 1995). *PYY* and *PP* genes are 10 kb apart in tandem near the *HOX2* (=*HOXB*) cluster of genes on chromosome 17q21.1 (Hort *et al.* 1995). The localization of the *NPY* and *PYY* genes supports the theory of a common ancestral origin. Also the close proximity of the *PYY* and *PP* genes is consistent with the theory of a more recent separate duplication of the *PYY* gene (Hort *et al.* 1995; Larhammar 1996) (Figure 2.2). Plenty of studies have been done to convince the evolutional NPY/PYY ancestor gene, but still Susumu Ohno's (1970) book "Evolution by Gene Duplication" is under controversial discussion (Pennisi 2001). Differing opinions about NPY/PYY ancestor and the two tetraploidizations theory in the early vertebrate history are under a vivid debate (Hoegg & Meyer 2005; Hughes 1999; Larhammar 1996; Larhammar *et al.* 2002; Lundin *et al.* 2003; Pennisi 2001).

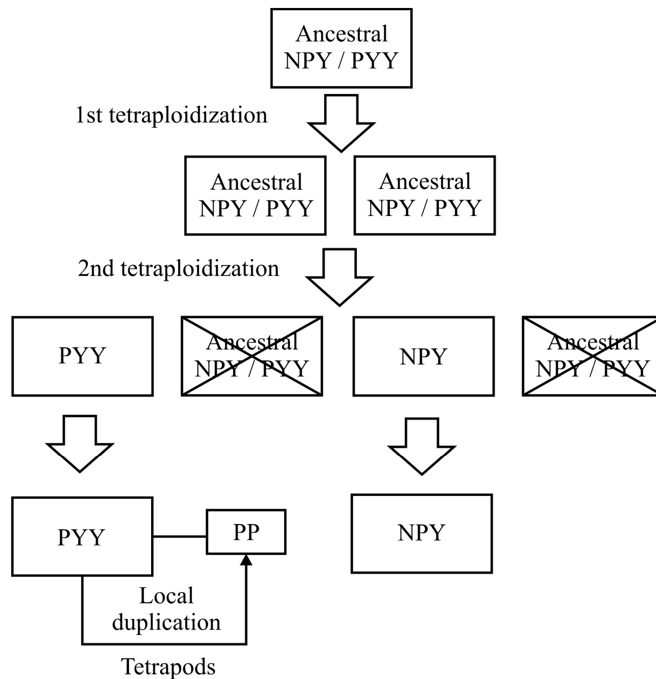

Figure 2.2. Current opinion of the evolution of NPY, PYY and PP. Modified from (Larhammar 1996; Soderberg *et al.* 2000; Sundström *et al.* 2008).

2.1.2 Processing and metabolism of NPY

2.1.2.1 The *NPY* gene promoter and transcription of the *NPY* gene

Transcriptional and post transcriptional factors that control the *NPY* gene expression have been studied by Lerchen et al. (1995). The data suggest that the NPY mRNA stability could play an important role in controlling the *NPY* gene expression in response to food or body weight status (Good 2000; Lerchen *et al.* 1995). The transcription unit of NPY is approximately 8 kilobase pairs and consists of four exons and three intervening sequences (Minth *et al.* 1986). The first exon contains nontranslated DNA and the second exon starts from methionine (AUG start codon) and ends at residue 63 (Minth *et al.* 1986). The third exon codes for aa's 64 to 90 and the fourth exon codes for aa's 91 to 97 of the preproNPY (Minth *et al.* 1986) (Figure 2.3).

The promoter region facilitates the transcription of a particular gene. It is usually located near the gene it regulates, and upstream of it. In the promoter region of the *NPY* gene there are only a few areas that have a critical role in the transcription of NPY. Especially important for the expression of the *NPY* gene are the residues from -63 to -51, particularly the CCCCTC sequence in this region (Minth & Dixon 1990). The region is protected from digestion and it is apparent that this area is important for transcriptional activation. Another important region is located from -143 to -118 and the CCCCTC sequence can be found in this region as well. The deletion of this region causes a two fold decrease in the expression of the *NPY* gene (Minth & Dixon 1990). It has also been suggested that a negative regulatory element exists in the distal NPY promoter between -798 to -498 (Minth-Worby 1994), but no further investigations of this area have been performed. Different transcription factors have been shown to influence the transcriptional activity of NPY. The nerve growth factor (NGF), which belongs to a family of neurotrophins, increases the transcription of NPY in a pheocromocytoma (PC12) cell line

(Higuchi *et al.* 1996; Minth-Worby 1994; Wernersson *et al.* 1998). NPY is transactivated by leptin via the *NPY* gene promoter region. Leptin induced transactivation of the *NPY* gene requires activation of JAK1, JAK2 and STAT3 especially in neural cells (Muraoka *et al.* 2003).

2.1.2.2 The kozak sequence and translation of preproNPY

A kozak sequence is a short recognition sequence, which includes the AUG start codon coding for methionine. The kozak sequence facilitates the initial binding of a small subunit of the ribosome to the mRNA (Kozak 1997). Translational initiation sites in eukaryotes are extremely regulated and they can hardly ever be changed to another codon from AUG. Kozak's consensus sequence is GCCRCC<u>AUG</u>G where the R at position -3 is preferably adenine (A) (numbering starting from the first A of the AUG codon) (Kozak 1997). In this consensus sequence the most conserved positions are the +4 position (G) and -3 position (R preferably an A) (Kozak 1986b). The first-AUG rule states that translation is most likely to initiate at the nearest AUG in the 5' end of mRNA (Cigan *et al.* 1988). There are many mechanisms by which the preference of the initiation site can be changed to another AUG codon (Burgess-Beusse *et al.* 1999; Cigan *et al.* 1988; Gross *et al.* 1998). In eukaryotes the initiation of translation occurs mainly via a scanning mechanism. This means that when AUG appears in a "strong" context, such as ANN<u>AUG</u>N or GNN<u>AUG</u>G, almost all ribosomes stop and initiate translation at that point (minimally leaky kozak sequence), but when AUG appears in a "weak" context, such as lacking R in –3 position and/or G in +4 position, most ribosomes continue scanning and initiate further downstream (maximally leaky kozak sequence) (Kozak 2001a; Wang & Rothnagel 2004). This process is called leaky scanning and it enables the production of multiple proteins with different molecular weights from one mRNA (Kozak 1986a; Kozak 1986b). The kozak sequence can be divided between exons, as is the case with the kozak sequence in preproNPY. The first exon includes part of the first kozak sequence which has a great importance in the translation of preproNPY even though the first exon contains only non-translated DNA.

PreproNPY is further processed to proNPY, NPY and fragments of NPY (Figure 2.3). The 28 aa signal peptide is cleaved by a signal peptidase enzyme that produces 69 residue proNPY. The proNPY usually travels to the Golgi complex and further to the *trans*-Golgi. This routing is common with all neuropeptides. Prohormone convertase enzymes, PC1/3 and PC2, are the most important enzymes for the processing of proneuropeptides and prohormones. The proNPY undergoes cleavage by proconverting enzymes, PC1/3 and/or PC2, releasing the C-PON (Brakch *et al.* 1997). Some kinetic studies have shown that PC1/3 cleaves proNPY more efficiently than PC2 and undergoes a cleavage at a single dibasic site Lys38-Arg39 (Brakch *et al.* 1997). NPY_{1-39} is further processed to NPY_{1-37} by carboxypeptidase like enzyme and amidated by peptidyl-glycine-α-amidating mono-oxygenase to NPY_{1-36} (Pedrazzini *et al.* 2003). Of the NPY modifying enzymes dipeptidyl peptidase 4 (DPP4; CD26; ADCP2; MIM 102720) is expressed in various cell lines, such as epithelial cells, ECs, fibroblasts and leukocytes (Silva *et al.* 2003) and it is co-localized with NPY (Zukowska-Grojec *et al.* 1998). DPP4 is a member of a prolyl oligopeptidase (POP) family of serine proteases (Rawlings *et al.* 1991). Mature NPY may be truncated to NPY_{2-36} by amino peptidase P enzyme or to NPY_{3-36} by DPP4 enzyme. Hook and others have studied a cysteine protease known as prohormone thiol protease (PTP) especially in chromaffin cells. PTP has been proved to cleave proNPY with rather high efficiency (Hook *et al.* 1996). More specifically cathepsin L has been identified as the cysteine protease component of purified PTP (Yasothornsrikul *et al.* 2003) and furthermore, it cleaves the proNPY at its dibasic prohormone processing site (Funkelstein *et al.* 2008). Other DPP4-like enzymes such as dipeptidyl peptidase 8 (DPP8; MIM 606819) and dipeptidyl peptidase 9 (DPP9; MIM 608258) have also been found to cleave NPY, but with lower efficacy than DPP4 (Frerker *et al.* 2007). Variation has been observed in the amounts of NPY fragments *in vivo* and some have been proved to be functionally active (Nilsson *et al.* 1998).

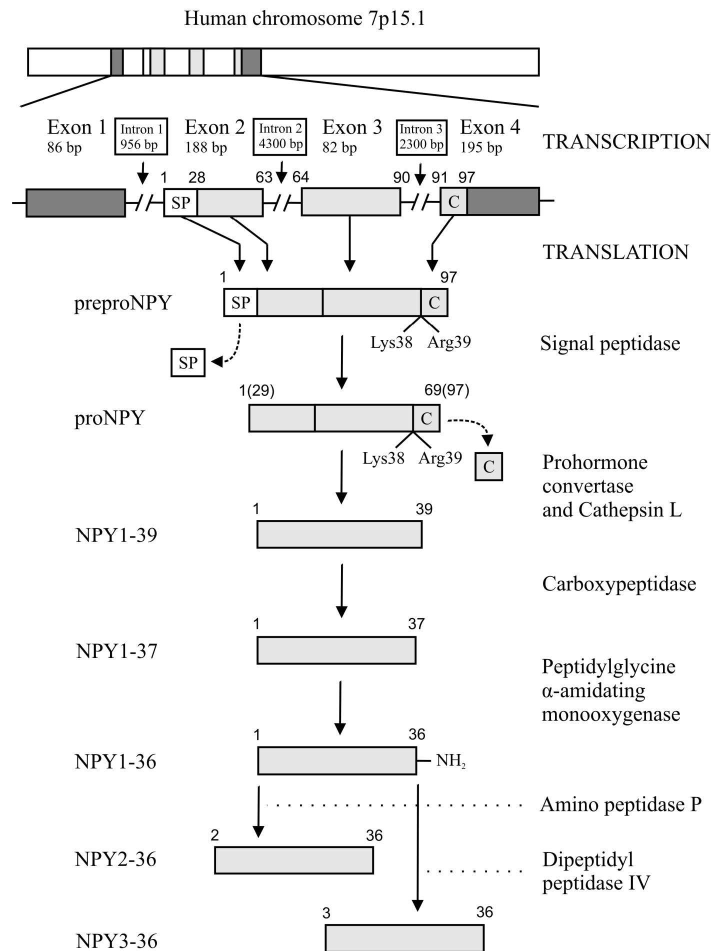

Figure 2.3. Synthesis and processing of the NPY in humans starting from the *NPY* gene in the human 7p 15.1 chromosome. The *NPY* gene is transcribed from the total of four exons of which exons 2, 3 and 4 contain the coding regions. PreproNPY containing 97 aa's is a result of translation. The signal peptide containing aa's from 1 to 28 are cleaved with a signal peptidase enzyme. Prohormone convertase and Cathepsin L enzymes cleave the C-PON (C) at a single dibasic site Lys38-Arg39. NPY_{1-39} is processed to NPY_{1-36} with two modifying enzymes; carboxypeptidase and peptidylglycine α-amidating monooxygenase. NPY_{1-36} can be truncated to NPY_{2-36} with the amino peptidase P enzyme or to NPY_{3-36} with the dipeptidyl peptidase IV enzyme. Modified from (Pedrazzini *et al.* 2003; Silva *et al.* 2002).

2.1.2.3 Fragments of NPY

Several fragments of NPY have been found *in vivo*. In the human cerebrospinal fluid most abundant peptides were NPY_{1-36}, NPY_{3-36} and NPY_{18-36} (Nilsson *et al.* 1998). Multiple other fragments have also been known to exist and function as NPY receptor agonists. NPY $_{2-36}$ and NPY_{13-36} have been known to have high affinity on the Y2 receptor (Foti *et al.* 2007; Gerald *et al.* 1995). The existence and effects of, for example, NPY_{13-36} fragment in humans have not been confirmed, but it has been thought to mediate an antiseizure activity via Y2 receptors (Foti *et al.* 2007). Another study suggested that at least in mice the Y2 receptor, as well as the Y5 receptor regulated the hippocampal seizures *in vitro,* while activation of Y5 receptors in extra-hippocampal regions reduced generalized seizures *in vivo* (Woldbye *et al.* 2005). Y2 and Y5 receptors have been studied to mediate the effects of the truncated NPY fragments

(Gerald *et al.* 1995; Gerald *et al.* 1996). It has been reported that NPY_{17-36} has been identified in rat cardiac cells (Sheriff & Balasubramaniam 1992). NPY_{17-36} exhibited inhibitory and stimulatory effects on cardiac adenylate cyclase activity (Balasubramaniam & Sheriff 1990). N-terminally truncated NPY peptides have been observed to be routed to mitochondria (Frerker *et al.* 2007; Silva *et al.* 2005). Some C-terminally truncated peptides have been found but overall, the studies concerning the C-terminally truncated peptides appear to be very few (Bouali *et al.* 1994; Stenfors *et al.* 1997). It has been reported that a C- terminally truncated NPY_{1-30} has been found in the rat brain (Wahlestedt *et al.* 1990) and it decreased spontaneous locomotor activity and induced hypothermia (Bouali *et al.* 1994). NPY fragments are presented in Table 2.1.

Table 2.1. NPY and fragments of NPY in order of potency to different NPY receptors. Modified from (Dumont & Quirion 2006; Merten & Beck-Sickinger 2006).

NPY receptor subtype	Endogenous NPY agonists in order of potency
Y1	NPY1-36 ≥ [Leu31, Pro34]NPY >> NPY2-36, NPY3-36, NPY13-36
Y2	NPY1-36 ≥ NPY2-36, NPY3-36, NPY13-36, NPY18-36, NPY22-36, >>[Leu31, Pro34]NPY
Y4	[Leu31, Pro34]NPY > NPY1-36 >> NPY2-36, NPY3-36, NPY13-36
Y5	NPY1-36, [Leu31, Pro34]NPY, NPY2-36, NPY3-36

2.1.2.4 Routing and release of NPY

Traditionally, the 28 aa signal peptide is believed to be responsible for proper folding, packaging and routing of NPY. First, NPY is routed to the ER via Golgi. The posttranscriptional processing, including glycosylation, disulphide bond formation and phosphorylation, takes place in the ER. NPY is routed to the cell surface in clathrin coated vesicles (Silva *et al.* 1995). NPY is mainly released from sympathetic nerve endings and it is stored and co-released with noradrenaline (NA) from the same vesicles (Donoso *et al.* 2006; Fried *et al.* 1985; Fried *et al.* 1986). NPY needs much more neuronal activation than NA to be able to be released from sympathetic nerve endings. The release of NPY presumably happens with a mechanism called kiss-and-run (Tsuboi & Rutter 2003; Zhu *et al.* 2007). In this mechanism the vesicles fuse transiently with the plasma membrane and release their contents through a partially opened fusion pore (Tsuboi & Rutter 2003). In the kiss-and-run model of exocytosis the vesicle does not exchange membrane components with the plasma membrane (Taraska & Almers 2004). NPY has also been speculated to be exocytosed with a method called cavicapture (Figure 2.4). In cavicapture the lipid bilayer of the membrane mixes with the plasma membrane briefly before resealing (Perrais *et al.* 2004; Taraska & Almers 2004). Cavicapture has been studied in chromaffin cells and interestingly, it was noticed that NPY may be retained in some granules for quite some time in comparison with other NPY granules. In fact it may be that NPY does not leave the granule at all (Perrais *et al.* 2004). In approximately 20% of the granules NPY was retained in the granule. A reason for this may be that the fusion pore is not large enough for NPY to be released from the granule (Perrais *et al.* 2004). The reason for the protein retention in the granule is not yet known.

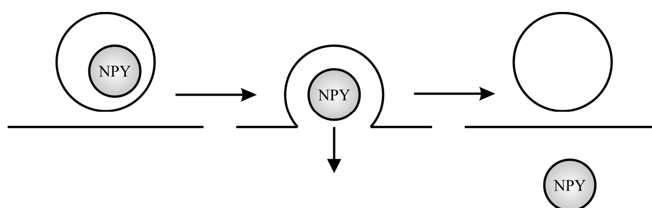

Figure 2.4. A hypothetical model of NPY exocytosis with the cavicapture mechanism. The membrane of the granule mixes with the plasma membrane briefly before resealing and NPY is released.

NPY has also been found in the nucleus, particularly in the perinucleoplasmic space of the human endocardial endothelial cells (EECs) (Jacques *et al.* 2003). Furthermore, NPY receptor (Y1) was also localized on the perinucleoplasmic space (Jacques *et al.* 2003). It has been speculated that nucleoplasmic NPY may be released to the nuclear envelope space (perinucleoplasm) and activate the inner nuclear membrane Y1 receptors (Jacques *et al.* 2003). It has also been suggested that nucleoplasmic NPY may be released inside the nuclear envelope space by Ca^{2+} dependent exocytosis and increase the Ca^{2+} amount (Jacques *et al.* 2006). Moreover, NPY can also be released into the cytosol to activate NPY receptors that are present on the outer nuclear envelope membrane (Jacques *et al.* 2006). In addition to nucleus, NPY immunoreactivity (NPY-ir) has been localized in mitochondria of human umbilical vein endothelial cells (HUVECs) (Silva *et al.* 2005), but not much is known about the mitochondrial NPY.

2.1.3 NPY receptors in humans

The study of NPY and its receptors in humans has not been widely conducted. NPY receptor selective analogs have been used as ligands in NPY receptor expression studies in human tumors. It has been found that for example breast carcinomas and adrenal cortical tumors have a very high receptor density of NPY Y1 receptors while paragangliomas and pheochromocytomas have a relatively high density of NPY Y2 receptors (Körner & Reubi 2007).

NPY receptors belong to the G-protein-coupled receptors (GPCRs) and thus consist of seven-α-helix transmembrane regions (7TM). NPY receptors have variable affinity to different NPY fragments as well as to PYY and PP. The affinity to a certain receptor varies depending on the ligand and the receptor subtype. NPY receptors can be divided into three subfamilies, Y1, Y2 and Y5, according to the sequence identity (Table 2.2). Y3 receptor has been characterized only by pharmacological studies and most likely this receptor does not exist as a separate gene (Blomqvist & Herzog 1997).

2.1.3.1 The Y1 subfamily

The Y1 subfamily includes Y1, Y4, Ya, Y6 and Yb receptor subtypes. The best characterized receptor in humans is Y1 (MIM 162641), which is located on the chromosome 4q31.3-q32. The Y1 receptor is a 384 aa protein and it has all the typical characteristics of a GPCR, such as potential glycosylation sites, certain extracellular cysteins and possible phosphorylation sites. The Y1 receptor is internalized, as typical for GPCRs, and recycled to the cell surface in 60 minutes. All mammalian Y1 receptors have 90-95% homology with human Y1 receptor. It has been shown that Y1 receptors are able to form homodimers (Dinger *et al.* 2003). NPY_{1-36} binds with nanomolar activity to the Y1 receptor but truncated forms of NPY, such as NPY_{2-36} or NPY_{3-36}, do not have high affinity for the Y1 receptor. The first selective agonist to Y1 receptor was [Leu^{31},Pro^{34}]NPY (Fuhlendorff *et al.* 1990a). Y1 receptors appear to be located in the

central nervous system (CNS) especially in the hypothalamus, cerebral cortex and amygdala, which has to do with the anxiolytic effects of NPY (Fuhlendorff *et al.* 1990a). The Y1 receptor has also been located in the colon, kidney, adrenal gland, heart, placenta, coronary and arterial smooth muscle, intestine, spleen, testes and lymphocytes (Allen *et al.* 1993; Petitto *et al.* 1994; Wharton *et al.* 1993). The main physiological functions mediated by the Y1 receptors are vasoconstriction (Wahlestedt & Hakanson 1986), blood pressure (Fuhlendorff *et al.* 1990a) anxiolysis and depression (Heilig *et al.* 1993; Kask *et al.* 2001), GHRH secretion (Kalra *et al.* 1990), leptin and insulin release (Gamba & Pralong 2006; Morgan *et al.* 1998), catecholamine release (Cavadas *et al.* 2006), ethanol intake (Thiele *et al.* 2002; Wetherill *et al.* 2008) and regulation of feeding (Kanatani *et al.* 2001). The Y1 receptor has also a role in the thermogenesis in rats (Lopez-Valpuesta *et al.* 1996a; Lopez-Valpuesta *et al.* 1996b).

The human Y4 (MIM 601790) receptor has 43% homology with the human Y1 receptor. The sequence homology between the human Y4 receptor and other species Y4 receptors is one of the lowest homologies, only 75%. The Y4 receptor is located on the chromosome 10q11.2-q21 (Wraith *et al.* 2000). It is a 375 aa protein and has all the characteristics of a GPCR. The Y4 receptor is unique in the sense that it binds PP with higher activity than NPY and PYY. In humans the Y4 receptor is located mainly in the pancreas, intestines, prostate (Lundell *et al.* 1995), gastrointestinal tract (Bard *et al.* 1995), heart (Gregor *et al.* 1996b), CNS and blood vessels (Bard *et al.* 1995). The Y4 mediated physiological functions are, for example, gall bladder contraction, gastrointestinal motility, gastric acid secretion and corticosteroid secretion (Andreis *et al.* 1993; Fuhlendorff *et al.* 1990b; McTigue *et al.* 1993; Schwartz 1983). Food intake is also mediated by the Y4 receptor (Balasubramaniam *et al.* 2006).

The NPY Y6 receptor is functional in rabbit and mouse but it is a pseudogene in humans due to a frameshift mutation (Gregor *et al.* 1996a). The deletion causes a truncated protein to be formed and thus the ligands can not properly bind to the receptor (Gregor *et al.* 1996a). The Y6 receptor has been cloned and characterized in chicken, but no functional response has been demonstrated (Bromee *et al.* 2006). The Y6 receptor is sometimes indicated as y6 receptor due to the unknown physiological effects (Michel *et al.* 1998). Although the human Y6 receptor is a pseudogene, its mRNA is expressed in several tissues including heart, skeletal muscle, spleen, prostate and small intestine (Matsumoto *et al.* 1996). The Yb and Yc receptors have been cloned in teleost fishes, but the functionality of these receptors is still unknown (Bromee *et al.* 2006).

2.1.3.2 The Y2 subfamily

The Y2 subfamily includes Y2 and y7 receptor subtypes. Y2 (MIM 162642) receptor has been cloned in various species and it has 90-95% homology between species. It consists of 381 aa's and possesses a seven transmembrane helix receptor structure. Interestingly, the overall homology between Y1 and Y2 receptor is only 31%. The Y2 receptor gene is located on the 4q31 chromosome near the Y1 and Y5 genes. In contrast to the Y1 receptor, the Y2 receptor does not appear to be internalized following agonist stimulation or the internalization is a slow process (Parker *et al.* 2001). The truncated forms of NPY, such as NPY_{2-36}, NPY_{3-36}, NPY_{13-36}, NPY_{18-36} and even NPY_{22-36}, have been shown to bind with high affinity to the Y2 receptor (Michel *et al.* 1998). The Y2 receptors are believed to be presynaptic (Wahlestedt *et al.* 1986) and involved in suppression of transmitter release (Westfall *et al.* 1990). The Y2 receptors have been found in the endothelium (Zukowska-Grojec *et al.* 1998). In the CNS the Y2 receptors are located mainly in the hippocampus (Dumont *et al.* 1992; Westfall *et al.* 1990), but also in other mammalian CNS areas such as substantia nigra, brainstem and hypothalamus (reviewed by Dumont & Quirion 2006). As Y2 receptor densities and expression levels were studied in brain tumors, it was detected that the Y2 receptor is prominent in especially glioblastomas (Korner &

Reubi 2008). The Y2 receptor has also been shown to be involved in the increase of food intake (Batterham *et al.* 2002), angiogenesis (Lee *et al.* 2003; Zukowska *et al.* 2003a), anxiety (Nakajima *et al.* 1998), gastrointestinal motility (Chen *et al.* 1997), circadian rhythms (Gribkoff *et al.* 1998; Huhman *et al.* 1996), cardiovascular regulation (Morton *et al.* 1999), neuronal excitability (Colmers & Bleakman 1994), regulation of bone formation (Baldock *et al.* 2002) nociception (Chen *et al.* 1997) and slowing the gastric emptying (Ishiguchi *et al.* 2001). It has been suggested that the Y2 receptor is involved in the formation of diabetic retinopathy (Koulu *et al.* 2004). Diabetic retinopathy in T2D patients with NPY signal sequence L7P polymorphism was over-represented (Koulu *et al.* 2004). Moreover, the development of retinal neovascularization in neonatal mice and rats was prevented in $Y2^{-/-}$-mice and in rats treated with the Y2-receptor antisense oligonucleotide (Koulu *et al.* 2004). Another finding on the association of Y2 receptor with T2D has also been done (Campbell *et al.* 2007).

The NPY y7 receptor has been cloned in teleost fish and amphibians (Fredriksson *et al.* 2004). Recently the y7 receptor has been cloned in chicken but the functional role of the y7 receptor is still unknown (Bromee *et al.* 2006). The y7 mRNA expression in zebrafish has been found in the GI tract, eye and brain and in chicken the y7 mRNA was detected in the adrenal gland (Bromee *et al.* 2006).

2.1.3.3 The Y5 subfamily

The single receptor of the Y5 subfamily is the Y5 receptor. The Y5 (MIM 602001) receptor gene has been located in the 4q31-q32 chromosome. The human Y5 receptor is transcribed in opposite direction to the Y1 gene and the Y5 receptor gene overlaps the promoter region of the Y1 receptor gene. The common promoter regions of the Y1 and Y5 receptors suggest a partial coordinated transcriptional regulation (Herzog *et al.* 1997). The Y5 receptor is a 446 aa protein and has all the characteristics of a GPCR. The sequence homology between the Y5 and other receptor subtypes is only 30% (Larhammar & Salaneck 2004). The Y5 receptor is mainly expressed in the brain, hypothalamus, hippocampus and amygdala (Parker & Herzog 1999; Weinberg *et al.* 1996). It has also been found in blood vessels and the intestine (Goumain *et al.* 1998). In the periphery the Y5 receptor has been found in organs such as the testes, spleen and pancreas (Statnick *et al.* 1998). The main physiological function of the Y5 receptor has been found to be in the regulation of feeding behaviour (Parker & Herzog 1999). It also regulates luteinizing hormone (LH) secretion (Raposinho *et al.* 1999) and kidney function (Bischoff *et al.* 1997). This receptor has been proved to be involved in sedation (Sorensen *et al.* 2004) and epileptic seizure formation (Woldbye & Kokaia 2004). Anxiolytic and orexigenic effects are mediated via Y1 and Y5 receptors, which appear to be colocalized in many brain regions and these two receptors have been detected to function as heterodimer receptor (Gehlert *et al.* 2007).

Table 2.2. The receptor subtype and size in humans, endogenous NPY agonists, receptor location and effects mediated through different NPY receptors, H=verified in humans.

Receptor subtype and size	Agonists	Antagonists	Receptor localization	Effects	References
Y1 **384**	NPY, PYY, PP [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY, [Arg^{6}, Pro^{34}]NPY, [Phe^{7}, Pro^{34}]NPY PYY3-36,	BIBP3226, BIBO3304, GR231118, GR231118-OMe, GI264879A, LY357897, SR120819A, 1229U91	**Periphery:** colon, kidney **(H)**, adrenal gland, heart **(H)**, placenta, coronary and arterial smooth muscle **(H)**, blood vessels **(H)**, intestine, spleen, testes, lymphocytes, adipocytes **(H)**, dental pulp tissue **(H)** CNS: hypothalamus **(H)**, cerebral cortex **(H)**, limbic and neocortical regions **(H)**, amygdala in the frontal brain areas	vasoconstriction **(H)**, blood pressure, anxiolysis, depression, GHRH secretion, leptin release, insulin release, catecholamine release, ethanol intake **(H)**, food intake, analgesia	(Caberlotto *et al.* 1997; Cavadas *et al.* 2006; Dumont & Quirion 2006; El Karim *et al.* 2008; Fuhlendorff *et al.* 1990a; Gamba & Pralong 2006; Heilig *et al.* 1993; Jacques *et al.* 2003; Jacques & Abdel-Samad 2007; Kalra *et al.* 1990; Kamiji & Inui 2007; Kanatani *et al.* 2001; Kask *et al.* 2001; Merten & Beck-Sickinger 2006; Morgan *et al.* 1998; Petitto *et al.* 1994; Thiele *et al.* 2002; Wahlestedt & Hakanson 1986; Wetherill *et al.* 2008; Wharton *et al.* 1993)
Y2 **381**	NPY, PYY, NPY2-36, NPY3-36, PYY3-36, NPY13-36, C2-NPY, CycloS-S[Cys^{20}, Cys^{24}]NPY	BIIE0246, JNJ-5207787	**Periphery**: endothelium **(H)**, blood vessels **(H)**, nerve fibres CNS: hypothalamus, brainstem, spinal cord, human cerebral cortex **(H),** frontal brain: hippocampus **(H)**, substantia nigra, amygdala **(H)**, striatum **(H)**, nucleus accumbens **(H)**	suppression of transmitter release, feeding behaviour **(H)**, angiogenesis **(H)**, anxiety, gastrointestinal motility, circadian rhythms, cardiovascular regulation **(H)**, neuronal excitability, regulation of bone formation, nociception, diabetic retinopathy **(H)** and T2D **(H)**	(Baldock *et al.* 2002; Batterham *et al.* 2002; Campbell *et al.* 2007; Colmers & Bleakman 1994; Dumont *et al.* 1992; Dumont & Quirion 2006; Huhman *et al.* 1996; Ishiguchi *et al.* 2001; Kamiji & Inui 2007; Koulu *et al.* 2004; Lee *et al.* 2003; Merten & Beck-Sickinger 2006; Michel *et al.* 1998; Morton *et al.* 1999; Westfall *et al.* 1990; Zukowska *et al.* 2003a; Zukowska-Grojec *et al.* 1998)
Y4 **375**	NPY, PP, [Leu^{31}, Pro^{34}]PYY, [Leu^{31}, Pro^{34}]NPY, PYY3-36, PP2-36, PP20-36, GR231118	1229U91	**Periphery**. Pancreas **(H)**, prostate **(H)**, uterus **(H)**, gastrointestinal tract **(H)**, heart, blood vessels **(H)**, skeletal muscle **(H),** kidney **(H)** CNS: hypothalamus **(H)**, frontal brain **(H)**, especially hippocampus **(H)** and preoptic area	gall bladder contraction, gastrointestinal motility, gastric acid secretion and corticosteroid secretion, food intake, LH secretion	(Andreis *et al.* 1993; Balasubramaniam *et al.* 2006; Bard *et al.* 1995; Dumont & Quirion 2006; Foti *et al.* 2007; Fuhlendorff *et al.* 1990b; Gerald *et al.* 1995; Gregor *et al.* 1996b; Kamiji & Inui 2007; Lundell *et al.* 1995; McTigue *et al.* 1993; Merten & Beck-Sickinger 2006; Schwartz 1983)
Y5 **445 or 455**	NPY, PYY,NPY2-36, NPY3-36, PYY3-36, [Leu^{31}, Pro^{34}]PYY, [Leu^{31}, Pro^{34}]NPY, PP, GR231118	CGP71683A, JCF 109, NPY5RA, GW438014A, L-152.804, CP732925, FMS586	**Periphery:** intestine, periphery; testes, spleen, skeletal muscle, pancreas, blood vessels, kidney **CNS:** hypothalamus **(H)**, especially arcuate nucleus **(H)**, substantia nigra **(H)**, hippocampus, amygdala **(H)**, preoptic area	feeding behaviour **(H)**, reproduction, LH secretion , kidney function, sedation, epileptic seizure formation, anxiolysis and orexigenesis	(Bischoff *et al.* 1997; Dumont & Quirion 2006; Gehlert *et al.* 2007; Goumain *et al.* 1998; Jacques *et al.* 1996; Merten & Beck-Sickinger 2006; Nichol *et al.* 1999; Parker & Herzog 1999; Raposinho *et al.* 1999; Sorensen *et al.* 2004; Statnick *et al.* 1998; Weinberg *et al.* 1996; Woldbye & Kokaia 2004)

2.2 PHYSIOLOGICAL FUNCTION OF NPY

2.2.1 NPY and the central nervous system

NPY is generally expressed in the central and peripheral nervous systems and it is involved in many physiological functions. Shortly after the discovery of NPY, it was linked to blood pressure regulation (Pernow *et al.* 1987; You *et al.* 2001). NPY and its expression have been extensively studied in the CNS where it serves as a neurotransmitter and/or a modulator of the neuronal function. In the earliest studies of NPY distribution in human brain, NPY-ir was found to be highest in basal ganglia, nucleus accumbens and amygdala, moderate amounts of NPY-ir was found in the hypothalamus, hippocampus, septal nuclei, cortex and periaqueductal grey (PAG) (Adrian *et al.* 1983; Dawbarn *et al.* 1984; Schwartzberg *et al.* 1990). The distribution of NPY-ir has been widely studied in animal studies as well. The parallel human and rat brain studies have given a different result of the distribution of NPY-ir. In rats the NPY-ir was highest in PAG, nucleus accumbens, hypothalamus, septum and amygdala, lower amounts of NPY-ir were found in basal ganglia, globus pallidus, hippocampus and cortex (Adrian *et al.* 1983). NPY has also been identified in human, lemur and rat spinal cord where it appears to be concentrated in the dorsal horn region (Allen *et al.* 1984; Gibson *et al.* 1984).

NPY has an important role in the feeding behaviour. The “axis of hunger” is from arcuate nucleus of the hypothalamus to the paraventricular nucleus of the hypothalamus (de Quidt & Emson 1986). Feeding behaviour has mainly been studied in animals. For example, injection of NPY into the hypothalamus of rats potently stimulates food intake and decreases energy expenditure while it simultaneously induces lipogenic enzymes in liver and white adipose tissue (WAT) (Schwartz *et al.* 2000). Continuous or repeated central administration of NPY leads to obesity (Schwartz *et al.* 2000). Leptin and insulin are closely related to the *NPY* gene expression. In the state of starvation leptin and insulin levels are low and this results in increased NPY expression and secretion in the hypothalamus, especially in the arcuate nucleus (Elmquist *et al.* 1998). For example leptin deficient knockout (KO) mice and rats with insulin deficient diabetes are characterized by high NPY synthesis and hyperphagia (Beck 2000; Schwartz *et al.* 1996). Human studies have not been conducted in large scale studies. Recently NPY signal peptide L7P polymorphism has been associated with increased food intake in humans (Ding *et al.* 2005). NPY and its receptors have also been of interest in the development of anti-obesity drugs. Data from the antisense oligodeoxynucleotides or blockade of NPY action by intraventricular infusion of NPY antibody has provided rather convincing evidence that appropriate antagonism of NPY action could lead to useful therapies for treating obesity (reviewed by Kamiji & Inui 2007; MacNeil 2007). Numerous studies concerning Y1, Y2, Y4 and Y5 receptors and the putative anti-obesity drugs have already been conducted. Nevertheless, further investigations involving simultaneous activation and inhibition of NPY receptors are still required (Kamiji & Inui 2007; MacNeil 2007).

NPY has also an important role in the neuroendocrine regulation as it influences the secretion and release of many hormones such as CRH, TRH, LH, GnRH, and GHRH (reviewed by Pedrazzini *et al.* 2003). The activity of the neuroendocrine system is closely associated with the energy homeostasis (Pedrazzini *et al.* 2003). The reproductive axis and the sexual behaviour have been studied in animal models and the results have been parallel. As NPY promotes hunger, it simultaneously shuts off the sexual drive and reproduction (reviewed by Kalra & Kalra 2004).

The effects of NPY and alcohol drinking have been studied mostly in animal studies, for example, using Indiana alcohol preferring (P) and non-preferring (NP) rats. In P rats the amount of NPY in amygdala, frontal cortex and hippocampus was significantly lower in comparison to NP rats, but higher levels of NPY were found in the hypothalamus (Ehlers *et al.* 1998). NPY possesses anxiolytic properties when infused into the area of amygdala (Heilig *et al.* 1989; Heilig *et al.* 1992; Heilig *et al.* 1993) and it has a role in alcohol intake, dependence, and withdrawal (reviewed by Thorsell 2007). Some human studies connecting NPY and alcohol dependence have been performed. Clinical studies of alcohol dependence show an association between initial anxiety and alcohol abuse possibly due to the anxiolytic action of alcohol (Pandey *et al.* 2003a; Pandey *et al.* 2003b). Furthermore, NPY expression was decreased in alcoholics and that may also play a role in human alcoholism (Mayfield *et al.* 2002).

Preclinical and clinical studies show increasing evidence that NPY is involved in mood disorders such as depression (reviewed by Thorsell 2007). Heilig et al. have found decreased plasma levels of NPY from depressive and suicidal patients (Heilig *et al.* 2004; Heilig 2004). When the depression was treated with repeated electroconvulsive shock treatments, NPY levels in the depressed patients were elevated (reviewed by Thorsell 2007).

NPY possesses sedative effects and it plays a role in epilepsy as well (Naveilhan *et al.* 2001; Silva *et al.* 2002). NPY over-expression in the hippocampus has been reported in various seizure models such as status epilepticus, kindling and genetically determined seizures (Noe *et al.* 2007). Noe et al. (2008) have provided evidence that intra-hippocampal injection of recombinant adeno-associated viral vector expressing the human NPY gene results in long-lasting peptide over-expression in neurons, decreases spontaneous seizure frequency and arrests seizure progression.

NPY and its receptors (Y1 and Y2) have been suggested to have a role in acute and chronic pain as well as neuropathic pain. Pain has mainly been studied in the rodent models. In models of acute pain, early behavioural studies have shown that spinal administration of NPY and Y2 receptor agonists decrease nociception (Smith *et al.* 2007). Y2 receptors may contribute to the antinociceptive actions of NPY by inhibiting excitatory neurotransmitter release from primary afferent terminals in substantia gelatinosa (Smith *et al.* 2007). A dramatic increase of NPY in dorsal root ganglion neurons has been detected after peripheral nerve injury (Hökfelt *et al.* 2007). Spinal administration of a Y1 (BIBO3304) or a Y2 (BIIE0246) receptor antagonist have dose-dependently shown to reverse the anti-allodynic actions of NPY, which could indicate that NPY acts at spinal Y1 and Y2 receptors to reduce spinal neuron activity and behavioural signs of inflammatory or neuropathic pain (Intondi *et al.* 2008).

2.2.2 NPY in the periphery

2.2.2.1 NPY and adipose tissue

There are two types of adipose tissue in humans, the white WAT and the brown adipose tissue (BAT). White adipose cells consist of a lipid droplet surrounded by the cytoplasm and the nucleus on the side. WAT has become an attractive study topic because of the increasing problem of obesity especially in the western world. One primary function of white adipocytes is to store excess energy (Avram *et al.* 2005). Brown adipocytes are polygonal in shape, and unlike white adipocytes, they contain a considerable amount of cytoplasm. Newborn babies have BAT as an energy source and the energy is released from the cells in the form of heat (Cinti 2006). In adults the amount of BAT is insignificant. The sympathetic nervous system

(SNS) innervates WAT and BAT and is partly responsible for thermogenesis and energy metabolism by relaying signals from the CNS (reviewed by Turtzo & Lane 2006).

The role of NPY in the adipocytes and adipose tissue is fairly complex and unclear. However, NPY has a clear role in the thermogenesis and energy metabolism. NPY has been proved to be expressed in the human adipose tissue and secreted by adipocytes (Kos *et al.* 2007). Leptin is produced mainly by WAT (Zhang *et al.* 1994), but small amounts of leptin are also produced by the hypothalamus (Morash *et al.* 1999), stomach (Bado *et al.* 1998) and pituitary (Jin *et al.* 2000; Popovic *et al.* 2001). In addition to the energy metabolism in adipose tissue, leptin has also been found to take part in several physiological functions such as bone formation, reproduction and cardiovascular functions (Sahu 2003). Leptin-sensitive neurons include those that produce NPY and several other peptides that take part in the energy metabolism (Sahu 2003). In the CNS, NPY is a potent orexigenic agent with leptin as the antagonistic agent (Schwartz *et al.* 2000). The central effects of NPY are thought to be mediated by the SNS (Turtzo & Lane 2006). Even though it has been experimentally shown that SNS innervates WAT, the exact way of the innervation is not yet known (Turtzo & Lane 2002). A number of possibilities have been suggested for the innervation of WAT. It may be that sympathetic fibres traverse adipose tissue while proceeding to other target tissues, or innervate only the vasculature of WAT (Turtzo & Lane 2006). However, direct innervation of adipocytes may also be possible (Turtzo & Lane 2006).

The data from the adipocyte/neuron co-cultures provides evidence that there is continuous cross talk between adipocytes and sympathetic neurons in the periphery (Turtzo & Lane 2006). Furthermore, in co-culture studies NPY strongly inhibits lipolysis (Turtzo *et al.* 2001). It has been found that when adipocytes are exposed to equimolar concentrations of NPY and leptin, the lipolytic rate is equivalent to the basal rate. This result indicates that there is a local homeostatic mechanism between leptin and NPY (Martínez *et al.* 2000). When insulin was added to the medium, the secretion of NPY by neurons was diminished in co-cultures (Turtzo *et al.* 2001). Insulin is an antilipolytic agent and if insulin is present, a need for another antilipolytic agent, such as NPY, is diminished (Turtzo & Lane 2006) (Figure 2.5).

2.2.2.2 NPY and bone

The bone tissue represents about 15% of the human body weight. In the normal skeleton, bone is constantly remodeled with the removal of small volumes of bone by the bone-resorbing cells, osteoclasts, and then replaced by the bone-forming cells, osteoblasts. When the balance between osteoclasts and osteoblasts is disturbed, the mass of bone may be lowered and the microarchitechture of the bone may be damaged and a rather common disease, osteoporosis, may occur. Osteoporosis and the risk of bone fracture is an increasing problem among the postmenopausal women and the elderly. (Allison & Herzog 2006).

As stated previously, NPY and leptin are important factors in energy homeostasis. The main role of NPY is in the maintenance of energy homeostasis. It is commonly known that a relationship between the body weight and the bone density exists (Allison *et al.* 2007) and obesity caused by an imbalance in energy homeostasis, can protect against osteoporosis (Albala *et al.* 1996; Tremollieres *et al.* 1993). Furthermore, the regulation of bone remodelling in the CNS and peripherally is a complex system which is not yet fully understood. The central regulation of the bone remodelling can be divided into four phases, which links hypothalamus and afferent and efferent signaling (reviewed by Rosen 2008). (Figure 2.5).

The NPY-ir studies of the bone tissue have provided evidence that NPY is localized in the bone. This immunoreactivity seems to be associated with blood vessel walls in the bone rather than bone cells (Allison *et al.* 2007). However, immunoreactive fibers have also been found in the bone lining and marrow cells (Ahmed *et al.* 1994). The relationship between NPY and the bone formation has mainly been studied in KO animal models. Leptin KO and NPY receptor KO models have revealed the involvement of especially Y1 and Y2 receptors in the regulation of bone formation (Allison & Herzog 2006; Lundberg *et al.* 2007). For example the Y2 receptor pathway seems to be distinct from the antiosteogenic pathway regulated by leptin. It has also been studied that if the Y2 receptor is deleted in the germ line, Y1 receptors are over-stimulated (Lundberg *et al.* 2007). Y1 receptor expression within bone cells in the absence of Y2 receptors are a likely mechanism for the greater bone mineralization *in vivo* and *in vitro*. This might be one possible approach to potential new treatments for osteoporosis (Lundberg *et al.* 2007).

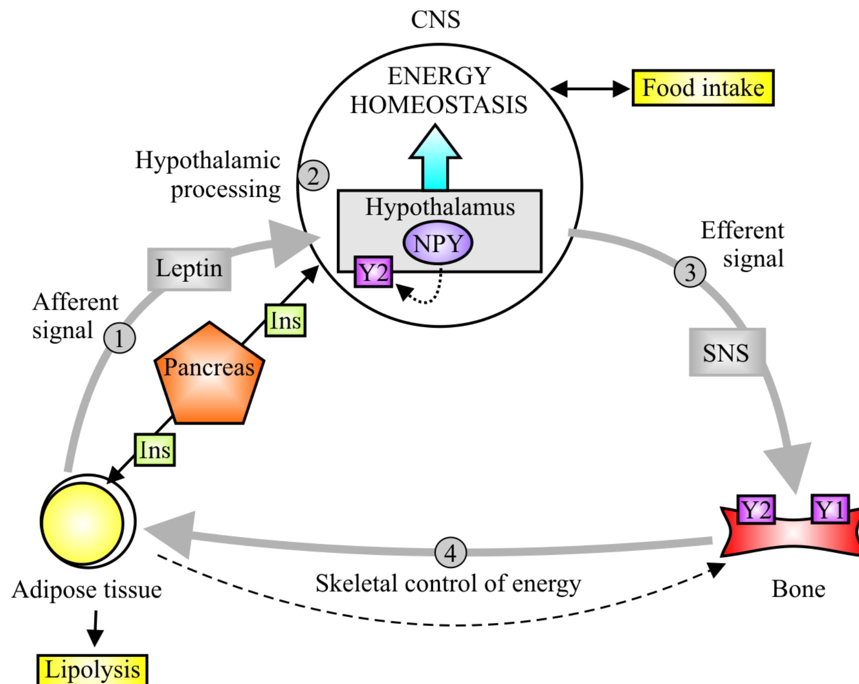

Figure 2.5. Proposed pathways to brain-adipocyte-bone axis. NPY is in a key role in the hypothalamus taking part of the feeding behaviour as well as in the communication between the SNS and the adipose tissue. Thermogenesis is regulated with co-operative actions between the SNS and the CNS, especially hypothalamus. In addition, central regulation of bone remodelling has been proposed to happen through the hypothalamus and it is determined by both afferent and efferent signaling. In phase 1 (indicated with number 1 in the picture) the afferent leptin signal originates from peripheral adipocytes. Additionally, insulin secreted from the pancreas is an important factor affecting fat metabolism. Phase 2 (indicated with number 2) involves the processing of the afferent signal in the hypothalamus NPY being one of the mediators in the afferent signal processing. Phase 3 (indicated with number 3) represents the efferent (sympathetic) output from the hypothalamus. Phase 4 (indicated with number 4) represents the putative skeletal regulation of adipocytes. The dotted line in phase 4 represents the theoretical possibility that adipocytes could regulate proliferation and differentiation of the cells in the bone tissue. Y2 receptors are thought to be the main signal mediating receptors in the hypothalamus and Y1 and Y2 receptors mediate the signalling in the bone tissue. Abbreviations: Ins, Insulin; Y1, NPY receptor 1; Y2, NPY receptor 2. Modified from (Turtzo & Lane 2006), (Allison & Herzog 2006) and (Rosen 2008).

2.2.2.3 NPY in kidney, adrenal gland and liver

Kidneys are organs that filter wastes from blood and excrete them with water and urine. Kidneys consist of about one million functional units called nefrons which are a part of the filtration system and osmoregulation. The kidney is also involved in hormonal actions and it contributes to the blood pressure regulation via renin-angiotensin-aldosterone mechanism. NPY has been found in the kidney of man, rat, monkey, mouse, hamster and guinea pig (Allen *et al.* 1985; Norvell & MacBride 1989; Reinecke & Forssmann 1988). It has been localized in adrenergic nerves of the renal arterial system as well as renal tubules of the human kidney (Norvell & MacBride 1989). The localization of the C-PON in the renal tubules suggests that the peptide may be generated also by the nephron and not only by the sympathetic nerve endings (Grouzmann *et al.* 1994). The mRNA of the Y1 receptor has been localized in the renal collecting ducts, loop of Henle and juxtaglomerular apparatus (Wharton *et al.* 1993). NPY agonist and antagonist studies have given an indication of the Y2 receptor existence in the kidney, but the results vary between species (Bischoff & Michel 1998) and the existence of the Y5 receptor in the kidney is still uncertain (Bischoff & Michel 1999; Gerald *et al.* 1996). The ability of NPY to alter the kidney function has been indicated in several studies. NPY may exert renal vasoconstrictor and tubular actions, but their functions depend on the species. NPY may also influence renin secretion (reviewed by Winaver & Abassi 2006). Renal blood flow and renal vascular resistance have been shown to be affected by NPY. In fact, the kidney seems to be uniquely sensitive to the vasoconstrictor effect of NPY (Bischoff & Michel 1998; el Din & Malik 1988; Minson *et al.* 1989).

The adrenal gland consists of two parts, adrenal medulla and adrenal cortex. The adrenal gland is one of the endocrine glands in mammals. Animal studies have revealed that the NPY concentration is higher in the adrenal medulla than in the cortex (Allen *et al.* 1983). At least two types of NPY receptors have been found in the adrenal gland; Y1 and Y4 (Sanabria & Silva 1994). NPY influences the adrenal cortex probably by an indirect mechanism that involves the local release of catecholamines (reviewed by Renshaw & Hinson 2001). On the contrary, in the adrenal medulla the secretion of catecholamines seems to be inhibited by NPY (reviewed by Renshaw & Hinson 2001).

The liver has multiple important functions such as detoxification, plasma protein synthesis, storage of glycogen, decomposition of red blood cells, etc. Liver and NPY have not been studied to a large extent. Some animal studies mainly on NPY and neurons that innervate liver have been performed. A study conducted with rats concluded that NPY gene expression is differentially regulated by caloric restriction in the hypothalamus and liver, as well as other abdominal organs (Sucajtys-Szulc *et al.* 2008). In the hypothalamus, the caloric restriction increased NPY mRNA level, while NPY mRNA level was decreased at the same time in the liver (Pesonen *et al.* 1992; Sucajtys-Szulc *et al.* 2008). The effect of NPY has also been studied on the hepatic arterial conductance (HAC) in the canine liver. NPY seemed to lower the HAC when it was infused alone into the hepatic artery (Mundinger & Taborsky 2000). This study concluded that NPY could act as an independent neurotransmitter in the liver (Mundinger & Taborsky 2000).

2.2.2.4 NPY and heart

In the heart NPY was first identified in intramural nerves associated with coronary blood vessels and muscle (Gu *et al.* 1984). NPY has been found in the sympathetic nerve endings of the human heart as well as in the heart of other species such as pigs, rabbits and rats (Onuoha *et al.* 1999). More specifically, NPY-ir has been located in the neurons of the endocardium (Marron *et al.* 1994), myocardium (Richardson *et al.* 2006), intracardiac ganglia (Richardson *et*

al. 2003) and postganglionic sympathetic nerves innervating blood vessels (Franco-Cereceda *et al.* 1985). It has been known for a while that NPY has multiple effects on the heart. It regulates the autonomic tone, cardiac contractility and circulation in animals (Allen *et al.* 1983; Balasubramaniam *et al.* 1988), as well as in humans (Franco-Cereceda *et al.* 1989; Franco-Cereceda 1989). More recently, NPY has been found to act as a local transmitter that produces inotropic and chronotropic effects (McDermott *et al.* 1993). Furthermore, NPY has been found to act as a prohypertrophic stimulus in cardiomyocytes (Millar *et al.* 1994). A correlation has been shown between plasma NPY levels and the amount of post-exercise ischemia in patients with coronary heart disease (Madsen *et al.* 1993). On the other hand, in acute heart failure the circulating NPY levels were raised in 25% of the patients (Hulting *et al.* 1990).

NPY receptors Y1 and Y2 have been found in the heart (Allen *et al.* 1993). Y1 and Y2 receptors have been located in human cardiac samples acquired from individuals without a history of cardiac disease (Jönsson-Rylander *et al.* 2003). NPY receptors have also been proved to take part in chronic heart failure in an animal model (Callanan *et al.* 2007). NPY and NPY Y1 receptor have been located in the human EECs. NPY has also been proved to regulate the function EECs (Abdel-Samad *et al.* 2007; Jacques *et al.* 2003). One important factor in the normal function of EECs is calcium. Calcium ion acts as an intracellular messenger and it creates electrochemical gradients between extracellular and intracellular spaces as well as intracellular compartments of the cell, such as mitochondria. Muscle contraction is one basic function caused by rapid rise in the cytosolic calcium level. The intracellular calcium amount has also been proved to be affected by NPY (Abdel-Samad *et al.* 2007; Silva *et al.* 2005). In EECs NPY causes a sustained increase of the intracellular calcium levels. The NPY-promoted release of calcium affects the excitation-contraction coupling of heart cells (Abdel-Samad *et al.* 2007). In addition to the effect of NPY, EECs control the contraction of cardiac myocytes by releasing nitric oxide (NO) (Brutsaert 2003; Shah *et al.* 1996). These results suggest that NPY has an important role in EEC and furthermore, heart function (Jacques & Abdel-Samad 2007).

2.2.2.5 NPY and smooth muscle

Smooth muscle cells (SMC) are found in multiple places through the human body. Smooth muscle is specialized in forceful contractions of short periods of time and also long lasting contractions of relatively low force. Smooth muscle is found, for example, in the walls of internal organs and most blood vessels. SMCs are in close contact with ECs and they mediate messages to and from ECs.

NPY has been studied extensively in VSMCs. In VSMC from human subcutaneous arteries and veins NPY has been shown to stimulate growth along with NA and ATP (Erlinge *et al.* 1994). NPY has been shown to be mitogenic also in the rat VSMCs and the receptors mediating this mitogenic effect seem to be Y1, Y2 and Y5 (Pons *et al.* 2003; Zukowska-Grojec *et al.* 1993). Further studies have shown that primarily the Y1 receptor mediates mitogenesis signals by activating Ca^{2+}-dependent and growth-promoting pathways (Pons *et al.* 2008). Cerebral arteries have been studied in rat as well as in human, and the effect of NPY is mediated through the Y1 receptor in SMCs (Bao *et al.* 1997; Nilsson *et al.* 1997; You *et al.* 2001).

NPY has an important role in blood pressure regulation. It induces constriction on the SMC side of the rat middle cerebral arteries (MCAs) but also dilation on the endothelial side of the MCAs when administered locally (Edvinsson *et al.* 1985; You *et al.* 2001). When NPY was administered to the endothelial side of MCAs, vessels were dilated through a mechanism that involved NO release (You *et al.* 2001). Constriction has also been studied in the human cerebral arteries (Nilsson *et al.* 1997). In cerebral arteries the constriction appears to be mediated by

changes in the smooth muscle membrane potential and also extracellular calcium influx (Edvinsson 1985). In situations of increased blood pressure, such as exercise or some forms of hypertension, circulating levels of NPY were raised in humans (Solt *et al.* 1990). When the blood pressure of NPY transgenic rats was studied, it was noticed that endogenously caused increase in the expression of NPY did not affect resting blood pressure, but it increased total vascular resistance and enhanced blood pressure responsiveness to NA (Michalkiewicz *et al.* 2001). Another finding of the same study was detected after acute hypotensive hemorrhage; an endogenously upregulated NPY played an important role in the spontaneous recovery of the blood pressure (Michalkiewicz *et al.* 2001). Hence, Michalkiewicz et al. (2001) concluded that endogenous NPY has an important role in the blood vessel tone regulation. In addition to the studies on the vascular tone, the effect of NPY to the SMC has been investigated in the female reproductive system, in human intestinal circular and longitudinal smooth muscle, tracheal vessels and rat colon. In all of these studies NPY induced a muscle contraction (Félétou *et al.* 1998; Laitinen *et al.* 1987; Markiewicz *et al.* 2003; Misra *et al.* 2004; Misra *et al.* 2005). The NPY KO and overexpressing (OE) animal studies are discussed in more detail in section 2.3.

2.2.3 Endothelial function and NPY

2.2.3.1 Endothelium

Endothelium lines all vessels, wheather small or large, with a single layer of cells. In the smallest vessels, capillaries, the vessel consists of only one EC layer that is able to surpass gases and molecules. The underlying vascular smooth muscle cell layer is a multilayered structure that is in close relationship with the endothelium. The main functions of endothelium are blood vessel permeability, blood pressure homeostasis, blood coagulation, fibrinolysis, transmigration adhesion and maintaining the physiological homeostasis (Bouis *et al.* 2001; Brandes *et al.* 2005). ECs do not replicate often and the degeneration of endothelium starts approximately at the age of 30. After that the risk for development of atherosclerosis increases substantially. ECs are basically in a stage called replicative senescence, i.e. they remain quiescent (Foreman & Tang 2003; von Zglinicki 2003). This means that the cells are viable and functioning but they do not divide. ECs divide approximately once every 3 years under normal conditions (Foreman & Tang 2003).

The surface of the endothelium is quite large and not all ECs function similarly. They maintain different functions according to their place and the size of the vessels (Bouis *et al.* 2001). One of the most commonly used models to study human ECs is HUVECs. The first protocol for culturing ECs was created by Jaffe and Nachman in 1973 (Jaffe *et al.* 1973). They also characterized the phenotype of the cells and concluded that after approximately 10 passages the cells do not divide *in vitro* anymore (Jaffe *et al.* 1973). The senescent cells are polynuclear, they contain vacuoles, are increased in granularity and giant in size. HUVECs (Silva *et al.* 2003; Zukowska-Grojec *et al.* 1998), human EECs (Jacques *et al.* 2003), as well as some other EC types, such as rat retinal ECs (Alvaro *et al.* 2007) and rabbit vascular ECs (Loesch *et al.* 1992) have been proved to produce NPY.

2.2.3.2 NO and NOS

NO is a gas synthesized by a family of nitric oxide synthase (NOS) enzymes. The half life of NO is few seconds. There are several isoforms of NOS found in humans and other organisms. Three isoforms have commonly been recognized, neuronal NOS (nNOS, NOS-1), inducible NOS (iNOS, NOS-2) and endothelial NOS (eNOS, NOS-3) (Napoli *et al.* 2006). There has also been discussion about the existence of mitochondrial NOS (mtNOS). NOS enzymes all produce

NO and citrulline in a five electron oxidation cascade of guanidine-nitrogen terminal of L-arginine (Lacza *et al.* 2006). Of these NOS isoforms nNOS and eNOS are regulated by calcium and calmodulin (CaM) and by post-translational modification of these enzymes. Inducible NOS is regulated by cytokine stimulation and this isoform is the biggest NO producer. NO synthases require several cofactors for proper function, such as tetrahydrobiopterin, nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide and flavin mononucleotide (Napoli *et al.* 2006). NOS enzymes are usually referred as being dimeric. In their active form they require CaMs and strictly speaking, the active form is a tetramere consisting of two NOS monomers associated with two CaMs (Alderton *et al.* 2001).

The issue of mitochondrial NOS (mtNOS) is controversial. Some studies convince that mtNOS exists (Ghafourifar & Richter 1997; Giulivi 1998) while others give a reason to believe it to be a different form NOS transported to mitochondria (reviewed by Brookes 2004). One of the controversial questions has been whether or not there are mitochondrial mechanisms which produce NO (reviewed by Lacza *et al.* 2006). In this paper Lacza states that based on their own research data and data also by others, it can be reasoned that mitochondria do not contain genuine NOS isoform (Lacza *et al.* 2006). On the other hand mtNOS has been described in rat liver (Ghafourifar & Richter 1997; Giulivi 1998), thymus (Bustamante *et al.* 2000) and brain (Riobo *et al.* 2002).

NO and eNOS are important components in the function of the endothelium. It has been said that this versatile and multifaceted NO and eNOS system is extremely vulnerable and the balance of the system can be disturbed easily. Disturbing factors can be such as inflammation, atherosclerosis and hypertension, psychological and physical stress (reviewed by Napoli *et al.* 2006). Endothelial NOS is localized in caveolae (Garcia-Cardena *et al.* 1996; Shaul *et al.* 1996), which are invaginations of 50-100 nm in size at the surface of the plasma membrane (Frank *et al.* 2003).

NO and eNOS have also been associated with ageing but there has been some controversy about the kind of changes that happen in ageing. Some studies have shown that the production of NO declines with age (Barton *et al.* 1997; Csiszar *et al.* 2002; Tanabe *et al.* 2003; Tschudi *et al.* 1996) and other studies have proved that eNOS expression has increased during ageing (Goettsch *et al.* 2001; van der Loo *et al.* 2000). NO has been described as the primary endothelium derived autacoid (Radziszewski *et al.* 1995) and also antiatherosclerotic principle (Fleming & Busse 1999). In vascular homeostasis NO has a protective role against atherosclerotic changes. For example, it suppresses abnormal proliferation of VSMCs, controls homeostasis, fibrinolysis, platelet and leukocyte interactions with the arterial wall, regulates vascular tone and growth and homeostasis of blood pressure. NO has a regulator role in granule exocytosis and it also modulates the immune system. (reviewed by Napoli *et al.* 2006).

ECs can initiate contraction on the surrounding SMCs (reviewed by Vanhoutte *et al.* 2005). It is known that NPY modulates the release of different neurotransmitters such as NO (Silva *et al.* 2005). NO is one of the factors affecting the state of relaxation and contraction in vascular walls (reviewed by Vanhoutte *et al.* 2005). It has been hypothesized that NPY receptor activation enhances NO release and this leads to the inhibition of NA release (Bitran *et al.* 1999). This hypothesis is supported by the study of Bitran et al. where it was shown that NPY enhanced stimulation-induced NO release. Furthermore, NPY did not inhibit NA release when NO synthesis was prevented with a NOS inhibitor (Bitran *et al.* 1999). A study by Chen et al. suggested that the Y1 receptor activation mediates ischemic injury *via* NO overproduction. When the Y1 receptor function was inhibited, excessive NO production was suppressed and ischemic injury was diminished (Chen *et al.* 2002). In a recent study NPY was found to stimulate retinal neural cell proliferation through NO, cGMP and ERK pathways (Alvaro *et al.*

2007). NO and NPY may also contribute to a regulatory loop between the immune and the adrenal systems and hence contribute to pathological conditions such as infection, stress and hypertension (Rosmaninho-Salgado *et al.* 2009).

2.2.3.3 Endothelial dysfunction and mitochondrial theory of ageing

NO has an important role in the development of vascular endothelial dysfunction (VED). VED refers to an impairment of endothelium dependent vasodilation, which is caused by the decreased bioavailability of NO in the vessel wall (Rush *et al.* 2005). The decreased bioavailability of NO may be caused by different factors affecting eNOS production and accelerated NO degradation caused by reactive oxygen species (ROS) such as oxygen ions, free radicals and peroxides (Cai & Harrison 2000). VED has also been characterized as partial or complete loss of balance between vasorelaxation and vasoconstriction and thrombosis and thrombolysis (reviewed by Balakumar *et al.* 2008). NO degradation and increased ROS levels may damage the endothelium and thus initiate cardiovascular dysfunction (Taniyama & Griendling 2003). It was hypothesised already over 30 years ago that ROS and oxidative damage affect ageing and dysfunction (Harman 1972). Moreover, ROS affects vascular remodelling, EC migration, inflammatory cascade and apoptosis (Yung *et al.* 2006). It has also been found that VED is a key event in the pathogenesis of a variety of diseases, such as hypertension (Savoia & Schiffrin 2007), atherosclerosis (Davignon & Ganz 2004), heart failure (Desjardins & Balligand 2006), myocardial infarction (Pesic *et al.* 2006), diabetes (Mangoni *et al.* 2005) and stroke (Papaharalambus & Griendling 2007).

Endothelial progenitor cells (EPCs) are formed in the blood marrow and they circulate in the blood stream. They tolerate oxidative stress better than endothelium itself due to manganese superoxide dismutase (MnSOD) (He *et al.* 2004). MnSOD is a mitochondrially located SOD and it is a critical insintric mechanism for the EPC survival against oxidative stress (He *et al.* 2004). When ECs age they finally reach the state of senescence (Brandes *et al.* 2005). Senescent cells are metabolically active, but morphologically altered (Foreman & Tang 2003). Acidic β-galactosidase (SA-β-Gal) is a senescence-associated enzyme which increases in aging ECs (Brandes *et al.* 2005). SA-β-Gal activity has been observed to be increased in atherosclerotic plaques especially in ECs (Minamino *et al.* 2002). In ageing cells mitochondrial dysfunction increases and the leakage of the superoxide (O_2^-) and H_2O_2 increases (Brandes *et al.* 2005). MnSOD is the enzyme that causes O_2^- radicals to be processed further to H_2O_2. In ECs especially, there is NO present in high concentrations. NO reacts with O_2^- and forms peroxynitrate ($ONOO^-$) and the reaction cascade continues further with possible inactivation of MnSOD (Brandes *et al.* 2005). $ONOO^-$ can change the NO synthase from an NO-generating enzyme to an O_2^- -generating enzyme (NO synthase uncoupling) via the oxidation of BH_4 (Brandes *et al.* 2005). The result of the MnSOD inactivation and NO synthase uncoupling is an increase in O_2^- concentration which again leads to $ONOO^-$ formation and further to mitochondrial DNA damage. This is often called as a vicious circle which leads to mitochondrial dysfunction and ageing (Brandes *et al.* 2005) (Figure 2.6).

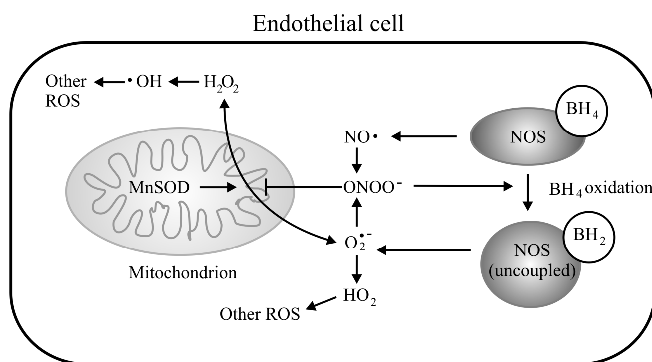

Figure 2.6. Potential mechanisms of ageing induced by oxidative stress in ECs. Modified from (Brandes *et al.* 2005; Jezek & Hlavata 2005).

The role of NPY in the endothelial dysfunction and ageing is not completely determined. Endothelial dysfunction is an early marker of atherosclerosis (Davignon & Ganz 2004) caused by a prolonged endothelial or smooth muscle damage and lipid accumulation (Badimon *et al.* 1993). NPY is potently angiogenic *in vitro* and *in vivo* and it stimulates EC adhesion to matrix and differentiation into capillary-like tubes (Zukowska *et al.* 2003a; Zukowska-Grojec *et al.* 1998). In many occasions NPY has been promoted as a pro-atherogenic factor, due to its potent vasoconstrictive effects and ability to accelerate vascular events such as restenosis (Kuo *et al.* 2007). Angiogenic effects of NPY are probably mediated via non-Y1 receptors, primarily Y2 receptors (Kuo *et al.* 2007). In aging, angiogenesis mediated via Y2 receptors were clearly reduced as well as the DPPIV amount (Kitlinska *et al.*2002).

2.2.3.4 NPY and NPY receptors in the endothelium

NPY has been shown to act on vascular ECs. NPY potentiates the effects of NA induced vasoconstriction especially in the endothelium of human saphenous veins (Fabi *et al.* 1998). EC migration, proliferation and adhesion on the extracellular matrix have also been proved to be promoted by NPY as well as the capillary tube formation *in vivo* and *in vitro* (Zukowska-Grojec *et al.* 1998). Immunoreactive NPY was first detected in HUVECs in 1993 (Cai *et al.* 1993a; Cai *et al.* 1993b). Later on, the production of NPY by ECs such as HUVECs, EECs and human lung microvascular endothelial cells (HMVEC-L) has been proved (Jacques *et al.* 2003; Silva *et al.* 2005; Zukowska-Grojec *et al.* 1998). HUVECs produce, store and respond to NPY (Silva *et al.* 2005; Zukowska-Grojec *et al.* 1998). The subcellular localization and immunoreactivity studies of NPY have shown that NPY is localized in the cytoplasm, nucleus, mitochondria and vesicle like structures in HUVECs (Silva *et al.* 2005).

HUVECs express NPY Y1 and Y2 receptor mRNA (Silva *et al.* 2005). Y1, Y2 and Y5 receptors are stimulated by NPY_{3-36} as well as NPY_{1-36} (Ghersi *et al.* 2001). It has been proposed that Y1, Y2 and Y5 receptors may act independently or as hetero-oligomeric complexes in human ECs (Movafagh *et al.* 2006). Furthermore, NPY and NPY Y1 receptor are expressed by EECs (Jacques *et al.* 2003). EECs line the cavity surface of the heart (Abdel-Samad *et al.* 2007). They differ from other cardiac and vascular ECs by being larger and very sensitive to circulating factors, such as angiotensin I (Jacques *et al.* 2003), endothelin-1 (Jacques *et al.* 2000) and NPY (Jacques *et al.* 2003). In addition to NPY, also NPY Y1 receptor has been localized in the cytosol and the nucleus of human and rat EECs (Jacques *et al.* 2003). Thus NPY acts on its receptors on the plasma membrane and the nuclear membrane levels (Jacques *et al.* 2003).

2.3 TRANSGENIC AND KNOCK OUT ANIMALS IN NPY STUDIES

2.3.1 NPY overexpressing and knock out animals

Transgenic OE and KO animal models have been a powerful way of studying the functions of the *NPY* gene (Lin *et al.* 2004). However, in transgenic OE and KO animal studies the possibility for compensatory mechanisms has to be considered. Several NPY and NPY receptor OE mice and rat models have been generated and analyzed. Inui et al. generated a transgenic mice model with 18% net increase in the arcuate NPY levels, but NPY levels did not show any increase in food intake or bodyweight (Inui *et al.* 1998). Thorsell et al. generated a hippocampal NPY OE rat model, which had decreased anxiety-related behaviour and spatial memory. This rat model gave more information about NPY-related stress and fear behaviour (Thorsell *et al.* 2000). Another NPY OE rat model proved that endogenous NPY is an important factor in the regulation of blood vessel tone, but body weight and food intake remained unchanged when compared to the controls (Michalkiewicz *et al.* 2001). Thiele et al. reported about the effects of NPY to ethanol intake. NPY OE mice had lower preference to ethanol and were more sensitive to the sedative and hypnotic effects of ethanol than wild type control mice (Thiele *et al.* 1998). The recent NPY OE mouse model by Ruohonen et al. (2008) showed that even a moderate increase in NPY levels in noradrenergic neurons leads to glucose and lipid metabolism problems that are key factors in the metabolic syndrome (Ruohonen *et al.* 2008a). However, the food intake was not changed in these OE mice (Ruohonen *et al.* 2008a). Furthermore, it was found that the overactive noradrenergic NPY system has a role in the regulation of blood pressure and stress response due to changes in the catecholamine concentrations in plasma and adrenal gland (Ruohonen *et al.* 2008b).

The first studies of the NPY KO mice ($NPY^{-/-}$) were generated by Erickson et al. (Erickson *et al.* 1996a; Erickson *et al.* 1996b). Under normal conditions the NPY KO mice did not show changes in food intake or body weight, but after fasting they showed hyperphagic behaviour (Erickson *et al.* 1996b). When NPY KO mice were treated with leptin, food intake and bodyweight were reduced when compared to the control mice (Erickson *et al.* 1996a; Erickson *et al.* 1996b). Furthermore, when these NPY KO mice were crossed onto leptin deficient *ob/ob* mice, significant phenotype change in the obese *ob/ob* mice was seen (Erickson *et al.* 1996a). These double KO mice had reduced food intake, increased energy expenditure and putatively developing diabetes (Erickson *et al.* 1996a). Several other double KO mice have been generated. For example NPY/agouti related peptide (AgRP) and NPY/yellow obese (A^y) double mutant mice. AgRP is an orexigenic peptide which is co-expressed with NPY in arcuate neurons (Qian *et al.* 2002). A^y mice have a defect in melanocortin 4 receptor pathway (Hollopeter *et al.* 1998). Neither NPY/AgRP nor NPY/A^y double mutant influenced feeding behaviour or weight gain under normal conditions (Hollopeter *et al.* 1998; Qian *et al.* 2002). However, double mutant mice lacking NPY and galanin eat significantly more and resulted in 30% heavier weight than the control mice (Hohmann *et al.* 2004). Moreover, leptin, insulin and glucose levels were elevated and chronic leptin treatment caused weight loss in the NPY/galanin mutant mice in comparison with controls (Hohmann *et al.* 2004). In a study by Imai et al. NPY KO mice showed changes in glucose homeostasis and had enhanced basal and glucose stimulated insulin secretion (Imai *et al.* 2007). When behavioural effects of the $NPY^{-/-}$ mice were studied, it was shown that $NPY^{-/-}$ mice were anxiogenic (Bannon *et al.* 2000). Furthermore, NPY deficient mice showed increased ethanol consumption when compared with the wild type mice (Thiele *et al.* 1998). NPY deficient mice were also less sensitive to the sedative and hypnotic effects of ethanol (Thiele *et al.* 1998). Recently, a new genetic mouse model for NPY deficiency has been introduced (Karl *et al.* 2008). Both sexes had suppressed

motor activity and exploration, and increased anxiety (Karl *et al.* 2008). However, the behavioural effects were more prominent in male NPY KO mice (Karl *et al.* 2008).

2.3.2 NPY receptor knockout animals

After the generation of NPY deficient mice, several NPY receptor KO models have been generated. Y1 receptors are widely expressed in the CNS, especially hypothalamus, which is thought to be responsible for the energy homeostasis. Interestingly, the Y1 receptor KO models do not seem to have any major abnormalities regarding food intake or body weight under normal feeding conditions (Kanatani *et al.* 2000; Pedrazzini *et al.* 1998). However, an obese phenotype was seen in the older female Y1 deficient mice (Pedrazzini *et al.* 1998). Furthermore, fasting-induced re-feeding is decreased in these Y1 receptor KO mice (Pedrazzini *et al.* 1998). Another study showed moderate obesity and mild hyperinsulinemia in the Y1 receptor KO mice (Kushi *et al.* 1998). The basal blood pressure and heart rate were unaffected in the Y1 receptor deficient mice, but the vasoconstrictor action was completely eliminated (Pedrazzini *et al.* 1998). After intracerebroventricular injection of NPY and its analogues, food intake was stimulated and mediated through the Y1 receptor (Kanatani *et al.* 2000). When the Y1 receptor deficient mice were crossed to leptin deficient *ob/ob* mice, body weight was significantly reduced when compared to the *ob/ob* mice (Pralong *et al.* 2002). In the study by Cavadas et al. plasma levels of catecholamines were shown to be increased possibly via increased tyrosine hydroxylase activity (Cavadas *et al.* 2006). The Y1 receptor seemed to be responsible for mediating voluntary alcohol consumption (Thiele *et al.* 2002). Male Y1 KO mice were less sensitive to the sedative effects of alcohol than the control mice and the KO mice also recovered more rapidly from ethanol induced sleep (Thiele *et al.* 2002). Interestingly, when the Y1 receptor KO mice brains were studied, it was discovered both *in vivo* and *in vitro*, that the cell proliferation and also the number of newly generated neurons were significantly reduced (Howell *et al.* 2003).

Y2 receptors are widely expressed in the arcuate nucleus in the CNS. The germline Y2 receptor KO mice showed reduced bodyweight gain and adiposity in male gender mice (Sainsbury *et al.* 2002a). The food intake in males was unaltered, while it was increased in female Y2 KO mice (Sainsbury *et al.* 2002a). Re-feeding was strongly increased in both sexes. With $Y2^{-/-}$ and *ob/ob* double KO mice the typical phenotype of *ob/ob* mice was changed (Sainsbury *et al.* 2002b). Adiposity was reduced; hyperinsulinemia, hyperglycemia and increased hypothalamic pituitary adrenal (HPA) axis activity were attenuated on the double KO mice. However, food intake and body weight gain were not affected (Sainsbury *et al.* 2002b). Behavioural studies on the Y2 KO mice by Redrobe et al. and Tschenett et al revealed that Y2 receptor has an inhibitory role on the anxiolytic like effects of NPY (Redrobe *et al.* 2003; Tschenett *et al.* 2003). Basal blood pressure of the Y2 KO mice was not changed, but heart rate was increased. Angiogenesis was not induced in the Y2 KO mice even though NPY is potent angiogenic factor *in vivo* (Ekstrand *et al.* 2003). Additionally, NPY induced aortic sprouting and re-vascularization of ischemic muscles was reduced in Y2 receptor KO mice (Lee *et al.* 2003). Furthermore, retinal neovascularization was prevented in Y2 KO mice (Koulu *et al.* 2004). A study by Woldbye et al. reported that in Y2 KO and Y5 KO mice, NPY partially inhibited magnesium-induced epileptiform activity in hippocampal slices (Woldbye *et al.* 2005). Moreover, in Y2/Y5 double KO mice NPY had no effect on the epileptiform activity (Woldbye *et al.* 2005). This study suggested that both receptors Y2 and Y5 separately regulated hippocampal seizures *in vitro*.

Sainsbury et al. have generated a germline Y4 KO mouse model (Sainsbury *et al.* 2002c). The Y4 receptor KO mice showed reduced food intake and body weight (Sainsbury *et al.* 2002c).

Contrary to $Y2^{-/-}$ and *ob/ob* double mutant mice, $Y4^{-/-}$ and *ob/ob* double KO mice did not show improvement of the obese and diabetic phenotype when compared with leptin deficient *ob/ob* mice (Sainsbury *et al.* 2002c). However, $Y4^{-/-}$ and *ob/ob* double KO mice showed significant improvement in fertility (Sainsbury *et al.* 2002c). The Y2 and Y4 double KO mice showed elevated food intake, but retained the lean phenotype (Sainsbury *et al.* 2003). Bone volume was increased significantly in Y2 and Y4 double KO mice suggesting that there is a synergistic interaction between Y2 and Y4 receptor pathways to regulate the bone volume and adiposity (Sainsbury *et al.* 2003). Interestingly, the Y4 receptor had a clear effect on heart. The heart size and weight of the Y4 KO mice were reduced and moreover, they had lower basal blood pressure than the control mice (Smith-White *et al.* 2002).

Mice depleted of Y5 receptor fed and grew normally until at over 30 weeks old they developed late onset obesity (Kanatani *et al.* 2000; Marsh *et al.* 1998). Y5 receptor and *ob/ob* double KO mice did not seem to have any beneficial consequences derived from food intake or body weight in comparison with *ob/ob* mice (Marsh *et al.* 1998). In addition, fertility was not changed in these double KO mice (Marsh *et al.* 1998). When voluntary ethanol consumption of the $Y5^{-/-}$ mice was studied, no difference between the KO mice and the control mice was seen (Thiele *et al.* 1998).

In conclusion, when food intake and body weight of the NPY receptor KO animals was considered, Y1 receptor KO animals did not seem to have any changes in the food intake or body weight under normal feeding conditions. In the Y2 receptor KO animals the female gender seemed to have increased food intake. Y4 receptor KO animals had reduced body weight and food intake and Y5 receptor KO animals developed late-onset obesity. Interestingly, Y2 receptor KO animals had increased heart rate, but the blood pressure was changed. Furthermore, Y4 receptor KO animals had reduced heart size and lower blood pressure than the control mice. Table 2.3 summarizes the major phenotypes of the NPY-OE transgenic animal models, NPY single KO and NPY receptor single KO mouse models.

Table 2.3. Summary of the major phenotypes observed in NPY-OE transgenic animal models, NPY single KO mouse models and NPY receptor single KO mouse models.

Transgenic animal model	Stimulatory effects	No effect	Inhibitory effects	References
NPY-OE mouse	impaired glucose tolerance, hyperinsulinemia, plasma catecholamines, ethanol induced sedation	body weight, food intake	voluntary ethanol intake, adrenal gland catecholamines	Inui *et al.* 1998; Thiele *et al.* 1998; Ruohonen *et al.* 2008a; Ruohonen *et al.* 2008b
NPY-OE rat	blood pressure	body weight, food intake	anxiety, blood pressure	Thorsell *et al.* 2000; Michalkiewicz *et al.* 2001
NPY-KO	anxiety, insulin secretion, ethanol consumption	body weight, food intake	fast-induced re-feeding, algesia, motor activity	Erickson *et al.* 1996a; Erickson *et al.* 1996b; Bannon *et al.* 2000; Thiele *et al.* 1998; Imai *et al.* 2007; Karl *et al.* 2008
NPY1r KO	adiposity (old females), body weight, insulin secretion, catecholamine secretion	basal blood pressure and heart rate	NPY-induced food intake and blood pressure, ethanol induced sedation, cell proliferation	Kushi *et al.* 1998; Pedrazzini *et al.* 1998; Kanatani *et al.* 2000; Thiele *et al.* 2002; Howell *et al.* 2003; Cavadas *et al.* 2006
NPY2r KO	body weight, bone volume, food intake (females)	food intake (males)	body weight, anxiety, angiogenesis, retinal neovascularization	Sainsbury *et al.* 2002a; Redrobe *et al.* 2003; Tschenett *et al.* 2003; Lee *et al.* 2003; Koulu *et al.* 2004
NPY4r KO		bone mass	body weight, blood pressure	Sainsbury *et al.* 2002c; Smith-White *et al.* 2002
NPY5r KO	body weight, adiposity, food intake	food intake, voluntary ethanol consumption		Kanatani *et al.* 2000; Marsh *et al.* 1998; Thiele *et al.* 1998

2.4 HUMAN ASSOCIATION STUDIES

2.4.1 L7P polymorphism in the prepro NPY

Leucine 7 to Proline 7 (L7P; GenBank NM_00905.2:c.20T>C; rs16139) polymorphism in the signal sequence of NPY was originally found in 1998 by Karvonen et al. (Karvonen *et al.* 1998). NPY L7P polymorphism is a single nucleotide polymorphism (SNP) which is a result of a single base substitution in the signal peptide part of the *NPY* gene. Base change from thymidine to cytosine (T1128C) causes a leucine to proline change on the aa level. This aa change supposedly causes quite radical change in the tertiary structure of signal sequence of NPY (Pesonen 2008). L7P polymorphism was found to alter the secretion and packaging of NPY (Mitchell *et al.* 2008). Furthermore, L7P polymorphism was found to increase peptide synthesis and secretion (Mitchell *et al.* 2008).

The NPY signal sequence P7 allele frequency among different populations varies widely. In Caucasian populations the frequency of the L7P polymorphism varies from 6% to 15% (Pesonen 2008) and for example in the Asian population this polymorphism is extremely rare (Ding *et al.* 2002; Drube *et al.* 2001; Jia *et al.* 2005; Makino *et al.* 2001). The most studied population regarding the preproNPY L7P polymorphism is the Finnish population. The NPY P7 allele variant frequency is approximately 12%. About 9000 individuals of the Finnish population have been genotyped to determine the P7 allele frequency (Erkkila *et al.* 2002; Heikkinen *et al.* 2004; Helisalmi *et al.* 2000; Järvisalo *et al.* 2003; Kaarniranta *et al.* 2007; Karvonen *et al.* 1998; Karvonen *et al.* 2000; Karvonen *et al.* 2001; Kauhanen *et al.* 2000; Koulu *et al.* 2004; Niskanen *et al.* 2000a; Pettersson-Fernholm *et al.* 2004; Pihlajamäki *et al.* 2003; Salminen *et al.* 2008; Ukkola & Kesäniemi 2007; Zhu *et al.* 2003). Approximately 9% of the Swedish population (Ding 2003b; Ma *et al.* 2007; Nordman *et al.* 2005; Wallerstedt *et al.* 2004; Zhu *et al.* 2003) and 6-7% of the German (Koehnke *et al.* 2002; Kölsch *et al.* 2006; Pesonen *et al.* 2003; Zill *et al.* 2008) and European Americans (Lappalainen *et al.* 2002; Ma *et al.* 2007; Skibola *et al.* 2005) carry this mutation. In addition to the Finnish population, 11% of the Polish population was found to carry the P7 allele (Tomaszewski *et al.* 2004). Ding et al. have suggested that the origin of the L7P allele variant could be in the northern Europe since the allele frequency shows a geographical north to south gradient of decreasing frequency (Ding 2003a). This hypothesis has been tested in Indian populations (Bhaskar *et al.* 2007). In this study 14 different ethnical populations were genotyped for the L7P allele variant and a weak tendency for P7 allele to decrease from north to south was found (Bhaskar *et al.* 2007). There was an outlier in the study, namely the Kota population, which had a 23% P7 allele frequency. The Kota population is said to be an isolated population and the L7P allele variant frequency was explained by that fact (Bhaskar *et al.* 2007). The homozygote P7P variant carrier frequency has been found to be quite rare, in the Finnish population the homozygote P7P carriers are approximately 0.4% and therefore in the studies conducted the homozygous P7P subjects were combined with the L7P group (Erkkila *et al.* 2002; Järvisalo *et al.* 2003; Karvonen *et al.* 1998; Karvonen *et al.* 2000; Karvonen *et al.* 2001; Niskanen *et al.* 2000a; Pettersson-Fernholm *et al.* 2004; Pihlajamäki *et al.* 2003). In all studies the allele frequencies followed the Hardy-Weinberg equilibrium. (Figure 2.7).

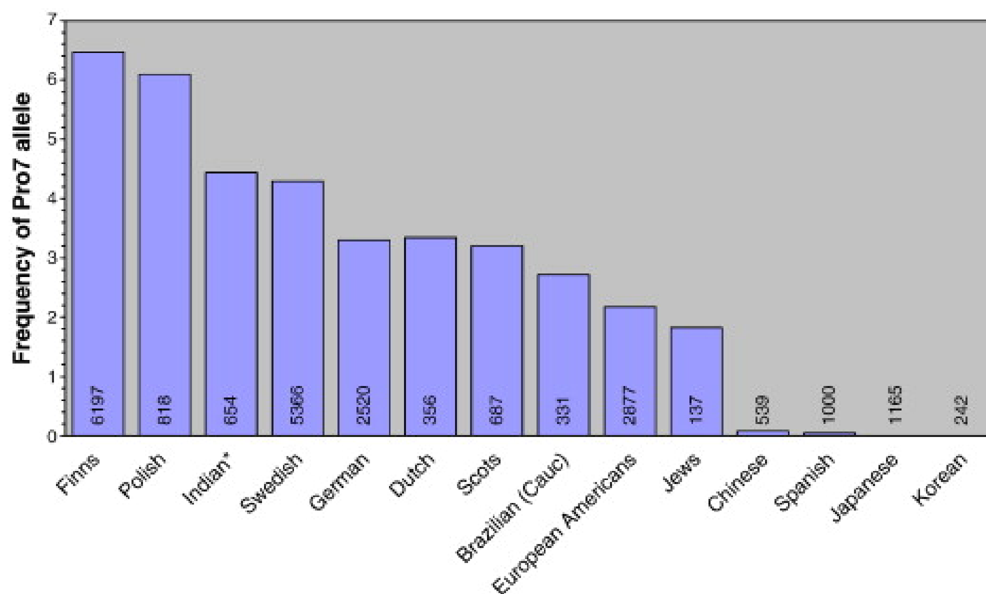

Figure 2.7. Frequency of the P7 allele of L7P variation in the *NPY* gene in different populations. Only those populations, where over 100 unrelated individuals are genotyped, have been included. The figures inside the columns indicate the pooled number of individuals genotyped in the literature (http://www.ncbi.nlm.nih.gov). (Pesonen 2008).

2.4.1.1 Association of L7P polymorphism with cardiovascular disease and serum lipid levels

In the first association study the NPY signal sequence L7P polymorphism was associated with high serum and LDL cholesterol levels in non-diabetic obese men (Karvonen *et al.* 1998). This study was conducted in the Finnish and in Dutch populations. So far multiple association studies of the NPY signal sequence L7P polymorphism have been carried out in several independent study populations. The NPY signal peptide L7P polymorphism has also been associated with accelerated atherosclerosis and intima media thickness (IMT) in middle aged men (Karvonen *et al.* 2001) as well as in patients with T2D (Niskanen *et al.* 2000a). Elevated serum cholesterol and triglyseride (TG) concentrations in youth are considered to be important determinants for fatty streak formation and possible atherosclerosis at later age (Pesonen 2006). Children at the ages 5 and 7 were studied for total and LDL cholesterol levels and TG levels (Karvonen *et al.* 2000). Boys with the L7P polymorphism had constantly higher TG levels in the early childhood, but this difference was not noticed in girls (Karvonen *et al.* 2000). The gender difference was clear in preschool aged children. In another study with middle aged subjects, the P7 allele was associated with higher serum total cholesterol concentration in women with coronary heart disease (CHD) who did not use lipid lowering drugs (Erkkila *et al.* 2002). P7 allele has also been associated with CHD in type 1 diabetes (T1D) patients and in patients with hypertension (Pettersson-Fernholm *et al.* 2004; Wallerstedt *et al.* 2004). On the other hand, a study in Polish population resulted in a negative association of LDL cholesterol levels with the [p.L7]+[p.P7] genotype in hypertensive patients (Tomaszewski *et al.* 2004). NPY and NA concentrations have been associated with higher blood pressure and significantly higher heart rate in subjects with P7 allele (Kallio *et al.* 2003; Karvonen *et al.* 2001). It has been suggested that subjects with the [p.L7]+[p.P7] genotype may have increased cardiovascular autonomic regulation due to different ratio of NPY and NA and, hence, this may correlate to the

increased heart rate (Jaakkola *et al.* 2005; Kallio *et al.* 2003). Sympathetic and parasympathetic heart rate variability were significantly increased with the [p.L7]+[p.P7] genotype subjects when compared with matched controls with the [p.L7]+[p.L7] genotype (Kallio *et al.* 2003).

Enhanced flow mediated endothelial function has been shown in the middle aged men and in children at the age of 5-10 (Järvisalo *et al.* 2003). On the contrary, in another study the NPY P7 allele was found to be protective against coronary artery atherosclerosis and possibly decrease the risk of acute coronary events in the middle aged men (Ilveskoski *et al.* 2008). P7 allele may also be associated with a dietary response to LDL concentration in overweight boys with a family history of early onset CVD (Salminen *et al.* 2008). Obesity at the earlier age and furthermore, a risk of CVD in obese subjects has been shown in subjects with L7P genotype (Jaakkola *et al.* 2007; van Rossum *et al.* 2006).

2.4.1.2 Other association studies of L7P polymorphism

Several association studies on preproNPY L7P polymorphism to diabetes mellitus have been conducted. In the T1D patients the P7 allele may contribute to nephropathy as well as CHD (Pettersson-Fernholm *et al.* 2004). The P7 allele has been associated with diabetic retinopathy (DR) in T2D patients but not with T1D patients (Koulu *et al.* 2004; Niskanen *et al.* 2000b). The onset and early onset age of T2D has been shown in Finnish subjects with the [p.L7]+[p.P7] genotype (Jaakkola *et al.* 2006; Ukkola & Kesäniemi 2007). Study of the L7P polymorphism in Swedish subjects showed association with T2D and with impaired glucose tolerance (Nordman *et al.* 2005). Cortisol concentrations and diurnal glucose concentrations have been proved to be significantly different in T2D patients due to the preproNPY genotype (Jaakkola *et al.* 2007) as well as decreased insulin secretion, delayed ghrelin suppression and increased cardiovascular responsiveness to NA during oral glucose tolerance test in healthy volunteers (Jaakkola *et al.* 2005).

The previously mentioned studies indicate that the L7P polymorphism affects the secretion of several hormones and neurotransmitters (Jaakkola *et al.* 2007). The role of the L7P polymorphism was studied during rest in healthy volunteers (Kallio *et al.* 2003). In this study it was found that subjects with the [p.L7]+[p.P7] genotype had significantly lower NPY and NA concentrations, but there was no difference in the systolic or diastolic blood pressure, free fatty acid (FFA) and leptin concentrations between the [p.L7]+[p.P7] and the [p.L7]+[p.L7] genotypes. However, subjects with the [p.L7]+[p.P7] genotype had significantly higher glucose concentrations, lower insulin concentrations and lower insulin/glucose ratios than control subjects with the [p.L7]+[p.L7] genotype (Kallio *et al.* 2003). The association of the [p.L7]+[p.P7] genotype with body mass index (BMI) and body weight has been proved in several studies (Ding *et al.* 2005; Karvonen *et al.* 2000; Mattevi *et al.* 2002; Nordman *et al.* 2005; van Rossum *et al.* 2006).

The association of preproNPY L7P polymorphism with the alcohol dependence has been controversial. Some studies have indicated that preproNPY P7 allele could be a risk factor for alcohol dependence (Koehnke *et al.* 2002; Lappalainen *et al.* 2002; Mottagui-Tabar *et al.* 2005) or it might predispose to alcoholism but retard the transition to alcoholism (Ilveskoski *et al.* 2001). In a population of Eastern Finnish middle aged men carriers of the P7 allele had 34% higher alcohol consumption than men with the [p.L7]+[p.L7] genotype (Kauhanen *et al.* 2000). On the contrary, some reports show that P7 allele is not associated with alcoholism in Caucasian populations or Asian populations, especially the Japanese population (Drube *et al.* 2001; Zhu *et al.* 2003; Zill *et al.* 2008). However, the P7 allele carrier frequency in the Asian population is extremely low and that probably explains the negative association of the P7 allele

with alcoholism. Furthermore, Heilig et al. have studied the association of the L7P polymorphism with depression and suggested that this polymorphism may contribute to depression (Heilig *et al.* 2004).

PreproNPY P7 allele has also been associated with increased risk for follicular lymphoma (Skibola *et al.* 2005), and it may affect favourably to bone mineral density in postmenopausal women (Heikkinen *et al.* 2004). In children P7 allele does not have an effect on macronutrient consumption (Karvonen *et al.* 2006), but it has been associated with higher birth weight (Karvonen *et al.* 2000). Also, a study of Schwab et al. suggested that P7 allele might cause compositional differences in the lipoprotein particles (Schwab *et al.* 2002).

2.4.2 Other NPY polymorphisms

There are only two nonsynonymous sequence variants found in the signal peptide part of the *NPY* gene up to date. NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) reports another single base alteration in addition to L7P change, namely L22M (c.64C>A; rs5571), in the signal sequence of NPY where aa 22 of the preproNPY, leucine, is changed to methionine (Pesonen 2006). In the L22M polymorphism an average allele frequency of the M22 allele has been reported to be 2.1% in the dbSNP database, but this allele has not been studied further (Ding *et al.* 2005). In the Finnish population (n=60) the L22M polymorphism has not been found and the existence of this sequence variant needs to be confirmed (Pesonen 2006). A part of the promoter region, the complete human *NPY* coding sequence and neighboring intronic sequences were screened in a selected population of Swedes (n=30). Altogether Ding et al. found eight SNPs of which one was nonsynonymous, previously reported L7P, and two synonymous sequence variants were found in the coding part. Five sequence variants were found in the intron and untranslated sequences (Ding *et al.* 2005). The only SNP found to be associated with physiological changes, higher BMI, was the previously reported L7P (Ding *et al.* 2005). Bray et al. have identified a -968_-969delTG (rs3037354) also found by Ding et al. (Bray *et al.* 2000; Ding *et al.* 2005). This -968_-969delTG polymorphism was associated with body fat patterning in non obese Mexican Americans (Bray *et al.* 2000). The existence of -968_-969delTG polymorphism was also tested in the Swedish population and the insertion frequency was found to be 0.75, which was similar to Mexican Americans (Ding *et al.* 2005). However, association to BMI in Swedish population was not found (Ding *et al.* 2005).

At least two studies have introduced SNPs in the *NPY* gene that has been associated with alcohol behaviour (Mottagui-Tabar *et al.* 2005; Okubo & Harada 2001). Five SNPs were compared between a Nordic population of alcohol dependent individuals and ethnically matched controls (Mottagui-Tabar *et al.* 2005). It was found that an NPY promoter region SNP, -688G>T (rs17149106) was associated with alcohol dependence (Mottagui-Tabar *et al.* 2005). In another study *NPY* gene coding region mutation; 204C>T (rs5574) was associated to alcohol withdrawal when compared to subjects without this polymorphism (Okubo & Harada 2001).

An *NPY* gene promoter region SNP -485T>C (rs16147) has been associated with developing depressive illness (Heilig *et al.* 2004). The L7P polymorphism was also associated with depression by Heilig et al. (2004). In a separate study nine *NPY* gene SNPs were detected, of which one was found to be relevant in schizophrenia (Itokawa *et al.* 2003). This SNP found by Itokawa et al. (2003) was the same SNP associated with depression by Heilig et al. (2004). It was shown that the -485T>C change significantly reduced transcriptional activity (Itokawa *et al.* 2003). It may be that this reduced transcriptional activity is connected to schizophrenia and depression (Heilig *et al.* 2004; Itokawa *et al.* 2003). In contrast, in a Danish population study no

association was found between the SNP -485T>C and schizophrenia, depression or panic disorder (Lindberg *et al.* 2006). Furthermore, SNP -485T>C has been studied by Zhou et al. among two other SNPs (Zhou *et al.* 2008). The *NPY* gene haplotypes were shown to predict NPY expression levels and plasma concentration levels. The explanation to the variation of the NPY expression levels was found to be the SNP -485T>C (Zhou *et al.* 2008). Another previously identified SNP -968_-969delTG (rs3037354) was claimed to contribute to the NPY expression level, but not as significantly as the SNP -485T>C (rs16147) (Zhou *et al.* 2008). Also, L7P polymorphism was associated with the NPY expression level, however, Zhou et al. were not fully able to account for the effects of p.L7P variant because of the low frequency of this allele in the study subjects (Pesonen 2008; Zhou *et al.* 2008). The study of Zhou et al. (2008) linked the haplotype-predicted NPY expression to emotion and stress resiliency. Another recent study identified a SNP in the promoter region of the *NPY* gene, namely 1450C>T, that has been suggested to be involved in differences in regulation of catecholamine secretion (Nishimura *et al.* 2008).

NPY receptor SNPs have also been studied. For example C→T polymorphism in the intervening segment of the genes encoding the Y1 and Y5 receptors was found by Blumenthal et al. (2002). This polymorphism is located in the intron 1 segment and associated with decreased TG and increased high density lipoprotein concentrations (Blumenthal *et al.* 2002). Another Y5 receptor polymorphism was found in the 3' UTR region (Jenkinson *et al.* 2000). This 1523T>C SNP was associated with body weight in Pima Indians (Jenkinson *et al.* 2000). A polymorphism in the intron segment of the Y1 receptor gene (rs7687423) was found to be associated with IgA nephropathy in the Japanese population (Ito *et al.* 1999). Altogether NPY receptor polymorphisms have not been studied to the extent of NPY polymorphisms.

3 AIMS OF THE STUDY

The NPY signal sequence L7P polymorphism has been associated with several physiological and pathophysiological responses. Yet, the impact of the polymorphism is not known at the cellular level. The current studies were undertaken to investigate the effect of the NPY signal sequence L7P polymorphism on the processing and functionality of the protein at the cellular level. Furthermore, the translation and mobility of the mitochondrial form of NPY was under investigation.

The specific aims of the studies were:

1. to elucidate the [p.L7]+[p.P7] and the [p.L7]+[p.L7] genotype effects on the localization of NPY and proNPY proteins.

2. to clarify the effect of the NPY signal sequence L7P polymorphism on the intracellular functions such as LDL uptake and LDL receptor amount, proliferation, apoptosis and furthermore, cellular senescence.

3. to reveal the effects of the [p.L7]+[p.P7] and the [p.L7]+[p.L7] genotypes on the nitric oxide synthase function and thus to nitric oxide production.

4. to study the two kozak sequences of the NPY gene and to clarify the effects of the first and the second AUG of the NPY gene.

5. to reveal the translation and routing of the NPY protein and the mitochondrially located protein and furthermore, specify the translational preferences of the NPY depending on the origin of the cell line.

6. to study the mobility of the NPY and the mitochondrially located protein and reveal the possible genotype differences of the mobility due to the [p.L7]+[p.P7] and the [p.L7]+[p.L7] genotypes.

4 MATERIALS AND METHODS

4.1 CELL CULTURE AND CELL LINES

All the cells were grown in cell culture flasks or plates in a suitable cell culture medium. The cells were detached from the flasks with 0.2 ml of 0.25% trypsin/0.02% K-EDTA. Phosphate buffered saline (PBS) was used as a buffer in the cell culture studies unless stated otherwise. The cells were grown at 37°C and 5% CO_2.

4.1.1 Primary cultured endothelial cells (I, II, III V)

Primary cultured HUVECs were isolated from freshly delivered umbilical cords. The Joint Ethics Committee of Turku University and Turku University Central Hospital approved the protocol for preparing a primary culture from human tissue samples.

Approximately 600 cell lines were isolated from the umbilical cords during these studies. Isolation of HUVECs was done with collagenase types II and IV (0.3 mg/ml) enzymes (Sigma, St Louis, MO, USA). The enzymes were diluted in PBS and the umbilical cords were incubated at 37°C for 15 min with the enzymes. Detached cells from veins of the umbilical cords were seeded to 0.2% or 0.1% gelatine coated (Sigma) cell culture flasks or cell culture plates. (Figure 4.1). All experiments were performed with cells between cell divisions (passages) 2 and 9. Experiments were done directly after the isolation of the cells or the cells were frozen in liquid nitrogen and used later.

Each HUVEC cell line with the [p.L7]+[p.P7] genotype was cultured with the [p.L7]+[p.L7] genotype cell line simultaneously and the experiments were performed with both genotypes at the same time to maintain the similarity and reliability of the cell culture and experimental conditions.

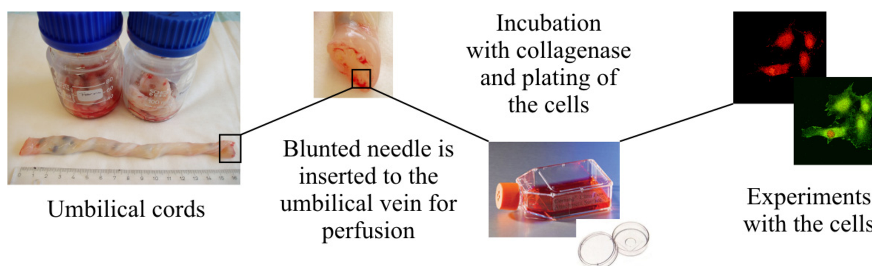

Figure 4.1. A schematic representation of the isolation of the HUVECs.

The basic growth medium for HUVECs, also referred as the maintenance medium, consisted of Medium 199 (M199) (Life Technologies Ltd., Paisley, England) supplemented with sodium heparin (Sigma), 10% fetal bovine serum (FBS) (Autogen Bioclear, Wiltshire, England), penicillin-streptomycin solution (BioWittaker, Walkersville, MD, USA), L-glutamine (Life Technologies Ltd.), gentamycin (BioWittaker), and endothelial cell growth supplement (ECGS), Upstate Biotechnology, Lake Placid, N.Y.). Fresh medium was added every other day until 90% confluence was reached. At the stage of 90% confluence the cells were re-plated or

experiments were performed. The detailed cell culture protocol for HUVECs is presented in the original publication I.

In addition to the basic growth medium, some experiments were done in a medium containing 20% FBS, also referred as the rich medium. The starvation medium was supplemented with 1% FBS and the poor medium lacked FBS. Other supplements remained constant during all experiments. The different treatment methods are summarized in Table 4.1.

Method I, long term treatment (III, IV): HUVECs were growth arrested for 4 hours in the starvation medium. After the growth arrest, the cells were grown in the rich medium. The cells were treated for 20 hours before further experiments.

Method II, short term treatment (IV): HUVECs were starved for 2 hours in the poor medium. 1 h treatments were done in the maintenance medium.

Method III, short term treatment (IV): The cells were not starved prior to treatment. 1 h treatments were done in the maintenance medium. Some treatments (immunocytochemical staining, the NO measurements with DAN) were performed in serum free (poor) medium not to disturb the following experiments. The control (untreated) cells were grown under the same conditions as the treated cells.

Table 4.1. Summary of the treatments of HUVECs in different experiments in studies III and IV. All the supplements were from Sigma.

Treatments	NPY concentrations	Treatment method	Experiments	Study
NPY LDL (2,5 µg/µl)	10µM	6 h, maintenance medium	Thymidine incorporation	unpublished
NPY	1µM	5 min, maintenance medium	LDL receptor staining	unpublished
NPY VEGF (25 ng/ml)	10 nM, 100 pM, 1pM	Method I	Cell count	III
NPY	10nM, 100pM, 1pM	Method I	Tunel staining	III
NPY VEGF (25 ng/ml)	1µM, 10nM, 100pM, 1pM	Method I	Western blotting	IV
NPY VEGF (25 ng/ml)	1µM, 10nM, 100pM, 1pM	Method III	Real time-PCR	IV
NPY VEGF (25 ng/ml)	10nM, 1pM	Method I	Real time-PCR	IV
NPY CaI (1 µM) Carbacholine (1 µM)	1µM, 10nM, 100pM,	Method III poor medium	eNOS immunocytochemistry	IV
NPY CaI (1 µM)	1µM, 10nM, 100pM, 1pM	Method II	DAF-2DA staining	IV
NPY CaI (1 µM)	1 µM,10 nM, 100pM	Method III poor medium	NO measurements (DAN)	IV

4.1.2 Immortalized cell lines (II, V)

Immortalized cell lines, Chinese hamster ovary (CHO-K1) cells and human neuroblastoma (SK-N-BE(2)) cells were original cell lines from ATCC (ATCC, Manassas, Virginia, USA).

CHO-K1 cells were cultured in Ham's F12 medium (Euroclone, Milano, Italy) or a minimal essential medium (MEM) (Cambrex Bio Science, Verviers, Belgium), supplemented with 5% fetal bovine serum (Euroclone), L-glutamine (Cambrex Bio Science) and penicillin-streptomycin solution (Cambrex Bio Science). Cells were divided or used for further experiments when approximately 80% confluency was reached.

SK-N-BE(2) cells were cultured in a mixture of MEM supplemented with Earle's salts and Ham's F12 medium (Euroclone). Medium for SK-N-BE(2) cells was supplemented with 10% fetal bovine serum (Euroclone), non-essential aa's (Euroclone), L-glutamine (Cambrex Bio Science), penicillin (Cambrex Bio Science), and streptomycin (Cambrex Bio Science). Cells were divided or used for further experiments when approximately 80% confluence was reached. The cell culture protocols for CHO-K1 and SK-N-BE(2) cells are presented in detail in the original publication II.

4.2 THE STUDY SUBJECTS (I)

The Joint Ethics Committee of the Faculty of Medicine, University of Turku and Turku University Central Hospital approved the study. Written informed consent was obtained from each subject for genotyping and for participation in maximal oxygen consumption (VO_{2max}) determination and 80% VO_{2max} cycle ergometer exercise test (workload 80% of the determined VO_{2max} corresponding 100% workload). Participation for the genotype screening was offered to non-selected volunteers over 18 years of age with no diseases. Nine subjects having the [p.L7]+[p.P7] genotype and nine pair-matched controls (matched for age, sex, and BMI) with the [p.L7]+[p.L7] genotype were selected for VO_{2max} determination and the 80% VO_{2max} cycle-ergometer exercise test. Detailed medical history (including diseases, medication, smoking, trauma, alcohol consumption) was recorded from all study subjects. A physical examination (including ECG, cardiac and pulmonary auscultation, blood pressure measurement, thyroid palpation, screening for clinical signs of infection) was conducted, and basic laboratory measurements (blood hemoglobin, total cholesterol, LDL cholesterol, glucose, FFA and alanine amino transferase concentration, leukocyte count, and erythrocyte sedimentation rate) were performed. Body fat was determined by skin-fold measurement.

4.3 GENOTYPING (I-IV)

For genotyping of HUVECs, a tissue sample from the umbilical cord was taken and the DNA from the homogenized tissue sample was extracted. For confirming the genotypes of the HUVECs, the cells were harvested by scraping and isolating the DNA from the scraped cells (I, II, III, IV). For genotyping the study subjects, blood samples were drawn from an antecubital vein (I).

HUVEC DNA and the blood leukocyte DNA were extracted with DNA isolation kit, (Capture Column Kit, Gentra Systems, Minneapolis, MN, USA). The L7P polymorphism is caused by a T1128C change on the aa level. The genotyping was done with either of the two methods, the

BsiEI or the *Nci1* restriction enzyme method. Up to 450 batches of HUVECs the genotyping was done with the *BsiEI* restriction enzyme method and the last 150 batches of HUVECs were genotyped with *Nci1* restriction enzyme. The blood samples from the study subjects were genotyped with the *BsiEI* restriction enzyme method.

***BsiEI method*:** Thymidine to cytosine change generates a *Bsi*EI restriction site in the exon 2 of the *NPY* gene. The 237 bp long PCR product was digested with *BsiEI* restriction enzyme (New England Biolabs Inc.) and analyzed on 2% agarose gel. If the T1128C substitution was present, fragments of 189 bp and 48 bp were generated. This method was originally described by Karvonen et al. (1998).

Nci1 method: The preproNPY genotype was determined with restriction fragment length polymorphism (RFLP) method. The primers for the PCR amplification of the exon 2 region were: upper primer 5'-TTGCACTCTCGCCGCGCG-3' and lower primer 5'-TGGTGCGTCCTCGCTCGGG-3' (MedProbe, Oslo, Norway). The lower primer introduces a one pair mismatch which removes the naturally occurring *NciI* restriction site (New England Biolabs Inc. Beverly, MA, USA). If the T1128C was present, the 556 bp PCR product was cleaved into 3 fragments; 260 bp, 193 bp and 103 bp. Method was derived from Koulu et al. (2004).

4.4 GFP CONSTRUCTS (II, V)

The original preproNPY cDNA construct was a gift from Professor Larhammar, University of Uppsala, Sweden. The NPY cDNA without the C-PON was inserted in the pEGFPN1 plasmid (Clontech, Palo Alto, CA, USA) and was a gift from Dr. Unkila, Hormos Medical, Turku, Finland. The construct was inserted to the multiple cloning site between *EcoRI* and *BamHI* restriction sites. The linker sequence between NPY and the beginning of enhanced green fluorescent protein (EGFP) was RDPPVAT. The original constructs had two kinds of preproNPY signal sequences. The 7th aa of the construct was either leucine or proline. (Figure 4.2)

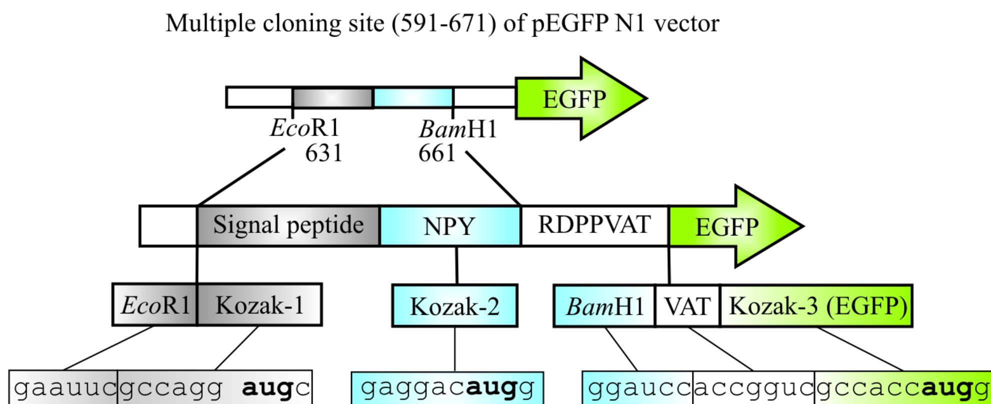

Figure 4.2. Structure of the GFP constructs. Modified from the original communication II.

A total of eight constructs were created (Table 4.2). The original construct was modified by site-directed mutagenesis method using *Pfu* DNA polymerase (1U/reaction) (MBI Fermentas GmbH, St. Leon-Rot, Germany) to amplify the plasmid. Entirely complementary 32 or 33 bp long primers (Eurogentec, Seraing, Belgium) were used to change the sequence of the signal

peptide-NPY-GFP construct. PCR products were purified with PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

The kozak-1 (GCCACCAUGC) sequence of the *NPY* gene was either added or destroyed. The AUG-2 (methionone, p.M45) of the kozak-2 (GAGGACAUGG) was destroyed by changing the AUG to GUG (valine, V45). The kozak-3 (the kozak sequence of GFP, GCCACCAUGG) remained constant through all modifications and experiments. Kzk-sp-NPY-GFP construct included both kozak sequences, kozak-1 and kozak-2, and therefore reminded the natural *NPY* gene sequence. In the sp-NPY-GFP construct the kozak-1 sequence was mutated to a non functional kozak sequence. Otherwise, it was the same construct as the previous one (kzk-sp-NPY-GFP). In the kzk-sp-NPY(V45)-GFP construct the AUG-2 was mutated to GUG changing the methionine at position 45 to valine (M45V). In the sp-NPY(V45)-GFP construct both kozak sequences (kozak-1 and kozak-2) were mutated. Kzk-sp-GFP construct included kozak-1 sequence and the signal peptide of the *NPY* gene, but lacked NPY and therefore lacked also kozak-2 sequence. The sp-GFP construct included AUG-1, but not the whole kozak-1 sequence. The only complete kozak sequence of the sp-GFP construct was kozak-3. In addition, NPY was missing from this construct. All constructs were sequenced with ABI Prism Sequencing System (Applied Biosystems, Foster City, CA, USA) to confirm the sequence.

Table 4.2. Summary of the constructs. Modified from the original communications II and V.

Construct	Description of the construct	Cell type	Kozak-1 GCCACCAUGC	Kozak-2 GAGGACAUGG	Kozak-3 (EGFP) GCCACCAUGG	Study
kzk-sp-NPY-GFP L7L	Includes the natural kozak (kozak-1 and kozak-2) sequences 7th aa of the signal sequence is leucine	SK-N-BE(2)	X	X	X	II, V
		CHO-K1	X	X	X	
kzk-sp-NPY-GFP L7P	Includes the natural kozak (kozak-1 and kozak-2) sequences 7th aa of the signal sequence is proline	SK-N-BE(2)	X	X	X	V
sp-NPY-GFP L7L	Excludes complete kozak-1 Includes complete kozak-2 7th aa of the signal sequence is leucine	SK-N-BE(2)		X	X	II, V
		CHO-K1		X	X	
sp-NPY-GFP L7P	Excludes complete kozak-1 Includes complete kozak-2 7th aa of the signal sequence is proline	SK-N-BE(2)		X	X	V
kzk-sp-NPY(V45)-GFP	Includes kozak-1 Kozak-2 is modified	SK-N-BE(2)	X		X	II
		CHO-K1	X		X	
sp-NPY(V45)-GFP	Kozak-1 is modified Kozak-2 is modified	SK-N-BE(2)			X	II
		CHO-K1			X	
kzk-sp-GFP	Includes kozak-1 Excludes NPY	SK-N-BE(2)	X		X	II
		CHO-K1	X		X	
sp-GFP	Kozak-1 is modified Excludes NPY	SK-N-BE(2)			X	II
		CHO-K1			X	

4.5 TRANSFORMATION AND TRANSFECTIONS (II, V)

Constructs were transformed to competent Escherichia coli DH5a strain and plated on LB plates supplemented with kanamycin (60 µg/ml, Sigma). The bacterial colonies were picked and grown in tubes overnight. Plasmids were purified with GenElute Plasmid Miniprep Kit (Sigma) for small scale (1-5 ml) plasmid purification and Nucleobond Pc 500 Kit (Macherey-Nagel, Düren, Germany) for larger scale (30-150 ml) plasmid purification.

Cells were prepared for transfection on glass-bottomed culture dishes and grown to 60% confluence. They were transiently transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), Fugene 6 (Roche Diagnostics GmbH, Mannheim, Germany) or TransFectin Lipid Reagent (Bio-Rad laboratories, Hercules, CA, USA) following the manufacturers' instructions. In short; 1-2 µg of plasmid DNA was added to the transfection reagent mix. After brief incubation at the room temperature the mixture was added on the cells. The medium was changed after 4 h of incubation or on the next day. The cells were incubated (5% CO_2, 37°C) for at least 24 h before microscopy.

For semi-stable transfections (V), neomycin (50 mg/ml, G418, Sigma) was used as a selection agent. The selection of the transfected cells lasted approximately three weeks. For microscopic experiments, the cells were plated on a 35 mm glass-bottomed culture dishes with 10 mm microwell (MatTek, Ashland, MA, USA).

4.6 IMMUNOCYTOCHEMISTRY (I-IV)

4.6.1 Immunostainings (I-IV)

NPY and C-PON (I, II, III): Before staining the cells were plated on glass coverslips at 1–2 $\times 10^4$ cells/cm^2 for 24–48 hours and fixed at room temperature for 20 min with 4% paraformaldehyde. Nonspecific binding was blocked by incubating the cells for 45 min with blocking buffer containing 0.2% Nonidet p-40 (Sigma) and 5% non-fat dry milk in 50 mM Tris-HCl, pH 7.6. For NPY and C-PON stainings polyclonal (goat anti NPY antibody and rabbit anti C-PON antibody, Affiniti Research Products Ltd. Mamhead, England) commercial antibodies were used. Primary antibody incubations (45 min) were performed simultaneously and after the incubation the cells were rinsed three times with PBS. Before secondary antibody incubations 5 min blocking was performed. Secondary antibody incubations (30 min) (FITC-conjugated sheep anti-rabbit IgG, Silenius Laboratories, Hawthorne, Australia and TRITC-conjugated rabbit anti-goat IgG, Sigma) were performed subsequently in the dark. Subsequent secondary antibody incubations were preformed to avoid false conjugation. After secondary antibody incubations the cells were rinsed three times with PBS and mounted for fluorescent microscopy onto a drop of anti-fade mounting medium containing 50% glycerol, 100 mg/ml DABCO® and 0.05% sodium azide in PBS on microscopic slides. The cells were stained at room temperature. Immunostainings were visualized with confocal microscope Leica TCS 4 D (100×/1.4 oil ICT:D objective, Heidelberg, Germany).

eNOS (VI): For immunocytochemistry cells were plated on glass coverslips at 1–2 $\times 10^4$ cells/cm^2 for 24–48 hours and fixed at room temperature for 15 min with 4% paraformaldehyde. Nonspecific binding was blocked and the cells were permeabilized as previously mentioned. The cells were incubated with mouse monoclonal anti human eNOS/NOS type III antibody (BD Biosciences, San Jose, CA, USA) for 1h. After the primary antibody incubation the cells were rinsed three times with PBS followed by 5 min blocking. Secondary antibody incubation (45 min) (Anti-mouse IgG Alexa Fluor 488 dye, Invitrogen, Paisely, UK) was performed in the dark. After secondary antibody incubation the cells were rinsed three times with PBS and mounted on microscopic slides for fluorescent microscopy onto a drop of anti-fade mounting medium, Vectashield (Vector Laboratories, Burlingame, CA, USA), containing DAPI for nuclear staining. The cells were stained at room temperature. Immunostainings were visualized with confocal microscope LSM 510 meta (Carl Zeiss, 63·/ 1.4 oil Plan Apochromat objective, Göttingen, Germany). In addition to NPY stimulation, a calcium ionophore (CaI/A23287, Sigma) was used for possible induction of the nitrite production and furthermore the eNOS production. Carbacholine (Sigma) was also used for eNOS production induction.

For the western blotting the membranes were incubated overnight at 4°C with mouse monoclonal human anti-eNOS/NOS type III (BD Biosciences). The secondary antibody incubation was performed with horse raddish peroxidase (HRP) conjugated anti-mouse IgG antibody (Sigma) for 90 min at room temperature. The western blotting protocol is presented in the section 4.11.

LDL receptors: LDL receptors were immunostained by incubating with polyclonal anti-human LDL receptor antibody raised in rabbit (Research Diagnostics Inc. Flanders New Jersey, USA) in CA-PBS-BSA for 30 min at 4°C. Cells were washed three times with Ca-PBS-BSA followed by incubation with the secondary antibody fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG raised in sheep (Sigma) diluted with Ca-PBS-BSA for 15-30 min at 4°C. After washing with $CaCl_2$ in PBS, the cells were fixed with 2% paraformaldehyde and analysed with FACS.

Negative controls for fluorescence consisted of treating the cells with fluoresceinated secondary antibody alone. Mitochondrial and Golgi markers were used in the immunocytochemical studies (Study II). The live cells were incubated with a mitochondrial marker MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) 1 µl per 2 ml of medium for 5 min at 37°C and 5% CO_2. After the incubation with the mitotracker the cells were washed three times with PBS and left to the culture medium for 30-45 min. After that the cells were either imaged or fixed with 4% paraformaldehyde. Golgi/ER marker BODIPY TR C5-ceramide (Molecular Probes) was activated according to the manufacturer's instructions. The cells were incubated for 30 min at 20°C and washed three times with PBS. After the Golgi/ER marker treatment the cells were imaged. The antibodies used in the current studies are presented in Table 4.3.

Table 4.3 Summary of the antibodies, dilutions and the origins of the antibodies used in the current studies.

Primary antibody	Dilution	Origin	Secondary antibody	Dilution	Origin	Study
Rabbit polyclonal anti human C-PON antibody	1:200	Affiniti Research Products Ltd.,	Sheep anti-rabbit IgG FITC conjugated	1:500	Silenius Laboratories	I
Goat polyclonal anti human NPY antibody	1:100	Affiniti Research Products Ltd.,	Rabbit anti-goat IgG TRITC conjugated	1:250	Sigma	I, II, III
Mouse monoclonal anti human eNOS antibody	1:200	BD Biosciences	Anti-mouse IgG Alexa Fluor 488 dye	1:750	Invitrogen	IV
	1:500	BD Biosciences	Anti-mouse IgG HRP conjugated	1:1000	Sigma	IV
Rabbit polyclonal anti human LDL receptor antibody	1:10	Research Diagnostics Inc.	Anti-rabbit IgG FITC conjugated	1:200	Sigma	III

4.7 PROLIFERATION EXPERIMENTS (III)

4.7.1 [^{3}H]-thymidine incorporation

HUVECs (1 x 10^4 cells) were plated to 96-well microtiter plates. The cells were cultured for 24 hours before adding the 1 μCi of [^{3}H]-thymidine. The cells were cultured in a medium with [^{3}H]-thymidine (Amersham Life Science, Buckinghamshire, United Kingdom) for 6 hours and harvested. The samples were dried overnight and counted in a DSA-based liquid scintillation counter (model 1409; Wallac Oy.) with 1 ml of OptiScint Hisafe (LKB Scintillation Products, Loughborough, England). The effect of LDL cholesterol and NPY on the [^{3}H]-thymidine incorporation of HUVECs was tested by incubating the cells with 2.5 μg/μl of LDL or 10 μm NPY for 6 hours before harvesting. In the [^{3}H]-thymidine incorporation experiments the passage of the cells was taken into account. Early and late passages were compared with each other (early passages 4-5 and late passages 6-9 respectively).

4.7.2 Cell count

HUVECs were plated on 6-well plates, each well containing approximately 70,000 cells. The cells were growth-arrested and treated with NPY and vascular endothelial growth hormone (VEGF). The cells were counted with a Bürker chamber (Hawksley & Sons Limited, Lancing, Sussex, UK).

4.8 APOPTOSIS EXPERIMENTS (III)

4.8.1 TUNEL staining

Apoptotic cells were detected with Terminal transferase-mediated deoxyuridine triphosphate-biotin end-labeling (TUNEL) assay (In situ cell death detection kit, Boehringer-Mannheim GmbH, Germany) according to the manufacturer's instructions. Briefly, PBS was used as a washing buffer and the cells were fixed with 2% paraformaldehyde. The permeabilization solution contained 0.1% Triton-X 100 in 0.1% sodium citrate and the cells were permeabilized for 2 min on ice. DNAse I, 300U/ml in Tris-HCl pH 7.5, 1 mg/ml BSA for 10 min at room temperature (Promega Corporation, Madison, Wisconsin, USA) was used as a positive control for apoptosis. Labelling solution (without the terminal transferase enzyme solution) was used as a negative control. The cells were incubated with the TUNEL reaction mixture containing the terminal deoxynucleotidyl transferase enzyme for 60 min at 37°C humidified chamber in darkness. The cells were treated with NPY and the amount of apoptotic cells was analyzed with FACS using FL1 (green) channel (Facscan and Facs Calibur, Becton Dickinson, Franklin Lakes, New Jersey, USA). The FACS settings (i.e. compensations and gain) were validated with the positive and negative control cells. In the TUNEL staining experiments the passage of the cells was taken into account (early passages 4-5 and late passages 6-9).

4.8.2 JC-1 staining

The mitochondrial redox potential change indicating apoptosis was measured with JC-1 mitochondrial membrane potential detection kit (Cell Technology Inc., Mountain View, California, USA) according to the manufacturer's instructions. Shortly, apoptosis was induced with 15 min incubation of 5 μM and 10 μM H_2O_2 16 hours before the JC-1 experiment. PBS was used as a washing buffer. The apoptotic and nonapoptotic cells were incubated with the JC-1 reagent for 15 min at 37°C, 5% CO_2, washed and resuspended in to the M199. The redox potential change was indicated by a fluorescence emission shift from red (~585 nm) to green (~530 nm). The ratio of the nonapoptotic (red) and apoptotic (green) cells was determined with FACS using FL2 (red) and FL1 (green) channels (Facscan and FACS Calibur) immediately after the staining. The FACS settings for the JC-1 staining were done according to the manufacturer's suggestions (FL1 PMT voltage 511, FL2 PMT voltage 389, Compensation: FL1-10.5% FL2, FL2-25.9% FL1) and validating the settings with H_2O_2 treated apoptotic cells and nontreated healthy cells.

4.9 LDL-UPTAKE AND LDL RECEPTOR DENSITY

In the LDL-uptake experiment, cells were detached from cell culture flasks and plated to U-shaped 96 well-plates. The cells were washed with Ca-PBS-BSA. The centrifugation speed did not exceed 1000 rpm at any time. Cells were incubated with Bodipy-LDL (Molecular Probes) which was diluted into Ca-PBS-BSA and incubated at 4°C for 30 min. The cells were washed at 4°C and fixed with 2% paraformaldehyde at 4°C for 20 min in the dark. The cells were analyzed with FACS using FL2 (red) channel by counting the mean immunofluorescence in 10,000 cells. In the LDL uptake experiment the passage of the cells was taken into account. Early passages (4-5) were compared with late passages (6-9).

The analysis of the LDL receptor stained cells was done with FACS using the FL1 (green) channel (Facscan). The effect of NPY (1 μM) was tested on the LDL receptor density. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG alone was used as a negative control for fluorescence. Unstained cells were used as control cells and for validation of the FACS settings. The immunocytochemical staining of the LDL receptors is presented in section 4.6.1.

4.10 RNA ISOLATION AND REAL-TIME PCR (IV)

HUVECs were pooled according to the genotype and the treatment. RNA was isolated with NucleoSpin RNA II (Macherey-Nagel Inc., Bethlehem, PA, USA) and RNA concentrations were determined with Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen, Paisley, UK) using the fluorescence spectrophotometer Wallac Victor^{2}V (PerkinElmer, Turku, Finland). Each sample was measured in triplicate and the RNA concentration of each sample was determined by using the high-range standard curve method, the RNA concentration varying from 20 ng /ml to 1 μg /ml.

Real time PCR was performed with TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA, USA) using ABI 7300 machinery. The eNOS primers and probe were planned with Primer Express software (Applied Biosystems): Forward primer, 5’-TCTCCGCCTCGCTCATG-3’; reverse primer, 5’-CATACAGGATTGTCGCCTTCAC-3’; probe,5’-CACGGTGATGGCGAAG-3’. The probe was tagged with FAM/MGB and the integral passive reference dye was ROX. Each sample contained 200 ng of total RNA isolated from HUVECs. The final concentration of the eNOS probe was 250 nM and concentrations of primers were 900 nM. Human GADP (GAPDH) (FAM/MGB Probe, Non-Primer Limited) endogenous control kit (Applied Biosystems) was used to normalize the amount of sample RNA. The reactions (total volume 25 μl) were incubated at 45 min at 48 °C, at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was measured as quadruplicate and the amplification plots were manually checked for integrity.

The comparative Ct method (=ΔΔCt method) was used to examine the relative quantification of non-treated and treated [p.L7]+[p.P7] and [p.L7]+[p.L7] genotype HUVECs. The results were analyzed with Relative Quantification computer software (Applied Biosystems) where the amount of target RNA was normalized with endogenous control (human GADPH) and expressed relative to a calibrator sample. The results were displayed on a logarithmic scale using mathematical equation $2^{-\Delta\Delta Ct}$. Non-treated samples were used as calibrator samples in analyses of the RT-PCR data.

4.11 WESTERN BLOTTING (IV)

HUVECs were pooled according to genotype and treatment. The cells were lysed and the protein was isolated and lysed with RIPA buffer. The protein amounts were determined with Bio-Rad Dc protein assay (Bio-Rad Laboratories Inc.). Five (5) μg of total protein samples were loaded to a 6% SDS-PAGE gel with a 4% stacker gel. The samples were transferred to Immobilon-P PVDF transfer membrane (30 min, 15 V) (Millipore Corporation, Bedford, MA, USA) with Trans-Blot semidry Electrophoretic transfer cell (Bio-Rad Laboratories Inc.). Nonspecific binding was blocked with blocking buffer containing 5% non-fat dry milk in 50 mM Tris-HCl, pH 7.6 and treated with primary antibody (mouse anti-human eNOS antibody)

overnight and secondary antibody for 90 min (anti-mouse IgG HRP conjugated). The antibodies for western blotting are presented in detail in section 4.6.1. PageRuler Plus prestained protein ladder (Fermentas Life Sciences, Glen Burnie, MD, USA) was used as a protein size standard. The protein bands were visualized with ECL Western Blotting Detection Reagents (Amershamn Biosciences, Buckinhamshire, UK) and Hyperfilm (Amershamn Biosciences). The optical signals were quantified with Image J 1.38x (NIH, USA, available at the internet address http://rsb.info.nih.gov/nih-image/).

4.12 NO DETERMINATIONS (IV)

HUVECs (6×10^4 cells/coverslip) were grown on coverslips overnight. The cells were starved in serum-free M199. Krebs-Ringer-Hepes (KRH) buffer was used for washing and incubations. The NO detection was preformed by incubating the cells with 5 µM diaminofluorescein (DAF-2DA) (Sigma) for 15 min at 37°C. In addition to the untreated cells, the cells were treated with NPY and CaI. N-Nitro-L-Arginine Methyl Ester (L-NAME, 1 µM, Sigma) was used as a nitric oxide inhibitor. Fixation was done with 4% paraformaldehyde (15 min) and fluorescence microscopy was performed.

For the 2,3-diaminonaphtalene (DAN) experiments 15,000 cells were plated on 6-well plates and cultured overnight. Cells were washed 3 times with PBS and grown in FBS and phenol red free M199 for 1h. The medium was collected and centrifuged to dispose all the cells left in the medium. The nitrate amounts were measured from the medium as suggested by the manufacturer (Calbiochem, Darmstadt, Germany). Sodium nitroprusside (0.1 mM) was used as a positive reference for NO production. Several intra-assays were performed to validate the method by repeating ten measurements of one sample. The DAN measurements were done with fluorometer Wallac Victor^{2}V (PerkinElmer Life) using 355 emission wavelength and 460 exitation wavelength. The nitrate standard curves were analyzed with GraphPad Prism 4 (GraphPad Software, San Diego, California, USA).

4.13 CONFOCAL MICROSCOPY (I-V)

4.13.1 Conventional confocal microscopy (I-IV)

For the fluorescence microscopy two types of microscopes were used: Leica TCS 4 D (100·/1.4 oil ICT:D objective) (Studies I and II) and LSM 510 meta (Carl Zeiss, 63·/ 1.4 oil Plan Apochromat objective) (Studies II-V). The CO_2 incubator chamber was used for live cell imaging of GFP, mitochondrial and Golgi/ER markers (Zeiss). Analyses of the microscopic data were performed with offline analyzer program LSM 510 meta (Zeiss). EGFP and FITC were detected with a 500–550 nm filter. Mitochondrial and Golgi markers were detected with a long pass filter starting from 560 nm (Zeiss) or with a 580–620 nm filter (Leica). The airy unit for the conventional confocal microscopy was set at 1 at all times. The assessment of fluorescence labels was done with Image J 1.38x (NIH, USA)

4.13.2 Fluorescence recovery after photobleaching (V)

Confocal microscope LSM 510 Meta with a CO_2 incubator chamber was used in FRAP experiments. The region of interest (ROI) was bleached with 100% laser power and the number of iterations for photobleaching was 100. The airy unit for the kzk-sp-NPY-GFP L7L and the kzk-sp-NPY-GFP L7P construct transfected cells was set at 2.24. For the photobleaching of the sp-NPY-GFP L7L and sp-NPY-GFP L7P construct transfected cells the airy unit was set at 1. The bleaching efficiency was between 69.2% and 98.9%. The fluorescence recovery was followed for approximately 3 minutes. The scanning laser intensity was set low (5% laser power) and the scanning speed fast to avoid photodamaging of the specimens. Analyses of the microscopic data were performed with offline analyzer program LSM 510 meta (Carl Zeiss) and the acquired recovery data were processed with FRAPCalc software (Courtesy of Dr. Rolf Sara, Turku Center for Biotechnology, Turku, Finland). Valinomycin was used in FRAP studies to increase the permeability of the inner membrane. The cells were treated with 1 mg/ml valinomycin (Sigma) for 20 min.

4.14 HUMAN VOLUNTEER EXERCISE STUDY (I)

Study subjects were asked to refuse any medication and alcohol-containing drinks for 48 h and from any caffeine-containing drinks or food for 12 h before the VO_{2max} measurement and before the 80% VO_{2max} cycle ergometer exercise test. They were asked to eat carbohydrate rich food and to avoid demanding physical exercise for 2 days before the tests. A light meal was offered 2 hours before the tests. VO_{2max} was determined by using a cycle ergometer (Model 800 S, Ergoline, Mijnhart, Netherlands). The followed protocol was continuously incremental. Direct paramagnetic O_2 and infrared CO_2 analysis of respiratory gases was done by using a PC-based automated system (Model 202, Medikro, Kuopio, Finland). The maximal VO_2 was determined, and the corresponding power level was considered as 100% VO_{2max}. In the 80% VO_{2max} exercise study, an intravenous cannula was inserted into an antecubital vein. After the exercise the subjects stayed at horizontal rest for 35 min. Before starting to run the cycle-ergometer (at 0 min timepoint), the subjects sat on it for 5 min. The exercise was started with a minimal workload. The workload was increased by 20% VO_{2max} steps in 2-min intervals until the 80% VO_{2max} workload was reached. The study subjects continued to exercise at 80% VO_{2max} level for 12 min. The exercise was followed by 10 min cooling with 20% VO_{2max} workload. The study subjects were monitored (ECG and blood pressure) by Datex Engstrom AS/3-system (Datex Ohmeda, Oulu, Finland) for a 30min baseline period before the exercise, during the 30 min exercise, and 50 min in a sitting position after the exercise. Nine blood samples (for the measurement of plasma NPY, A, NA, lactate, insulin, and serum FFA) were collected and heart rate was recorded at –30, –5, 0, 8, 16, 20, 30, 40, 60, and 80 min. Blood pressure was measured at –30, –10, 0, 16, 25, 30, 40, 60, and 80 min.

NPY, insulin, NA, A and FFA concentration determinations: Plasma NPY and insulin concentrations were determined by using radioimmunoassay kits EURIA-NPY (Euro-Diagnostica Inc., Malmö, Sweden) and INSIK-5 (DiaSorin s.r.l., Saluggia, Italy). NA and A concentrations in plasma were determined by using high-performance liquid chromatography (HPLC). FFA concentrations were determined with NEFA-C Reagent set (Wako Chemicals GmbH, Neuss, Germany), and plasma lactate concentrations with an enzymatic UV-method (Roche Diagnostics GmbH) by using Hitachi 917 Automatic Analyzer (Hitachi Ltd., Tokyo, Japan).

4.15 STATISTICAL ANALYSES

Study I

Baseline characteristics of study subjects were compared by using a two-sample *t*- test. Catecholamine (A, NA) values were log-transformed to normalize their distributions before further analysis. The means of each sequentially measured parameter between the genotypes [p.L7]+[p.P7] and [p.L7]+[p.L7] were compared by using repeated measures ANOVA for mixed models. In the case of statistically significant genotype-by-time interaction (ANOVA, overall difference) the Fisher least significant difference multiple comparison procedure was used to test equality of group means at each time point. These tests were carried out as linear contrasts by using the same statistical model. For correlation analysis, Pearson's correlation coefficients were calculated. All data are presented as mean ± SEM. Statistical analysis was performed with SAS software (Version 6.12, SAS Institute Inc., Cary, N.C.). A two-sided P-value of less than 0.05 was considered statistically significant.

Study III

The data were analyzed with unpaired t-test and with two-way ANOVA using Bonferroni post hoc test for multiple comparisons with GraphPad Prism 4 (GraphPad Software, San Diego, California, USA). ANOVA included the main effects of genotype and treatment or passage ([^{3}H]-thymidine incorporation experiment) and the interaction effect of genotype×treatment or passage ([^{3}H]-thymidine incorporation experiment). The results were presented as mean values ± SEM. Statistical significance was set at the value $p<0.05$.

Study IV

The real time PCR and DAF-2DA results were analyzed with two-way analysis of variance (ANOVA). The western blot results were analyzed using two-way repeated measures ANOVA to account for the correlation between the results measured within the sample batch. The DAN results were analyzed with repeated measures two-way ANOVA where treatment was used as a repeated factor. ANOVA models included the main effects of treatment and genotype and the interaction effect of genotype×treatment. The results in the graphs are presented as mean values ± SEM. Statistical analyses were done using SAS System for Windows, release 9.2 (SAS Institute Inc., Cary, NC). Statistical significance was set at the value $p<0.05$.

Study V

The data were analyzed with two-way ANOVA using Bonferroni post test for multiple comparisons with GraphPad Prism 4 (GraphPad Software, San Diego, California, USA). The results are presented as mean values ± SEM. Statistical significance was set at the value $p<0.05$.

5 RESULTS

5.1 DIFFERENCES BETWEEN THE [P.L7]+[P.L7] AND THE [P.L7]+[P.P7] GENOTYPES .

5.1.1 Immunocytochemistry of endothelial cells (I, II, III, IV))

5.1.1.1 NPY immunocytochemistry (I, II, III)

When HUVECs were stained with NPY and proNPY (C-PON) antibodies, it was revealed that there was a clear difference in the pattern of staining between HUVECs with the [p.L7]+[p.L7] genotype and the [p.L7]+[p.P7] genotype. The [p.L7]+[p.L7] genotype cells showed mostly proNPY-ir in contrast to the [p.L7]+[p.P7] genotype cells where mainly NPY-ir was seen. The amount of NPY-ir was also assessed from microscopic images using fire colour scale bar (Study III). Additionally, NPY-ir (green fluorescence) was assessed by quantification of the mean green values of single cells (III). The [p.L7]+[p.P7] genotype cells produced significantly more (p=0.027) NPY-ir than the [p.L7]+[p.P7] genotype cells. The NPY-ir amounts of the two genotypes are represented in Figure 5.1.

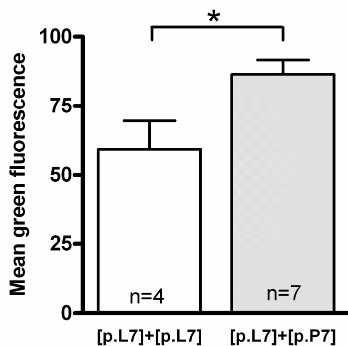

Figure 5.1. NPY-ir (green fluorescence) of 4 [p.L7]+[p.L7] genotype cells and 7 [p.L7]+[p.P7] genotype cells assessed with Image J 1.38x. The bars represent mean values ± SEM. * $p<0.05$ with unpaired t-test. Modified from the original communication III.

When HUVECs were stained with an anti-NPY antibody and a fluorescent mitochondrial marker, it was revealed that part of the NPY-ir was co-localized with mitochondria and part of NPY-ir was detected in intracellular vesicle-like compartments. These results indicated that in HUVECs the endogenously expressed NPY-ir is targeted to mitochondria, in addition to some vesicular compartments (Figure 5.2).

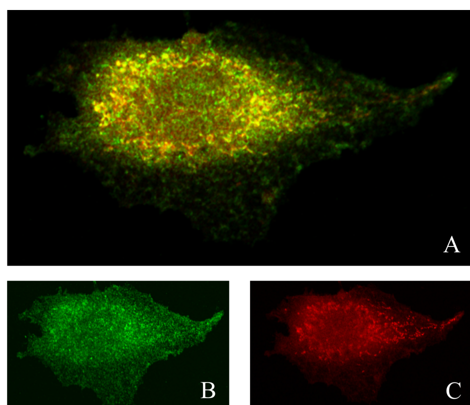

Figure 5.2. Endogenous NPY-ir in HUVECs.
A. An overlay picture of the NPY immunostained and mitochondrial marker stained HUVECs. NPY-ir is seen as green fluorescence and mitochondrial marker is seen as red fluorescence. The colocalization of NPY-ir and the mitochondrial marker (MitoTracker Red CMXRos, Molecular Probes) is seen as yellow colour.
B. NPY antibody stained cell (green fluorescence).
C. Mitochondrial marker stained cell (red fluorescence).

5.1.1.2 eNOS immunocytochemistry (IV)

The cells were immunocytochemically stained with the anti-human eNOS antibody. The eNOS protein production seemed to be elevated in the 1h NPY treated cells with the [p.L7]+[p.P7] genotype when compared to the [p.L7]+[p.L7] genotype. The difference in the basal level was not as clearly seen as in the NPY-treated cells. The difference in the eNOS staining between the [p.L7]+[p.P7] and the [p.L7]+[p.L7] genotypes was most clearly seen in 10 nM NPY and 1 µM NPY treated cells. Cells treated with the 100 pM NPY did not give a clear difference in the intensity of the staining between the two genotypes. The difference between CaI (1 µM) and carbacholine (1 µM) treated [p.L7]+[p.P7] and [p.L7] + [p.L7] genotype was not clearly detectable. The main difference was seen in the control cells in addition to 10 nM and 1µM NPY treated [p.L7]+[p.P7] and [p.L7]+[p.L7] genotype cells. It could be seen that in the [p.L7]+[p.L7] genotype cells the eNOS production was not raised after the NPY treatments in comparison to the control cells. (Figure 5.3)

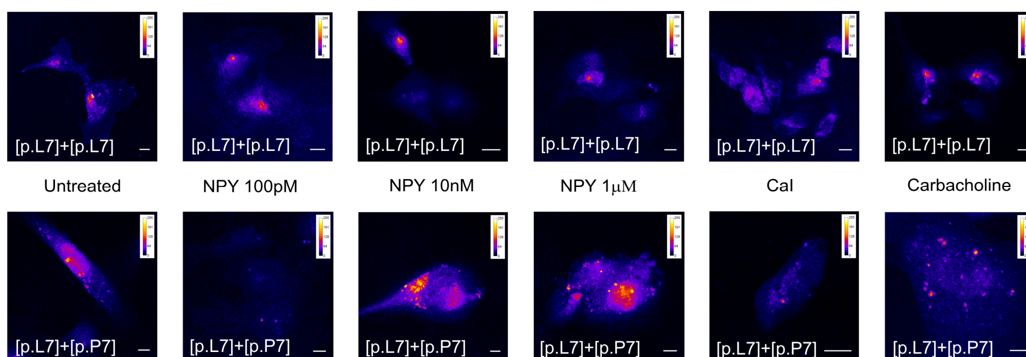

Figure 5.3. Immunoreactivity in HUVECs stained with eNOS antibody mouse anti-human-eNOS/NOS type III and anti-mouse IgG Alexa Fluor 488 dye. In addition to the untreated cells HUVECs were treated with 100 pM, 10 nM and 1 µM NPY, 1 µM CaI and 1 µM carbacholine. The pictures are intensity scaled to emphasize the difference between the genotypes. The [p.L7]+[p.L7] genotype cells are in the upper row and the [p.L7]+[p.P7] genotype cells are in the lower row. The scale bars are 10 µm and the calibration bars are in the upper right corners of each picture. Modified from the original communication IV.

5.1.2 Proliferation experiments (III)

5.1.2.1 [^{3}H]-thymidine incorporation

[^{3}H]-thymidine incorporation correlates with the rate of DNA synthesis and is an indirect measure of proliferation. The overall effect of the passage was found statistically significant ($p=0.0091$). The difference in the rate of the DNA synthesis between early and the late passages in cells with the [p.L7]+[p.L7] genotype was statistically significant ($p<0.05$), but the difference in the rate of the DNA synthesis between the two passages in the [p.L7]+[p.P7] genotype cells was not statistically significant. Neither LDL nor NPY stimulus had significant effect on the rate of the DNA synthesis measured with [^{3}H]-thymidine.

5.1.2.2 Cell count

The cells were counted to assess the cell division rate and the effects of NPY and VEGF treatments were tested. In the stimulation experiments the cell count of the control treatment (i.e. basal growth) was subtracted from the cell count of the respective treatment. VEGF was used as a positive control to increase the cellular growth rate. The overall effect of the treatment was statistically significant ($p=0.021$). Even though there was a tendency towards increased growth, there was no statistical significant stimulation of the growth in cells with the [p.L7]+[p.L7] genotype. NPY increased the growth of cells with the [p.L7]+[p.P7] genotype significantly ($p<0.05$), but there was no stimulation of growth by VEGF.

5.1.3 Apoptosis experiments (III)

5.1.3.1 *In situ* cell death (TUNEL)

The TUNEL assay is considered to measure the later phase apoptosis. The *in situ* cell death was assessed at early and late passages. There were no statistically significant differences in the extent of apoptosis between the genotypes and passages. The NPY treatments (10 nM, 100 pM, 1 pM) on late passage cells did not have any influence on the apoptosis in cells with either of the two genotypes.

5.1.3.2 Mitochondrial redox potential change (JC-1)

The mitochondrial redox potential change (JC-1 experiment) is a measure of very early apoptosis. The induction of cellular apoptosis was achieved with 5 µM or 10 µM H_2O_2 treatments. The basal change in the redox potential was measured in cells with [p.L7]+[p.L7] and [p.L7]+[p.P7] genotypes without treatments. The overall effect of the treatment was statistically significant ($p<0.0001$). The 5 µM H_2O_2 treatment of cells with the [p.L7]+[p.P7] genotype increased apoptosis by 210% ($p<0.01$) in comparison to the basal level. The cells with the [p.L7]+[p.L7] underwent only a 50% increase (ns) in the amount of apoptotic cells with a similar treatment. The 10 µM H_2O_2 induction increased the apoptosis by 190% ($p<0.001$) in cells with the [p.L7]+[p.L7] genotype and by 228% ($p<0.001$) in cells with the [p.L7]+[p.P7] genotype compared to the respective basal levels.

5.1.4 LDL-uptake and LDL receptor density

LDL uptake was significantly increased (p<0.05) in the late passage cells (6-9) compared with the early (4-5) passage [p.L7]+[p.L7] genotype cells, but not in cells with the [p.L7]+[p.P7] genotype. The increase of the LDL uptake was 110% in cells with the [p.L7]+[p.L7] genotype, but only 30% in cells with the [p.L7]+[p.P7] genotype. The overall effect of the passage was statistically significant (p=0.046) (Figure 5.4 A). In the LDL receptor density measurements there was not a statistical difference between the genotypes and the treatment with NPY did not have a significant effect on the LDL receptor density. (Figure 5.4 B).The LDL uptake seemed to rise in the [p.L7]+[p.L7] genotype, but the LDL receptor amount remained constant. This indicated that the plasmamembrane LDL turnover rate might be slower in the late passage HUVECs with the [p.L7]+[p.L7] genotype when compared to the early and late passage HUVECs with the [p.L7]+[p.P7] genotype.

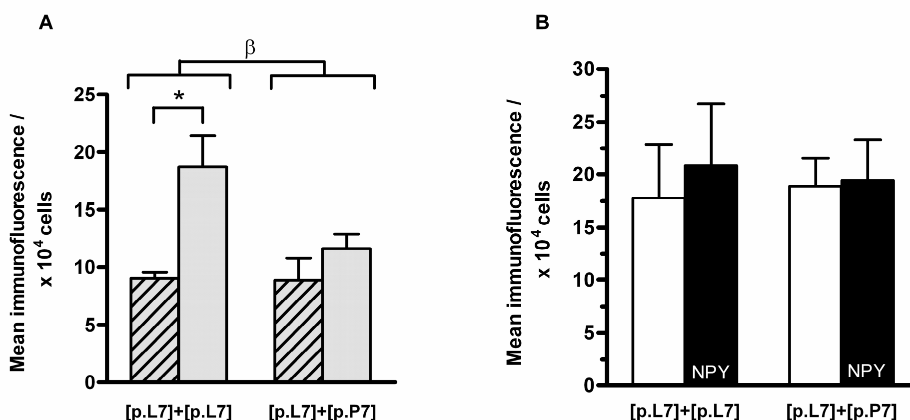

Figure 5.4. LDL uptake and LDL receptor amount in HUVECs.
A. LDL uptake of the [p.L7]+[p.L7] (n=15) and [p.L7]+[p.P7] (n=9) genotype cells grouped by early (4-5) (hatched bars) and late (6-9) (grey bars) passages. The statistical analysis was done with two-way ANOVA and Bonferroni's post hoc test. The bars represent mean values ± SEM. β represents p=0.046, * represents p<0.05.
B. LDL receptor density in [p.L7]+[p.L7] and [p.L7]+[p.P7] genotype cells (white bars). The NPY treatment (1 nM, 5 min) of cells with both genotypes (black bars). The statistical analysis was performed with two-way ANOVA and Bonferroni's post hoc test. The bars represent mean values ± SEM, n=7 in both groups.

5.1.5 Real time –PCR (IV)

In the real-time PCR experiments, differences were observed between the [p.L7]+[p.L7] genotype and the [p.L7]+[p.P7] genotype HUVECs in the 1 h (short term) NPY (1 µM, 10 nM, 100 pM, 1 pM) treatments. The overall trend was that in the [p.L7]+[p.L7] genotype cells all the treatments lowered the eNOS mRNA production while in the [p.L7]+[p.P7] genotype cells all the treatments raised the eNOS mRNA amount. The interaction effect (genotype×treatment) was very significant (p<0.0001) in the short term treated cells. Moreover, the overall genotype effect was statistically significant (p<0.0001). In [p.L7]+[p.L7] genotype cells all the treatments lowered the eNOS mRNA amount significantly in comparison to the untreated control cells, while in the [p.L7]+[p.P7] genotype cells the effect was completely opposite; the treatments

raised the eNOS mRNA amount significantly when compared to the untreated control cells. (Table 5.1).
The 20 h (long term) treated samples demonstrated very different results in contrast to the short term treated samples. The cells were treated with 10nM and 1pm NPY concentrations and VEGF. In the long term treated cells the interaction (treatment×genotype) effect was significant ($p<0.0001$). The overall genotype effect was also considered as significant ($p=0.0005$). In general, the treatments raised the eNOS mRNA amount in both genotypes. The 1 pM NPY treatment had quite a remarkable effect on the eNOS mRNA amount in both genotypes ($p=0.0094$). A statistical significance between VEGF treated [p.L7]+[p.L7] and [p.L7]+[p.P7] genotype cells was seen ($p<0.0001$). The original ΔΔCt±SD and $2^{-\Delta\Delta Ct}$±SD values for short term and long term treatments are presented in Table 5.1.

Table 5.1. Summary of the RT-PCR results: Treatment methods (I=short term treatment and III=long term treatment) and treatments of the [p.L7]+[p.L7] and the [p.L7]+[p.P7] genotype cells. The results are presented with values of ΔΔCt±SD and $2^{-\Delta\Delta Ct}$. Modified from the original communication IV.

Genotype / treatment method	Treatment	ΔΔCt±SD	$2^{-\Delta\Delta Ct}$±SD
[p.L7]+[p.L7] treatment method I n=4	untreated	0,0±0,79	1,00±0,79
	1 µM NPY	0,9±1,20	0,53±1,20
	10 nM NPY	1,1±0,78	0,48±0,78
	100 pM NPY	2,0±0,47	0,25±0,47
	1 pM NPY	1,8±0,99	0,28±0,99
	VEGF	1,8±0,69	0,28±0,69
	carbacholine	2,0±0,54	0,26±0,54
[p.L7]+[p.P7] treatment method I n=4	untreated	0,0±0,91	1,00±0,91
	1 µM NPY	-0,45±1,1	1,37±1,1
	10 nM NPY	-0,96±0,74	1,94±0,74
	100 pM NPY	-0,60±0,76	1,51±0,76
	1 pM NPY	-0,80±0,75	1,75±0,75
	VEGF	-0,87±0,86	1,83±0,86
	carbacholine	-0,50±0,60	1,42±0,60
[p.L7]+[p.L7] treatment method III n=4	untreated	0,0±0,51	1,00±0,51
	10 nM NPY	-0,16±0,46	1,11±0,46
	1 pM NPY	-0,87±0,55	1,82±0,55
	VEGF	-0,41±0,46	1,33±0,46
[p.L7]+[p.P7] treatment method III n=4	untreated	0,0±0,26	1,00±0,26
	10 nM NPY	-0,08±0,59	1,06±0,59
	1 pM NPY	-0,69±0,51	1,61±0,51
	VEGF	-1,14±1,37	2,20±1,37

5.1.6 Western blotting (IV)

Before the western blots, HUVECs were treated for 20 h (long term treatment) with NPY (1 µM, 10 nM, 100 pM, 1 pM) and VEGF. The primary antibody (mouse monoclonal anti human eNOS antibody) detected the 140 kDa protein band. Secondary antibody used in the western blot study was the HRP conjugated anti-mouse IgG. PageRuler Plus prestained protein ladder was used as a protein size standard. The interaction effect between the genotype and the treatment was not statistically significant. Nevertheless, the overall effect of the genotype was significant ($p=0.0099$) (model adjusted mean 19976 vs. 16398). The only statistically significant result between the genotypes was found to be in the 1µM NPY treatment ($p=0.0252$), when the treatments were compared separately.

5.1.7 NO determinations (IV)

The NO production was measured in the cells with a fluorescent dye, DAF-2DA. The statistical treatment×genotype interaction effect was not statistically significant but the trend could be seen ($p=0.0843$). The overall effect of the genotype was significant ($p=0.004$). In the untreated cells the difference between [p.L7]+[p.P7] and [p.L7]+[p.L7] genotype cells was significant ($p=0.0003$). The untreated [p.L7]+[p.P7] genotype cells produced significantly more NO than the untreated control cells of the [p.L7]+[p.L7] genotype. In addition to the untreated cells, statistical significance was also detected in the 10 nM NPY treated cells ($p=0.0335$).

The NO amount (DAN measurements) was measured from the serum-free media supplemented with NPY or CaI and the fluorescence intensity was compared between the two genotypes. The NO amount was measured from the serum-free medium and the fluorescence intensity was compared between the two genotypes. This experiment did not produce statistically significant differences.

5.1.8 Exercise study with healthy volunteers (I)

5.1.8.1 Baseline characteristics and VO_{2max} determinations

There were no differences in the mean baseline characteristics or VO_{2max} values between study subjects in the two genotype groups ([p.L7]+[p.L7] and [p.L7]+[p.P7]).

5.1.8.2 Heart rate

Subjects with the [p.L7]+[p.P7] genotype had significantly higher mean heart rate before and during the exercise study ($p<0.05$) than the subjects with the [p.L7]+[p.L7] genotype. No statistically significant differences were detected in mean heart rate at any separate time point between the groups and no differences were observed in systolic or diastolic blood pressures between the genotypes during the study period.

5.1.8.3 NPY and FFA concentrations

Subjects with the [p.L7]+[p.P7] genotype had higher overall plasma NPY concentration. The maximal NPY concentrations were found at 20 min timepoint. The statistically significant

differences in the NPY concentrations were found at 20 min timepoint and near significant differences in the NPY concentrations were found at 30 min and 40 min post exercise (p=0.05). The mean exercise induced increase of NPY between 0 min and 20 min was 90.4 ± 12.7 pmol/L in the subjects with the [p.L7]+[p.P7] genotype and 51.9 pmol/L ± 5.4 in the subjects with the [p.L7]+[p.L7] genotype.

There were no statistically significant differences in the concentrations of A and NA in plasma between the two genotype groups. The mean NA/NPY ratio in plasma was 84.9 ± 9.7 in subjects with the [p.L7]+[p.L7] genotype and 56 ± 5.3 in subjects with the [p.L7]+[p.P7] genotype. A clear difference was found in the overall FFA concentrations between the two groups ($p<0.05$). Subjects with the [p.L7]+[p.P7] genotype had significantly lower FFA concentrations when compared to subjects with the [p.L7]+[p.L7] genotype. The largest difference in post exercise NPY concentration was at the 40 min timepoint. NPY levels had a positive association with FFA concentrations in both genotype groups. Lower overall insulin concentrations were found in subjects with the [p.L7]+[p.P7] genotype but no clear exercise-induced reduction in insulin levels in this group. The statistically significant differences in concentrations were detected before the exercise at 0 min and after exercise at 60 min. Lactate levels were identical in the two genotype groups during the study.

5.2 THE PUTATIVE MITOCHONDRIAL NPY FRAGMENT (II, V)

The *NPY* gene was found to have two in-frame kozak sequences which do not perfectly fit to the kozak consensus sequence. The first kozak sequence (kozak-1) was found to be a part of the signal peptide which routes NPY normally to secretory vesicles. The sequence analyses of the *NPY* gene with a computer-aided sequence prediction program suggested that the second kozak (kozak-2) is part of a putative mitochondrial targeting signal. The kozak-2 sequence starts from the methionine at position 45 of preproNPY, which is the 17th aa of mature NPY (p.M45). The kozak-2 sequence was predicted with Mitoprot computer software to have a 64% probability to be routed to mitochondria. The ribosomes stop and start the translation process from the first kozak sequence more often in the neuroblastoma (neuronal) cells than in the fibroblast (non neuronal) cell line. (Summary of the localization of the constructs is presented in Table 5.2).

5.2.1 CHO-K1 cells

In the CHO-K1 cells, kzk-sp-NPY-GFP was co-localized with the Golgi/ER marker and the mitochondrial marker with some cytoplasmic fluorescence. The sp-NPY-GFP construct was co-localized with the mitochondrial marker, but not with the Golgi/ER marker. The kzk-sp-NPY(V45)-GFP construct was mainly localized in the Golgi/ER with some cytoplasmic staining, but no mitochondrial targeting was seen. Sp-NPY(V45)-GFP construct was detected in the cytoplasm (normal localization of the green fluorescent protein). The translation in this case supposedly started from the kozak-3 sequence (the kozak sequence of the EGFP) and therefore, only GFP was formed. This staining is comparable to that of plain GFP, which is known to be localized in the cytoplasm. Some localization was also detected the Golgi/ER compartment. The kzk-sp-GFP construct was co-localized with the Golgi/ER marker and some cytoplasmic staining was seen, but no mitochondrial targeting was detected. The sp-GFP construct was targeted to the cytoplasm, but some co-localization with the Golgi/ER marker was detected. Co-localization with the Golgi/ER marker was also seen with the sp-NPY(V45)-GFP construct (Table 5.2).

5.2.2 SK-N-BE(2) cells

In the SK-N-BE(2) cells, the kzk-sp-NPY-GFP construct was routed to the Golgi/ER compartment and no mitochondrial staining was detected. The sp-NPY-GFP construct was perfectly co-localized with the mitochondrial marker, but not with the Golgi/ER marker. However, the kzk-sp-NPY(V45)-GFP construct was not co-localized with the mitochondrial marker, but instead, it was localized in the Golgi/ER and in the cytoplasm. The sp-NPY(V45)-GFP construct was primarily seen in the cytoplasm and no co-localization with the mitochondrial marker was detected. In contrast to CHO-K1 cells, only a minimal amount of co-localization with the Golgi/ER marker was seen. The kzk-sp-GFP construct was not localized in mitochondria or in the cytoplasm; instead it was localized in the Golgi/ER. The sp-GFP construct routed mainly to the cytoplasm, but a small amount of targeting to Golgi was seen. This was, however, more uncommon than in the CHO-K1 cell line (Table 5.2).

Table 5.2. Summary of the cell type specific location of the constructs. Modified from the original communication II.

Construct	Kozak-1	Kozak-2	Kozak-3 (EGFP)	Cell type studied	Localization of the construct
kzk-sp-NPY-GFP	X	X	X	SK-N-BE(2)	TGN
				CHO-K1	TGN + mitochondria + cytoplasm
sp-NPY-GFP		X	X	SK-N-BE(2)	Mitochondria
				CHO-K1	Mitochondria
kzk-sp-NPY(V45)-GFP	X		X	SK-N-BE(2)	TGN + cytoplasm
				CHO-K1	TGN + cytoplasm
sp-NPY(V45)-GFP			X	SK-N-BE(2)	TGN + cytoplasm
				CHO-K1	Cytoplasm
kzk-sp-GFP	X		X	SK-N-BE(2)	TGN + cytoplasm
				CHO-K1	Cytoplasm
sp-GFP			X	SK-N-BE(2)	TGN + cytoplasm
				CHO-K1	Cytoplasm

5.2.3 Mobility of the GFP constructs with L7P (V)

The difference in the NPY mobility between L7P and L7L constructs in the cells with neuronal origin was studied with FRAP. In the cells transfected with the ER-routed kzk-sp-NPY-GFP L7L and kzk-sp-NPY-GFP L7P constructs, approximately 65% of the NPY protein was attached to some cellular structures, probably the trans Golgi network (TGN) and the secretory vesicles. Mobility of the kzk-sp-NPY-GFP L7P -produced protein was higher in comparison to the mobility of the kzk-sp-NPY-GFP L7L -produced protein (Table 5.3). The difference in the

mobility between the kzk-sp-NPY-GFP L7P and the kzk-sp-NPY-GFP L7L -produced proteins was not statistically significant. However, a trend could be seen and further studies are needed to confirm this observation. The putative mitochondrial proteins produced by the sp-NPY-GFP L7L and sp-NPY-GFP L7P constructs seemed to be strongly attached to some stable, undefined mitochondrial structures. Approximately 91% of the protein was attached to membranes or other stable structures. There was no significant difference between the constructs with the two different genotypes (L7L and L7P) (Table 5.3). Valinomycin treatment lowered the mean percentage of the mobile proteins from approximately 9% to 4% and, consequently, raised the attachment of the mitochondrial proteins.

When the ER-routed kzk-sp-NPY-GFP L7P construct mobility was compared with the same genotype mitochondrial construct sp-NPY-GFP L7P, the difference in the mobility of the ER-routed and mitochondria-routed P7 carrying constructs was significant. As valinomycin decreased the mobility percentage of the mitochondria-routed construct even further. When the ER-routed and the mitochondria (with and without valinomycin treatment) -routed L7 containing constructs were compared the difference was not statistically significant. There was a similar trend in the mobility of the proteins between the two genotype groups (L7L and L7P).

Table 5.3. Summary of the location and mobility percentages of the constructs in SK-N-BE(2) cells.

Construct	Localization of the construct in SK-N-BE(2) cell line	Mobility %
kzk-sp-NPY-GFP L7L	TGN	25 ± 6.3%
kzk-sp-NPY-GFP L7P	TGN	39 ± 7.8%
sp-NPY-GFP L7L	Mitochondria	10 ± 3.2%
sp-NPY-GFP L7P	Mitochondria	8 ± 1.9%

6 DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Cell lines

In the current series of studies two immortalized cell lines were used: A neuroblastoma cell line, SK-N-BE(2) (II, V) and a fibroblast cell line, CHO-K1 (II). The SK-N-BE(2) cells were human neuroblastoma cells from a male gender, while CHO-K1 cells were ovary cells from a Chinese hamster. The neuronal origin SK-N-BE(2) cells have previously been shown to have high affinity to NPY and contain especially Y2 receptors (Wahlestedt *et al.* 1992). SK-N-BE(2) cells have frequently been used in the NPY research (Kitlinska 2007). These cells with the neuronal origin allowed focusing on the neuroblastoma cell behaviour especially in the light of NPY and preproNPY L7P polymorphism. During these studies a putative mitochondrial NPY fragment was found (II, V). The putative mitochondrial NPY fragment was studied in the transfected SK-N-BE(2) and CHO-K1 cells. This comparison of the two cell lines gave important information on the intracellular processing differences of NPY in cells with a neuronal origin and in non-neuronal cells. However, despite the immortalization, it had to be ensured that the cells did not lose their differentiation. Therefore, the immortalized cells were cultured for maximum of 20-30 passages and discarded.

HUVECs were used as the primary cultured EC line. They contained the individual genome of a foetus and were easily obtainable. Therefore, HUVECs were a convenient material for studying genotype differences. HUVECs have previously been proved to produce NPY (Silva *et al.* 2003; Zukowska-Grojec *et al.* 1998). They grow in a cobblestone pattern and can be grown to confluence. They can be isolated and maintained as primary cell culture up to 10 passages (Jaffe *et al.* 1973). In the current studies HUVECs were extracted from fresh umbilical cords delivered from the Turku University Hospital. The cells of the umbilical cords were viable approximately 24 hours after the umbilical cord was cut. All the cell extractions were done so that the time from the cut of the umbilical cord to the extraction of the cells did not exceed 24 hours. In the studied HUVECs the amount of the two genotypes was neither in agreement with Hardy-Weinberg equilibrium ($p=0.0004$) nor the allele frequency of Finnish population (varies from 11 to 16%), (Karvonen *et al.* 1998; Karvonen *et al.* 2000). The carrier frequency of the L7P polymorphism varies between different populations. The HUVEC population in the current studies was most probably a mixed population. The ethnic background of the umbilical cord donors was not known due to the restrictions set by the Joint Ethics Committee of the Faculty of Medicine, University of Turku and the Turku University Central Hospital. All HUVECs in these studies were heterozygote. Only one homozygote ([p.P7]+[p.P7]) was found during these studies. The homozygous cells were not viable for reasons unknown.

All HUVECs used in the study had a genotype match from the other genotype group. The genotype-matched cells were isolated and seeded on the same day. It was made sure that the growth conditions remained as similar as possible at all times. Although the cells were seeded at the same time, their growth during the experiment was distinct and rather variable as often seen with the differentiated human primary cultured cells. This variability was noticed even with the control cells. The variability due to the primary cell culture lines was diminished with repeating each experiment approximately three times. The critical component of the cell culture medium was ECGS which contained growth promoting factors for vascular ECs of mammalian origin, especially HUVECs. This component was kept constant even though the overall cell culture conditions were changed during some experiments. The FBS concentration was changed to

promote a stressful condition for the HUVECs. Stress is an important factor in endothelial dysfunction and extracellular stress was under investigation in the studies III and IV. The extracellular stress to the cells was caused by starvation (nutrient deprivation) or by treatment with H_2O_2. In these studies also the treatment times with NPY and VEGF varied (1h and 20 h). This gave the possibility to compare rapid and slow responses to extracellular stimuli.

Although HUVECs can be cultured for several passages as functional ECs, they do undergo age-associated changes during the culture lifespan and start to lose their differentiation (Park *et al.* 2006). The ECs do not normally divide in adult vessels. The increased EC turnover has been considered as a risk for atherosclerosis (Foreman & Tang 2003). In these studies the cells were used at passages 2-9 (mostly at passages 4-9). Furthermore, the cells were divided into two categories according to the passage of the cells. Passages 2-5 were considered to be early passages and passages 6-9 were considered to be the late passages.

Regarding the studies done with primary cell lines, it has to be kept in mind that the growth and the overall behaviour of each individual cell line may vary considerably. In addition to repeating experiments, another way to avoid problems caused by the variability of the primary cultured cells is pooling, which is a useful way of getting reliable results especially when one single sample does not contain a sufficient amount of samples (Peng *et al.* 2003). Furthermore, pooling minimizes the amount of lost information in cases where single sample cannot be detected efficiently (Schisterman & Vexler 2008). Pooling was performed in Study IV to avoid the aforementioned problems.

6.1.2 Fluorescent methods and protein and organelle visualization

NPY, C-PON and eNOS were visualized with fluorescent antibodies. Antibodies and confocal microscopic studies gave the first indication of the putative mitochondrial NPY fragment. Furthermore, differences in the staining patterns between the genotypes were observed. Immunocytochemical detections of NPY and C-PON were carried out with polyclonal antibodies. The polyclonal antibodies have their advantages as well as disadvantages. Compared with monoclonal antibodies, polyclonal antibodies are not as specific as the monoclonal antibodies which are often harder to detect. Due to the polyclonal antibodies, the exact binding site of the antibodies was not known and furthermore, the different sizes of the NPY fragments were not known. The polyclonal anti-NPY antibody recognizes both NPY and proNPY and does not recognize C-PON which is present on proNPY. Therefore also C-PON specific antibody and double labelling techniques were used to differentiate between NPY and C-PON. Furthermore, the correct binding of the polyclonal anti-NPY antibody was verified with a commercially available NPY_{1-36} protein. Dr. Grouzmann's group has used monoclonal antibodies to detect the different parts of the NPY protein in HUVECs (Grouzmann *et al.* 1992; Silva *et al.* 2005). These results are in agreement with the results presented in this thesis. The eNOS antibody used in these studies was a monoclonal antibody. Two parallel methods (immunocytochemistry and western blotting) were used with the eNOS antibody. The use of two parallel methods was necessary in our Study IV since the eNOS immunostained cells were not quantified and hence, the result of the immunocytochemical staining can be considered as an indicative result. The specific binding to the right sized protein was confirmed and the quantification was done with the western blotting.

In addition to the immunocytochemical methods, full NPY and the mitochondrial NPY fragment were studied with EGFP constructs. EGFP is a fluorescent tag with excitation peak at 488 nm and emission peak at 509 nm. GFP is a universal tool in localization and trafficking

studies in live cell imaging (Lippincott-Schwartz *et al.* 2001) and is has allowed the real-time studying of neuropeptide proteins *in vitro* and *in vivo* (Levitan 2004; Levitan 1998). Furthermore, fluorescence photobleaching recovery experiments are easy to conduct with GFP-tagged neuropeptides (Levitan 2004). In Study V, the FRAP technique was used to study the mobility of the EGFP tagged NPY and mitochondria routed proteins. The traditional confocal microscopy allows very high quality pictures to be taken but in FRAP, the quality of the confocal pictures is poorer than in the traditional confocal microscopy. The poorer quality is due to the limitations of the FRAP technique. The EGFP fusion protein can be photodamaged and if the movement of the molecule in question is to be visualized in a living cell without doing harm to the fluorescence and the cell, the use of laser needs to be quick and relatively low-powered. It is beneficial that the cell is scanned only once in order to avoid harming the fluorescence intensity and the cell as little as possible. Each scanning with laser still bleaches the fluorescence. On the other hand, when the cell is scanned only once and rather rapidly, the quality of the image suffers. Nevertheless, as stated in the review article of Gerlich and Ellenberg: "For each biological application, it is crucial to find a suitable compromise between sufficient, but not toxic, illumination, spatial resolution, temporal resolution and the signal-to-noise ratio so that the maximum number of acceptable images can be acquired before the specimen is completely photobleached or damaged." (from Gerlich & Ellenberg 2003).

GFP itself is quite a large protein and it can potentially disturb intracellular trafficking. However this commonly used tag has previously been used in the NPY and NPY receptor studies (Bohme *et al.* 2007; Ding *et al.* 2005; Dinger *et al.* 2003; El Meskini *et al.* 2001; Mitchell *et al.* 2008). In addition, when the trafficking of the EGFP constructs was compared with the localization of the endogenously expressed NPY visualized by immunostaining, the localization was similar. GFP protein itself contains a kozak sequence. In our studies this kozak sequence was named kozak-3 to avoid ambiguity in results. The cellular localization of the EGFP was verified to be cytoplasmic and nuclear.

Quantification of the fluorescence is a common matter of controversy. There are multiple opinions about the quantitative measures of the fluorescence. In fluorescence quantification, the intensity of the confocal image can be quantified, but it has to be remembered that fluorescence intensity may change between individual cells in GFP construct transfected cells as well as immunocytochemically stained cells. Therefore the appropriate controls, such as untransfected cells or cells stained only with the secondary antibody, are valuable in the quantification of the fluorescence. The appropriate negative controls allow the background reduction and the positive controls and standards verify the detection of the correct signal. For example, in the current studies positive and negative controls were used in the experiments with FACS. The settings of FACS were validated with unstained (negative) control cells and positively stained cells. In the apoptosis experiments apoptosis-induced cells were used as positive control cells. The measurement of apoptosis itself divides opinions, since it is commonly known that for example in the TUNEL experiment necrotic cells may cause false positive results. Also, DNA strand breaks may be prominent in cell populations with high proliferative activity. These false positive results can be avoided with morphological examination of the cells, since apoptotic cells have a characteristic pattern. Furthermore, it is important to analyse each sample several times. In our Study III each of the samples in the TUNEL experiment was analysed in triplicates. Additionally another method to detect apoptosis (JC-1) was used in addition to TUNEL. Overall the optimization of methods is required for reliable results and care must be taken in analysing the results. In addition to the methodological aspects variability in the results may be due to the hardware settings. To minimize the variability in the results, the hardware settings were kept constant. In FACS the symmetry of the peak was checked each time, the PMT voltage and the compensation were kept constant. In confocal microscopic studies parameters such as laser power, pinhole, gain, scan speed and offset were kept constant.

6.2 DIFFERENCES FOUND BETWEEN [P.L7]+[P.L7] AND [P.L7]+[P.P7] GENOTYPES (I, III, IV, V)

The original article that introduced the T1128C SNP was published by Karvonen et al. (1998). The L7P polymorphism found in the signal peptide part of the prepro NPY was associated with higher serum cholesterol and LDL cholesterol levels in obese subjects (Karvonen *et al.* 1998). Later studies revealed that the T1128C change has pleiotrophic effects; it has been associated with multiple physiological and pathophysiological responses. The majority of the NPY signal peptide L7P polymorphism carriers were found to be heterozygous (Karvonen *et al.* 1998). Consequently, *in vivo* neurons are likely to contain preproNPY with L7 and P7 (Mitchell *et al.* 2008). The first Study of the thesis revealed a different distribution of the NPY-ir between the two genotypes. In HUVECs with the [p.L7]+[p.P7] genotype, the amount of NPY without C-PON was prominent. ECs with the [p.L7]+[p.L7] genotype contained almost exclusively proNPY. This finding suggested a difference in the processing of the preproNPY between the genotypes. Furthermore, in the clinical studies it has been shown that subjects with the [p.L7]+[p.P7] genotype have increased amount of NPY-ir in plasma after stimulation when compared with subjects with the [p.L7]+[p.L7] genotype. In the Study III it was verified that cells with the [p.L7]+[p.P7] genotype produce significantly more NPY-ir than the cells with the [p.L7]+[p.L7] genotype and furthermore, in the Study V it was discovered that P7 allele may cause increased mobility of the mature NPY_{1-36}. It was hypothesized that one reason for the increased mobility may have been the increased production of NPY_{1-36} in cells carrying the [p.L7]+[p.P7] genotype. Therefore NPY_{1-36} can also be found in the mobile fraction (V). On the contrary, cells with the [p.L7]+[p.L7] genotype contained probably less mature $NPY_{1-36,}$ and more proNPY was routed to the secretorial pathway. This idea was supported by the recent study of Mitchell et al. (Mitchell *et al.* 2008). They studied the L7P and L7L preprohormones in mouse anterior pituitary cells and mouse chromaffin cells. They also concluded that in the L7P and L7L NPY prohormones were sorted to the same dense core granules in neuronal cells and furthermore, to the secretory pathway (Mitchell *et al.* 2008). Similar expression was found in the hippocampal slices and the neuronal cell line (Mitchell *et al.* 2008). The major finding of their study was the increased peptide secretion from the cells expressing the P7 prohormone. In addition, the dense core granules contained higher amounts of P7 prohormone (Mitchell *et al.* 2008). The study of Mitchell et al. (2008) is in complete agreement with our studies. The difference in the cellular distribution was seen in Study I using NPY and C-PON recognizing antibodies and quantified in the Study III. However, we quantified the amount of NPY-ir and not the proNPY-ir. The kozak sequences were under investigation in the Study II and it was shown that in the cells with neuronal origin the mature NPY is produced and routed to Golgi, ER and the secretory pathway. Moreover, it was proved that the non-neuronal cell may produce less NPY_{1-36} and more truncated NPY fragments such as NPY_{17-36}. In the light of our studies and the study of Mitchell et al. (2008) it can be hypothesized that endogenous changes in the processing due to the L7P polymorphism have an effect on the exogenous NPY.

In addition to the original association study, the L7P polymorphism has been associated with enhanced carotid atherosclerosis in elderly patients with T2D and in control subjects (Karvonen *et al.* 2001; Niskanen *et al.* 2000a). Endothelial dysfunction has an important role in the pathogenesis of atherosclerosis (Davignon & Ganz 2004; Lusis 2000; Stoll & Bendszus 2006). Apoptosis has been observed in many inflammatory processes and it has been claimed that apoptosis contributes to plaque formation and rupture in atherosclerosis (Berk *et al.* 2001; Salvayre *et al.* 2002; Stoneman & Bennett 2004; Winn & Harlan 2005). Especially the uptake and accumulation of modified LDLs can induce apoptosis in ECs (Boyanovsky *et al.* 2003; Escargueil-Blanc *et al.* 1997). Based on the previously mentioned association studies, the genotype effects on apoptosis, proliferation, LDL uptake, and LDL receptor amount were

studied in ECs (Study III). No significant difference in the overall apoptosis between the two genotypes was found. It may be that cells with [p.L7]+[p.P7] genotype have a higher response to a milder apoptosis stimulus, although this is highly hypothetical. Exposure of ECs to high LDL levels evokes H_2O_2 production, which in turn may lead to cytoskeleton remodelling potentially causing endothelial dysfunction (Holland *et al.* 2001). Also, there was no difference in the basal level proliferation (rate of DNA synthesis) of HUVECs between the two genotypes at early passages. Nevertheless, the overall effect of the passage was statistically significant. Neither NPY nor LDL had any effect on proliferation between the genotypes; however the cells in this experiment were not syncronized which may decrease the sensitivity of the assay. Cells with the [p.L7]+[p.L7] genotype at late passages divided significantly faster than cells with the same genotype at early passages. A similar tendency in cells with the [p.L7]+[p.P7] genotype was found, nevertheless the difference between early and late passages was not statistically significant. This result may indicate that the [p.L7]+[p.P7] genotype cells undergo to the state of replicative senescence earlier in their lifespan than the [p.L7]+[p.L7] genotype cells. It is known that when human somatic cells age, they stop replicating and enter a state of growth arrest, replicative senescence (Foreman & Tang 2003). Senescent cells are viable and metabolically active but they have altered gene and protein expression (Foreman & Tang 2003). ECs are known to undergo to the senescent state *in vivo* and *in vitro* (Foreman & Tang 2003). Endothelial senescence may result in gene and protein expression change, and contribute to the development of age associated diseases such as atherosclerosis. However, it may also be that the proliferative lifespan is changed between the [p.L7]+[p.L7] and the [p.L7]+[p.P7] genotype cells. Therefore, when the [p.L7]+[p.P7] genotype cells are entering the stage of growth arrest, the [p.L7]+[p.L7] genotype cells may well be at the stage of active proliferation. It may be speculated whether the division to early and late passages is valid for both genotypes. This hypothesis remains to be confirmed.

In another set of proliferation experiments (cell count), where both early and late passage cell lines were included, NPY stimulated the growth. The overall effect of the treatment was statistically significant and the increase was statistically significant in the [p.L7]+[p.P7] genotype cells. The LDL uptake experiment showed, that when the passage of cells increased, the cells with the [p.L7]+[p.L7] genotype exhibited more LDL-uptake than the [p.L7]+[p.P7] genotype cells. The uptake and metabolism of LDL are critical in the formation of atherosclerotic plaques. The pathological effects of LDL on the arterial wall involve LDL crossing through ECs, retention in the arterial wall ("lipoprotein retention"), and modification to more atherogenetic forms of LDL, which in turn can be taken up by scavenger receptors on macrophages. LDL is delivered into the cell mainly by LDL-receptor mediated endocytosis (Jeon 2005). LDL receptor density did not differ between the genotypes. However, other means of LDL-uptake, such as lipoprotein lipase (LPL) mediated LDL binding to EC–derived extracellular matrix (ECM) layer (Saxena *et al.* 1992) or transcytosis (Vasile *et al.* 1983), could be stimulated. ECs also lose their barrier function and become leakier as they age (Boissonneault *et al.* 1990). This could lead to enhanced infiltration of small lipoproteins into the cells at the later passages, in other words, when cells age. Increased LDL-uptake into the cell without increased receptor mediated endocytosis could also be due to slower turnover rate of LDL in the late-passage ECs with the [p.L7]+[p.L7] genotype compared with early- and late-passage cells with the [p.L7]+[p.P7] genotype. This could lead to lipid accumulation in older ECs (i.e. higher passages), as has been reported previously with human pancreatic β-cells (Cnop *et al.* 2000). If the normal age-related increase of LDL uptake is repressed in cells with the [p.L7]+[p.P7] genotype at higher passages, it could lead to higher LDL levels in adults with the [p.L7]+[p.P7] genotype, as seen in the clinical studies (Karvonen *et al.* 1998).

NPY has been proved to mediate vasodilation through NOS pathways in several studies (Kobari *et al.* 1994; You *et al.* 2001). Endothelial dysfunction is in close relation to an impairment of

NOS dependent pathways and is an early marker of atherogenesis (reviewed by Napoli *et al.* 2006). Additionally, L7P polymorphism has been associated with atherosclerosis as stated previously. Hence, we decided to look at the possible association of the L7P polymorphism and the eNOS and NO expression in ECs. Our Study IV was the first study to prove that L7P polymorphism had an effect on the eNOS and NO amounts. Moreover, there was also a difference in the NPY dependent NO expression at the cellular level between the two studied genotypes. There was not a significant difference in the eNOS production in the long term treatments at the basal level. Yet, in the short term treatments, the NPY stimulus seemed to provoke a faster response in the [p.L7]+[p.P7] genotype cells. It is probable that the [p.L7]+[p.L7] genotype cells react slower to the changes in the environmental conditions than the [p.L7]+[p.P7] genotype cells. It may also be, that in a long time frame the [p.L7]+[p.L7] genotype cells seize the eNOS expression levels of the [p.L7]+[p.P7] genotype cells. The differences in the eNOS expression may be, again, one factor contributing to the physiological differences associated with the L7P polymorphism. It is also known that shear stress is a contributing factor to the endothelial NO production and contractility of the veins (Qiu *et al.* 2001). Additionally, NPY has been associated with stress response. Circulating plasma NPY levels increase during stress, particularly when the stress is severe or prolonged (Zukowska *et al.* 2003b). In fact, in stressed healthy [p.L7]+[p.P7] genotype subjects the plasma and overall NPY levels were significantly higher than in the healthy subjects with the [p.L7]+[p.L7] genotype (I). In addition to the association of the L7P polymorphism with the increased release of vascular NPY (I), the P7 substitution has also been associated with enhanced endothelial dependent vasodilation (Järvisalo *et al.* 2003). Based on these findings, it seems that L7P polymorphism affects the vasodilation system through a mechanism that involves NO.

The association of *in vitro* cellular studies with the clinical studies, as well as experimental animal studies, is not necessarily simple. Overall, it can be concluded that [p.L7]+[p.P7] genotype cells seemed to be more sensitive to the extracellular stimuli. The ability of a cell to sense and appropriately respond to adverse conditions is a result of multiple intracellular signalling pathways, which trigger the response to the signal in question (Leong *et al.* 2003; Martindale & Holbrook 2002). Cellular hyper-responsiveness is probably a contributing factor to hypertension and furthermore to cellular senescence and premature aging resulting in atherogenic changes (Vanhoutte *et al.* 2005). As stated previously, endothelial dysfunction and NOS dependent pathways contribute to atherogenic changes (reviewed by Napoli *et al.* 2006). The combination of shear stress, higher blood pressure, increased NPY levels and cellular hyperreactivity results in increased vasodilation and alternatively increased contractility following the vasodilation, could be causative factors to endothelial dysfunction and atherosclerosis in the carriers of the [p.L7]+[p.P7] genotype (Figure 6.1). The physiological effects may be a summary of many changes at the cellular level and fully understanding the potential hypersensitivity detected in these studies need further research. However, the hypersensitivity may be important in explaining, for example, the independent association of P7 allele with enhanced atherosclerosis in humans. As evidenced earlier, T1128C change has pleiotrophic effects. Multiple reasons which might be contributing factors to the physiological changes were found. It may also be speculated weather the actual cause of some changes is the L7P polymorphism or some other reason, such as a linked polymorphism. Furthermore, it has to be kept in mind that all the studied cells were heterozygous. It may be that differences between the genotypes would be clearer in homozygous cells.

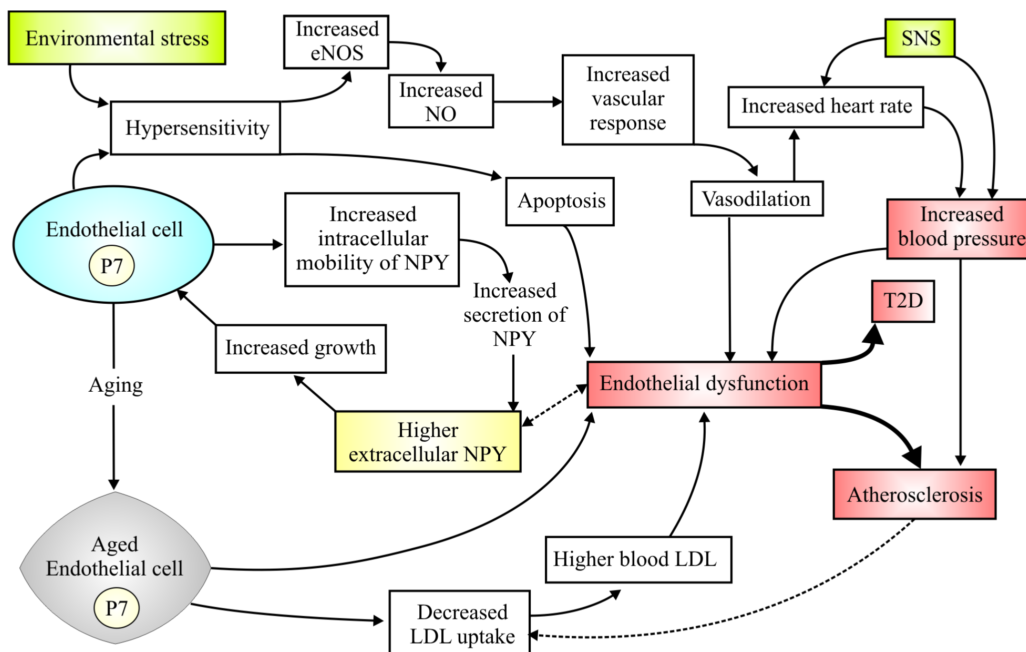

Figure 6.1. A hypothetical model of the effects of the preproNPY P7 allele based on the results of these studies and the clinical associations studies. P7 allele containing cells react to environmental stress and are hypersensitive. This, in turn, leads to an increase in NO production and increased vascular response. Increased vascular response contributes to vasodilation and increased heart rate. Vasodilation is a contributing factor in endothelial dysfunction but the role of the SNS should not be forgotten in the control of the heart rate and the blood pressure. Increased blood pressure, on the other hand, contributes to the development of atherosclerosis. Aging is a contributing factor to endothelial dysfunction. Furthermore, aged ECs may have decreased LDL uptake that leads to higher blood LDL concentration. Higher blood LDL concentration is also a contributing factor to the endothelial dysfunction. Endothelial dysfunction contributes to the development of atherosclerosis and T2D. Nevertheless, ECs with P7 allele have increased intracellular mobility of NPY, which leads to increased secretion of NPY and increased extracellular NPY. The higher extracellular NPY may increase the growth of the ECs. This again, may lead to a vicious circle of growth and again, hypersensitivity and ageing.

6.3 THE PUTATIVE MITOCHONDRION TARGETED FRAGMENT OF NPY (II, V)

In addition to the investigation of the L7P polymorphism, the other leading idea of the current studies (II, V) has been the investigation of the putative mitochondrion targeted fragment of NPY. The mitochondrial location of NPY-ir was introduced in Study II and studied further in Study V. Kozak sequences are important in the translation process of the protein. The importance of the kozak sequence has mainly been studied by Marilyn Kozak’s group (Kozak 1986a; Kozak 1997; Kozak 2001a; Kozak 2001b; Kozak 2002a; Kozak 2003; Wang & Rothnagel 2004). Furthermore, the categorization of the kozak sequences into strong and weak sequences has become an important issue in translation studies (Kozak 1983; Kozak 1989; Kozak 2001c; Kozak 2002b; Kozak 2003). Current studies prove the existence and investigate the mitochondrial NPY fragment. Furthermore, the mobility of the mitochondrial NPY fragment was studied. The kozak sequence leading to the mitochondrial targeting was named as the kozak-2 sequence with the thought of NPY protein translation initiation sequence of being

the kozak-1 sequence. The first studies of the mitochondrial effects of NPY were conducted already in 1988 by Balasubramaniam et al. (Balasubramaniam *et al.* 1988). Further studies identified a fragment NPY_{17-36} and investigated the ability of this fragment to exhibit a biphasic effect on cardiac adenylate cyclase activity (Sheriff & Balasubramaniam 1992). It was noticed that the biphasic effect of the NPY_{17-36} was specific to certain tissues (Sheriff & Balasubramaniam 1992). In our studies the mitochondrial routing of the kozak-2 sequence was originally predicted with sequence analyses program called Mitoprot. This analysis was supported by computer software called P-SORT, which also gave a high probability for the kozak-2 sequence to be routed to mitochondria (Brun *et al.* 2006). The first aa to start the translation of the mitochondrial targeted protein was predicted to be the 17th aa of the mature NPY (NPY_{1-36}). In addition to our study, the study of Silva et al. detected an endogenous NPY-ir in the mitochondria of HUVECs (Silva *et al.* 2005). Brun et al. (2006) have studied the N-terminal truncated NPY and proved that the targeting was indeed mitochondrial. They used an NPY midportion-sensitive antibody (Brun *et al.* 2006). This supports our study and indicates that even though EFGP tagged protein constructs were used to study the localization and the mobility of the protein, the targeting was not disturbed by the EGFP tag. The exact size of the protein is not known and it has to be kept in mind that when the mitochondrial localization in our study was found, EGFP constructs lacked the C-PON of the preproNPY. However, it is likely that the size of the mitochondrial protein is 20 aa's after post translational modifications. The initiation of translation *in vivo* is probably the 17th aa and the stop codon is likely to be the same stop codon as with the mature NPY protein. There is no reason not to believe that the post translational modifications take place in the similar way as with the mature NPY_{1-36}.

The novel finding of our study was the targeting differences of the mitochondrial protein in neuronal origin and non-neuronal cell lines. When the kozak-2 sequence was destroyed the routing to mitochondria was inhibited. In contrast, when only the kozak-2 was present, the mitochondrial targeting was seen in both cell lines. The kozak sequences are declared to be leaky when the start codon is sometimes passed by the ribosomes and other times the translation is started on the start codon in question. Furthermore, the kozak sequences can be divided into minimally leaky and maximally leaky kozaks (Kozak 1999; Kozak 2002b). Based on our results it is probable that the kozak-2 is a minimally leaky kozak sequence. It appears that in the non-neuronal cells the leaky scanning of the kozak-2 sequence is occurring more than in neuronal cells. Additionally, when the kozak-1 sequence of NPY was studied, it was noticed that the kozak-1 sequence was a requirement for protein targeting to Golgi and ER in the cells with neuronal origin, but not in non-neuronal cells. The specific targeting to the Golgi and ER in the neuroblastoma cells was a logical finding since NPY has a secretorial role in neuronal cells and in cells with neuronal origin. Also, when the kozak-1 and the kozak-2 sequences of the *NPY* gene are present, the kozak-1 seems to be the stronger kozak sequence. To conclude, in the cells with the neuronal origin the kozak-1 sequence seemed to be the preferred translation initiation site.

6.4 THE INTRACELLULAR MOBILITY OF NPY AND THE MITOCHONDRIAL NPY FRAGMENT (V)

Due to the lack of knowledge about the mitochondrial fragment of NPY, also the mobility of the fragment was characterized. Study V was conducted to learn more about the mitochondrial NPY fragment, NPY and also possible effects of the NPY signal sequence L7P polymorphism on the mobility of the NPY protein. The mobility of the mitochondrial protein was found to be extremely low. Valinomycin permeabilizes the inner membrane of the mitochondrion (Safiulina *et al.* 2006). Therefore valinomycin treatment was used to see if the mitochondrial mobility was

changed after permeabilization of the inner membrane. It was concluded that the mitochondrial fragment was not located in the intermembrane space or the inner membrane of the mitochondrion. In fact the mobility was even lower after the valinomycin treatment. The reasoning from that result was that the mitochondrial protein was possibly attached to the outer membrane of the mitochondrion. Of course, the mitochondrial fragment may have extra-mitochondrial targets as suggested by its receptor-mediated effects on rat cardiac adenylate cyclase activity (Sheriff & Balasubramaniam 1992). It can also be speculated that the mitochondrial fragment may have a role in oxidative phosphorylation. Oxidative phophorylation is mechanistically a complex process and results in ATP formation. In the study of Balasubramaniam et al. (1988), NPY was found to uncouple oxidative phosphorylation and block mitochondrial calcium uptake (Balasubramaniam *et al.* 1988). However, no evidence was found that the mature $NPY_{1\text{-}36}$ or the truncated $NPY_{17\text{-}36}$ would actually enter mitochondria (Balasubramaniam *et al.* 1988). To speculate even further, if the mitochondrial fragment had a role in oxidative phosphorylation, it could also affect the ageing and senescence of the cells. It has also been suggested that the NPY fragment might cause a mitochondrial membrane permeability transition (MPT), causing changes in Ca^{2+} balance between the endoplasmic reticulum and mitochondria, which may, in turn, affect the apoptosis (Kanno *et al.* 2004; Tsujimoto *et al.* 2006). Mitochondria containing $NPY_{17\text{-}36}$ has been shown to have 14 mV more negative membrane potential that mitochondria without NPY (Brun *et al.* 2006). This could have considerable effects on the mitochondrial function. It can be speculated that the mitochondrial fragment could affect cellular metabolism, energy expenditure and even apoptosis. It is also debatable whether the mitochondrial protein has any importance *in vivo*. When evolution is considered, the mature NPY sequence is rather conserved and this includes also the kozak-2 sequence (Larhammar 1996). The evolutional conservation may suggest that the mitochondria-targeted protein has an importance in the intracellular functions. The physiological importance of the mitochondrial fragment of NPY remains to be studied in the future.

7 CONCLUSIONS

These cellular studies on the effect of the NPY signal sequence L7P polymorphism on the processing and functionality of the protein and the mitochondrial form of NPY allow the following conclusions:

1. A clear difference was seen in the intracellular distribution of NPY and proNPY. In the [p.L7]+[p.P7] cells NPY-ir was prominent, while in the [p.L7]+[p.L7] genotype cells more proNPY-ir was detected.

2. In ECs the LDL-uptake was increased in cells with the [p.L7]+[p.L7] genotype at late passages when compared to early passages, no such increase was detected in cells with the [p.L7]+[p.P7] genotype. Differences between the genotypes in growth stimulation by NPY, VEGF and in early apoptosis stimulation were found. ECs with the [p.L7]+[p.P7] genotype react differently to growth stimulation and under stressful conditions they are more vulnerable than cells with the [p.L7]+[p.L7] genotype.

3. NPY was proved to have a significant effect on the eNOS and NO metabolism. L7P polymorphism in the signal sequence of preproNPY has a significant effect on the eNOS and NO production in ECs.

4. The mitochondria-targeted NPY fragment was proved to exist and the translation initiation sequence for the mitochondrial targeting was identified. Also translation differences were found between neuronal and non-neuronal cells.

5. The mobility of the mitochondrially targeted NPY fragment was minimal and therefore it is probable that the protein is attached to the outer membrane of the mitochondrion. The genotype did not have any major effect on the mitochondrial protein. The L7P change in the signal sequence of preproNPY may cause an increased mobility of the mature $NPY_{1\text{-}36}$.

These *in vitro* results support clinical findings where L7P polymorphism was associated with pathophysiological conditions such as atherosclerosis. At the cellular level, it can be concluded that the L7P polymorphism of preproNPY might cause a series of intracellular changes contributing to the state of pre-senescence, leading to endothelial dysfunction and even to atherosclerosis. Furthermore, the changes in the NO and eNOS expression levels between the [p.L7]+[p.L7] and the [p.L7]+[p.P7] genotypes may contribute to the vascular tone and even blood pressure, which are important factors in the development of cardiovascular diseases. The physiological relevance of the mitochondrial fragment needs further investigation.

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