TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 838

MEDICA - ODONTOLOGICA

TRANSGENIC MICE OVEREXPRESSING NEUROPEPTIDE Y: AN EXPERIMENTAL MODEL OF METABOLIC AND CARDIOVASCULAR DISEASES

by

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TURUN YLIOPISTO
Turku 2009

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ISBN: 978-951-29-3819-3 (PRINT)
ISBN: 978-951-29-3820-9 (PDF)
ISSN: 0355-9483
Painosalama Oy – Turku, Finland 2009

"Drug therapies are replacing a lot of medicines as we used to know it."

George W. Bush

ABSTRACT

Suvi Ruohonen

Transgenic mice overexpressing neuropeptide Y: An experimental model of metabolic and cardiovascular diseases

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Annales Universitatis Turkuensis, Medica-Odontologica, 2009, Turku, Finland

Neuropeptide Y (NPY) is an abundant neurotransmitter in the brain and sympathetic nervous system (SNS). Hypothalamic NPY is known to be a key player in food intake and energy expenditure. NPY's role in cardiovascular regulation has also been shown. In humans, a Leucine 7 to Proline 7 single nucleotide polymorphism (p.L7P) in the signal peptide of the *NPY* gene has been associated with traits of metabolic syndrome. The p.L7P subjects also show increased stress-related release of NPY, which suggests that more NPY is produced and released from SNS. The main objective of this study was to create a novel mouse model with noradrenergic cell-targeted overexpression of NPY, and to characterize the metabolic and vascular phenotype of this model.

The mouse model was named OE-NPY^{DBH} mouse. Overexpression of NPY in SNS and brain noradrenergic neurons led to increased adiposity without significant weight gain or increased food intake. The mice showed lipid accumulation in the liver at young age, which together with adiposity led to impaired glucose tolerance and hyperinsulinemia with age. The mice displayed stress-related increased mean arterial blood pressure, increased plasma levels of catecholamines and enhanced SNS activity measured by GDP binding activity to brown adipose tissue mitochondria. Sexual dimorphism in NPY secretion pattern in response to stress was also seen. In an experimental model of vascular injury, the OE-NPY^{DBH} mice developed more pronounced neointima formation compared with wildtype controls.

These results together with the clinical data indicate that NPY in noradrenergic cells plays an important role in the pathogenesis of metabolic syndrome and related diseases. Furthermore, new insights on the role of the extrahypothalamic NPY in the process have been obtained. The OE-NPY^{DBH} model provides an important tool for further stress and metabolic syndrome-related studies.

Keywords: Neuropeptide Y, transgenic mouse, sympathetic nervous system, stress, obesity, impaired glucose tolerance, hypertension, vascular remodelling

TIIVISTELMÄ

Suvi Ruohonen

Neuropeptidi Y:tä yli-ilmentävä siirtogeeninen hiiri: Kokeellinen metaboliasairauksien sekä sydän- ja verisuonitautien eläinmalli

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Neuropeptidi Y (NPY) on hermoston välittäjäaine, jota esiintyy runsaasti aivoissa ja sympaattisessa hermostossa. Hypotalaminen NPY on tärkeä syömistä ja energiankulutusta säätelevä tekijä. NPY:n on myös osoitettu osallistuvan sydämen ja verisuonten toiminnan säätelyyn. Ihmisillä on osoitettu *NPY* geenin signaalipeptidissä sijaitsevan Leusiini 7 / Proliini 7 polymorfian (p.L7P) eli yhden aminohapon poikkeaman olevan yhteydessä metaboliseen oireyhtymään liittyvien haitallisten muutosten kanssa. L7P alleelin kantajilla vapautuu myös enemmän NPY:tä verenkiertoon stressireaktion aikana, mikä viittaisi siihen, että sympaattinen hermosto tuottaa ja vapauttaa enemmän NPY:tä. Tämän väitöskirjatutkimuksen tavoitteena oli valmistaa siirtogeeninen hiirimalli, jossa NPY:tä yli-ilmennetään elimistön noradrenergisissa hermosoluissa. Lisäksi tavoitteena oli tutkia ja tunnistaa kyseisen hiirimallin metabolinen sekä sydän- ja verisuonitauteihin liittyvä ilmiasu.

Hiirimalli nimettiin OE-NPY^{DBH} hiireksi. NPY:n yli-ilmentäminen sympaattisessa hermostossa ja aivojen noradrenergisissa hermoissa johti hiirillä rasvan määrän lisääntymiseen ilman, että ruuminpaino nousi tai että syöminen lisääntyi. Hiirillä havaittiin jo nuorella iällä rasvan kertyminen maksaan, mikä myöhemmällä iällä yhdessä kasvaneen rasvaprosentin kanssa johti heikentyneeseen sokerinsietokykyyn ja liialliseen insuliinin eristykseen. Hiirillä havaittiin myös kohonnut keskivaltimopaine stressin aikana, lisääntynyt katekoliamiinien plasmapitoisuus sekä kohonnut sympaattisen hermoston aktiivisuus mitattuna GDP:n sitoutumisaktiivisuudella ruskean rasvan mitokondrioihin. Stressivasteinen NPY:n eritys oli erilainen naaraiden ja urosten välillä. Kokeellisessa verisuonivauriomallissa OE-NPY^{DBH} hiirille kehittyi selvästi verrokkihiiriä paksumpi valtimon sisäpinnan uudiskasvukerros (neointima).

Nämä siirtogeenisillä hiirillä saadut tulokset yhdessä kliinisten kokeiden tulosten kanssa osoittavat, että noradrenergisten solujen NPY:llä on tärkeä rooli metabolisen oireyhtymän ja sen liitännäissairauksien synnyssä ja etenemisessä. Lisäksi nyt tuotetun hiirimallin avulla on saatu uusia näkemyksiä ei-hypotalamisen NPY:n roolista kyseisten sairauksien kehittymisessä. OE-NPY^{DBH} hiirimalli tarjoaa hyvän työkalun stressin ja metabolisen oireyhtymän välisen suhteen tutkimukseen.

Avainsanat: Neuropeptidi Y, siirtogeeninen hiirimalli, sympaattinen hermosto, stressi, lihavuus, heikentynyt sokerinsieto, kohonnut verenpaine, verisuonten uudismuovaus

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ABBREVIATIONS

α-MSH	α -melanocyte stimulating hormone
ACh	acetylcholine
ACTH	adrenocorticotropic hormone
AgRP	agouti-related peptide
ANOVA	analysis of variance
AP-P	aminopeptidase P
ARC	arcuate nucleus
ATP	adenosine triphosphate
AUC	area under curve
BAT	brown adipose tissue
BMD	bone mineral density
BMI	body mass index
CART	cocaine and amphetamine-regulated transcript
CCK	cholecystokinin
CHD	coronary heart disease
CNS	central nervous system
CPON	carboxyterminal peptide of NPY
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
DA	dopamine
DIO	diet-induced obesity
DBH	dopamine-β-hydroxylase
DMH	dorsomedial hypothalamus
DPPIV	dipeptidyl peptidase IV
L-DOPA	L-dihydroxyphenylalanine
GABA	γ-aminobutyric acid
GDP	guanosine 5'-diphosphate
GH	growth hormone
GHRH	growth hormone-releasing hormone
GnRH	gonadotropin-releasing hormone
GLP-1	glucagon-like peptide-1
HDL	high density lipoprotein
H&E	hematoxylin and eosin staining
HPA axis	hypothalamus-pituitary-adrenal axis
i.c.v.	intracerebroventricular

i.p.	intraperitoneal
IGTT	intraperitoneal glucose tolerance test
IRES	internal ribosomal entry site
LC	locus coeruleus
LDL	low density lipoprotein
LH	luteinizing hormone
LHA	lateral hypothalamic area
LPL	lipoprotein lipase
MCH	melanin-concentrating hormone
mRNA	messenger ribonucleic acid
NA	noradrenaline
Nnt	nicotinamide nucleotide transhydrogenase
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
OE	overexpression
PBS	phosphate-buffered saline
p.L7P	Leucine 7 to Proline 7 polymorphism
PNMT	phenylethanolamine-*N*-methyltransferase
POMC	pro-opiomelanocortin
PP	pancreatic polypeptide
PVN	paraventricular hypothalamic nucleus
PYY	peptide YY
RIA	radioimmunoassay
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SNS	sympathetic nervous system
TH	tyrosine hydroxylase
VMH	ventromedial hypothalamus
VSMC	vascular smooth muscle cell
WAT	white adipose tissue
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III:

I Ruohonen ST, Pesonen U, Moritz N, Kaipio K, Röyttä M, Koulu M, Savontaus E. Transgenic mice overexpressing neuropeptide Y in noradrenergic neurons: A novel model of increased adiposity and impaired glucose tolerance. *Diabetes* 2008, 57: 1517-1525.

II Ruohonen ST, Savontaus E, Rinne P, Rosmaninho-Salgado J, Cavadas C, Ruskoaho H, Koulu M, Pesonen U. Stress-induced hypertension and increased sympathetic activity in mice overexpressing neuropeptide Y in noradrenergic neurons. *Neuroendocrinology* 2008, in press.

III Ruohonen ST, Abe K, Kero M, Toukola L, Ruohonen S, Röyttä M, Koulu M, Pesonen U, Zukowska Z, Savontaus E. Sympathetic nervous system-targeted neuropeptide Y overexpression in mice enhances neointimal formation in response to vascular injury. *Peptides* 2008, in press.

1 INTRODUCTION

Metabolic syndrome is a common name for a combination of medical disorders that increase an individual's risk for cardiovascular disease and type 2 diabetes (Fig. 1.1). The main features of metabolic syndrome include insulin resistance, impaired glucose tolerance, cholesterol abnormalities, hypertension and abdominal obesity. The first step in the development of metabolic syndrome is usually body weight gain. Risk for health consequences increases progressively as the body mass index (BMI) increases. The World Health Organization (WHO) defines *overweight* as a BMI equal to or more than 25, and *obesity* as a BMI equal to or more than 30. There are about 1.6 billion adults that are overweight, and 400 million adults are obese in today's world (www.who.int, 2008). The WHO's estimates for the year 2015 are 2.3 billion and 700 million adults, respectively. The prevalence of obesity in middle-aged Finnish population in 2005 was 23.5% and 28.0% in men and women, respectively (Saaristo et al. 2008). Due to changes in lifestyle and increasing consumption of food, the prevalence of these traits associated with "syndrome X" is continuously increasing throughout the world.

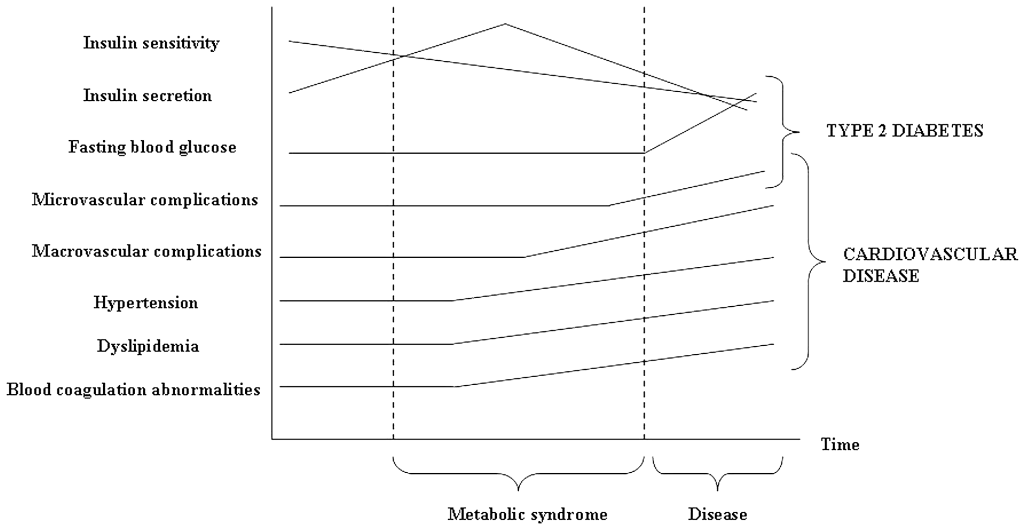

Figure 1.1. The role of metabolic syndrome in the genesis of type 2 diabetes and cardiovascular disease. Figure is modified from a review article (Cefalu 2006).

Neuropeptide Y (NPY) is one of the most common peptides in the brain and an abundant neurotransmitter in the peripheral sympathetic nervous system (SNS). NPY has been linked to several disorders associated with metabolic syndrome. It plays a well-established role in the hypothalamic control of body energy balance by promoting feeding and lipid storage in white adipose tissue (WAT). The latter is mainly a result of decreased sympathetic activity, which fits with one of the most dominating theories of obesity causation: the *Mona Lisa hypothesis*. It is an acronym for "**m**ost **o**besities **kn**own **a**re **l**ow **i**n **s**ympathetic **a**ctivity" (Bray 1991). Recently, it was

shown that peripheral stress-induced NPY increases abdominal obesity without affecting energy intake or body weight gain (Kuo et al. 2007). This is a novel aspect on stress: chronic SNS activation can promote adiposity via NPY-mediated mechanisms, and hence challenges the Mona Lisa theory. Thus, central and peripheral NPY act via distinct mechanisms to promote adiposity.

In addition to obesity, NPY has been related to type 2 diabetes and cardiovascular diseases in humans and experimental animal models. Most data available on NPY and its function in the progression of metabolic syndrome-related disorders comes from experimental animal models. The role of peripheral NPY in the regulation of energy homeostasis has not gained much attention, although NPY and its receptors are located in key peripheral tissues, such as adipose tissue, liver and pancreas. In humans, a rather common Leucine 7 to Proline 7 polymorphism (p.L7P) in the *NPY* gene has been associated with traits of metabolic syndrome especially in obese carriers of the p.L7P allele (Karvonen et al. 1998). Increased production of mature NPY is suggested to contribute to the development of metabolic disturbances associated with the polymorphism. The special interest of this thesis was to study the role of NPY in various physiological conditions related to human metabolic syndrome by using a novel mouse model overexpressing NPY in noradrenergic neurons of the brain and periphery.

2 REVIEW OF THE LITERATURE

2.1 Neuropeptide Y (NPY)

2.1.1 General background

NPY was originally isolated from porcine brain (Tatemoto et al. 1982) and its structure is closely related to that of the other peptides in the family (pancreatic polypeptide, PP and peptide YY, PYY). All the members of this family are 36 amino acids long include tyrosine residues at both ends of their amino acid chains and they are recognized by the same neuropeptide receptors, namely Y receptors. All of these peptides also have the ability to modulate food intake; NPY promotes energy intake whereas PP and PYY are known inhibitors of feeding. Sequences from many types of vertebrates show that NPY has remained extremely well conserved throughout vertebrate evolution with 92% amino acid sequence identity between mammals and cartilaginous fishes (Larhammar et al. 1993).

NPY, PP and PYY share a similar structure and activate the rhodopsin-like G-protein coupled NPY receptors with several subtypes: Y_1, Y_2, Y_4, Y_5, and possibly y_3, and y_6. PP and PYY are mainly found in the gastrointestinal tract released in response to nutrient stimuli, and PYY is additionally found in neurons (Ekblad and Sundler 2002). The high degree of amino acid homology between these three peptides suggested similar secondary structures, which was shown to be true in the early 1990s (Fuhlendorff et al. 1990). The tertiary structure of the peptides consists of two helical units, which are packed into a typical β-hairpin fold by hydrophobic interactions. In an inactive form, NPY exists as a dimer where the α-helical segments are in parallel and antiparallel orientation. Upon activation, a shift occurs towards the monomeric form (Merten and Beck-Sickinger 2006).

The human *NPY* gene consists of four exons (Fig. 2.1). The first exon contains a 5' untranslated region. The second encodes the signal peptide (1-28) and the main part of the mature NPY peptide (29-64). The third exon encodes amino acid residues 65-90 and the main part of the carboxyterminal (CPON). Exon four contains the end of the CPON and a 3' untranslated region (91-97). The mature NPY peptide is formed from amino acid fragment 29-64 after two fragments (1-28 and 67-97) have been cleaved off by prohormone convertases (Silva et al. 2002). Two further truncations at the C-terminal end lead to the biologically active amidated NPY. The amidation step is essential for the activity of NPY for it prevents degradation by carboxypeptidases (Silva et al. 2002). The mature NPY_{1-36} can be further processed in secretory granules by two neuroendopeptidases: dipeptidyl peptidase IV (DPPIV) (Mentlein et al. 1993; Medeiros and Turner 1994), and aminopeptidase P (AP-P) (Medeiros and Turner 1994; Mentlein and Roos 1996). The resulting products are $NPY_{3–36}$ and $NPY_{2–36}$, respectively. Cleavage of the N-terminal amino acids (tyrosine and tyrosine-proline) by DPPIV and AP-P results in less affinity to Y_1 receptor subtype, and higher binding

activity to Y_2 receptors as discussed in more detail in chapter 2.2. Neutral endopeptidase, mostly present in kidneys, results in several cleavage sites in both NPY and PYY that are considered as inactivating cleavages (Medeiros and Turner 1994).

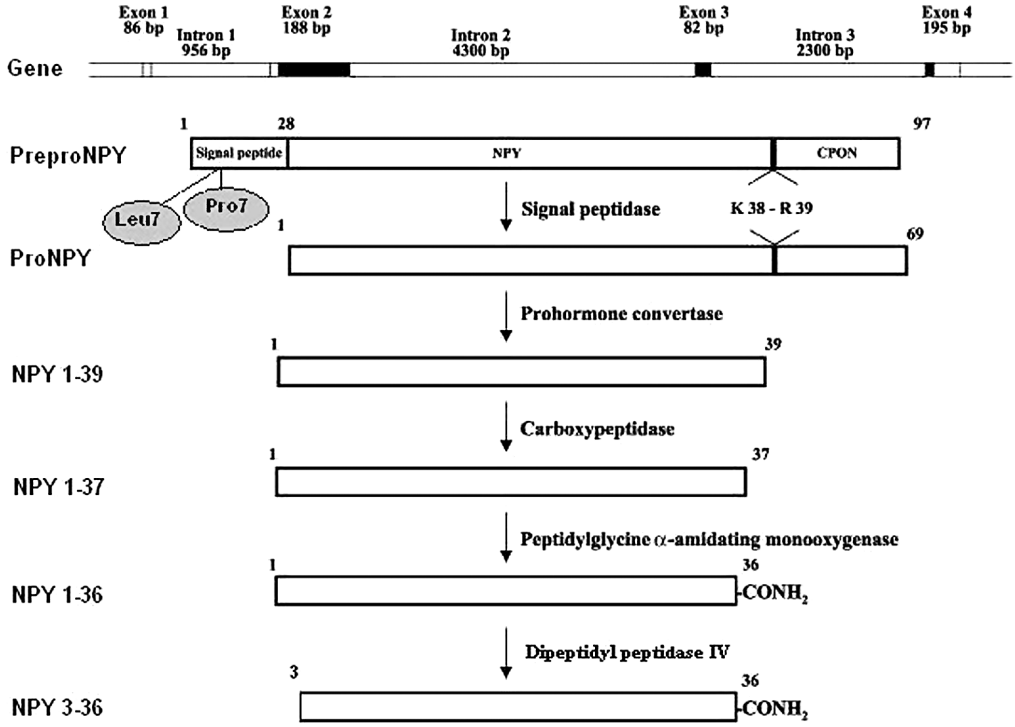

Figure 2.1. Biosynthesis of human NPY. Leu7 = amino acid number 7 in the protein; Pro7 = amino acid 7 in the protein (see chapter 2.5 for more); CPON = carboxyterminal; K 38 – R 39 = cleavage site for prohormone convertase 1/3 or 2. Figure is modified from Silva et al 2002.

In plasma, NPY is found mostly as NPY_{1-36}, but also as NPY_{3-36}. The disappearance curve for NPY from circulation is biphasic; the clearance halftime for the first phase is approximately 4 minutes and that of the second phase is 20 minutes (Pernow et al. 1987). Plasma NPY levels correlate with those of noradrenaline (NA) but not with adrenaline suggesting a sympathetic origin for the release of circulating peptide (Zukowska-Grojec et al. 1988). Gut and liver make a major contribution to the plasma NPY levels in humans (Morris et al. 1997).

2.1.2 NPY in the sympathetic nervous system (SNS)

2.1.2.1 Introduction to SNS

In the periphery, NPY is mainly expressed in NA-producing neurons. SNS is part of the autonomic nervous system, which innervates cardiac muscle, smooth muscle, and glandular tissues, such as spleen and pancreas. SNS governs the emergency reaction,

also referred to as the *fight-or-flight* reaction. It increases output to the heart and other organs, and peripheral vasculature to enable the person to respond efficiently to the sudden changes in the external or internal environment. Sympathetic neurons are divided into two subtypes: pre- and postsynaptic neurons (Fig. 2.2). Axons of the presynaptic neurons exit from the spinal cord. They form synapses with postganglionic neurons in sympathetic ganglia close to the spinal cord. The transmitter released into the synaptic cleft is acetylcholine (ACh), which binds to the nicotine receptors on the postsynaptic neurons. Postganglionic neuron releases sympathetic neurotransmitter NA. Neuropeptides co-stored with NA are also released upon sympathetic activation. These include NPY, chromogranin A and enkephalins. Target tissue contains respective receptors for transmitters to bind. Peripherally the most commonly present adrenergic receptors are alpha ($\alpha_{1\text{-}2}$) and beta ($\beta_{1\text{-}3}$). Dopamine (DA) receptors ($DA_{1\text{-}2}$) are also present in some peripheral tissues, mainly in kidney and heart. In general, sympathetic activation increases heart rate, blood pressure and insulin secretion and decreases gut motility. Thus, the body is in alert mode ready to utilize its energy stores.

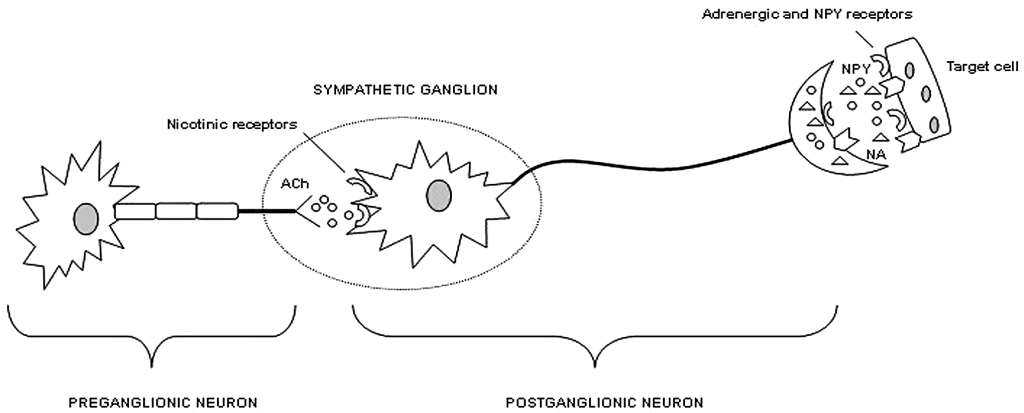

Figure 2.2. Sympathetic neurotransmission. A preganglionic neuron from the spinal cord synapses with a postganglionic neuron in a sympathetic ganglion. The ganglion contains the cell body, where protein synthesis takes place. The neurotransmitter released is ACh, which binds to nicotinic receptors on postsynaptic neuron. Postsynaptic neuron in turn releases sympathetic neurotransmitters mainly NA and NPY. Target tissue contains adrenergic receptors (α- and/or β-subtypes) and NPY receptors (Y_1-Y_6). Presynaptic α_2 and Y_2 autoreceptors regulate the release of transmitters into the synaptic cleft. ACh = acetylcholine; NA = noradrenaline; NPY = neuropeptide Y.

2.1.2.2 Catecholamine synthesis, storage and release

Catecholamines are a group of sympathomimetic amines that function as neural transmitters. Catecholamine biosynthesis begins with an uptake of the L-tyrosine into postsynaptic sympathetic nerve terminals (Nagatsu et al. 1964). Tyrosine hydroxylase (TH) catalyzes the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA) (Fig. 2.3), which is the rate-limiting step in catecholamine synthesis (Udenfriend 1966). The end-product of the biosynthetic pathway, NA, inhibits the production of TH

by negative feedback reaction (Nagatsu et al. 1964), whereas exposure to stressors augments the synthesis and concentration of TH in SNS and sympathetically innervated organs, as reviewed elsewhere (Wong and Tank 2007). L-DOPA is rapidly converted to DA by L-aromatic-amino-acid decarboxylase (L-AADC). Most L-DOPA is metabolized into DA, but some of the L-DOPA enters the circulation unchanged (Goldstein et al. 1987). Dopamine-β-hydroxylase (DBH) catalyzes the conversion of DA to NA. DBH is localized to large dense-core vesicles, mainly in a membrane-bound form, in nerve terminals of NA and adrenaline producing cells. By definition, dopaminergic neurons do not contain DBH. NA is stored at a high concentration in synaptic vesicles along with DBH, and both substances are released into synaptic cleft by exocytosis. In the adrenal medulla, phenylethanolamine-*N*-methyltransferase (PNMT) converts NA to adrenaline (Kirshner 1959). PNMT is also found in some parts of the human brain, but little is known about the role of adrenaline in central nervous system (CNS) (Stone et al. 2003).

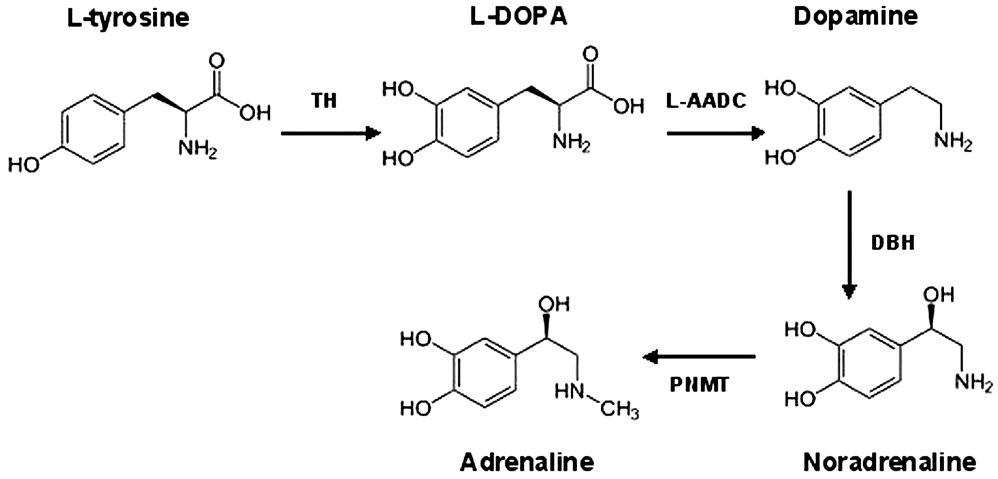

Figure 2.3. Catecholamine synthesis pathway. Synthesis of the endogenous sympathetic ligands, dopamine, noradrenaline and adrenaline, occurs in sympathetic neurons and in the adrenal chromaffin cells. Adrenaline is the primary catecholamine product of the chromaffin cells, whereas NA is mainly produced in sympathetic neurons, which lack PNMT. The rate-limiting enzyme in the pathway is TH, which catalyzes the conversion of L-tyrosine to L-DOPA. TH = tyrosine hydroxylase; L-AADC = L-aromatic-amino-acid decarboxylase; DBH = dopamine-beta-hydroxylase; PNMT = phenylethanolamine-*N*-methyltransferase.

SNS contains two types of cytoplasmic vesicles where catecholamines are synthesized and stored: small dense-core (diameter 40-60 nm) and large dense-core vesicles (diameter 80-120 nm). Cores of vesicles also contain adenosine triphosphate (ATP), and at least three types of polypeptides: chromogranin A, enkephalins and NPY (Neuman et al. 1984; Takiyyuddin et al. 1994). Ultracentrifugation experiments using nerve fibre extracts have shown that NPY co-sediments with NA in the same large dense-core vesicles (Fried et al. 1985). NPY is also co-released with NA into circulation upon sympathetic activation (Han et al. 1998) and feeding (Morris et al. 1997). In exocytosis, depolarization of sympathetic nerve terminals opens membrane

calcium channels resulting in discharging of the soluble vesicle contents into the extracellular fluid. From the extracellular space, catecholamines are inactivated by uptake into cells with subsequent intracellular deamination by monoamine oxidase, or translocation into storage vesicles.

2.1.2.3 NPY in SNS and other locations in peripheral nervous system

Sympathetic neurons containing NPY innervate vascular smooth muscles including cerebral vasculature (Edvinsson et al. 1983; Ekblad et al. 1984), heart (Gu et al. 1983), thyroid gland (Grunditz et al. 1984), respiratory tract (Sheppard et al. 1984), gut (Furness et al. 1983; Sundler et al. 1983), pancreas (Sundler et al. 1983), liver (Taborsky et al. 1994; McCuskey 2004) and eye (Terenghi et al. 1983). NPY is found in adrenal glands of humans and other species. It is produced by chromaffin cells and abundantly secreted by human phaeochromocytomas, the neuroendocrine tumours of the adrenal medullas (Majane et al. 1985; de Quidt and Emson 1986; Grouzmann et al. 1989). In adrenal medulla, most NPY is present in adrenaline-producing chromaffin cells demonstrated by co-localization of NPY and PNMT (Wolfensberger et al. 1995), but NPY is also present in NA-type adrenal cells (Gulbenkian et al. 1985).

In addition to SNS, NPY is located in non-sympathetic neurons in the periphery. These include the myenteric plexus and submucosal ganglion (Furness et al. 1983; Sundler et al. 1983). The former innervates other myenteric ganglia and the circular muscle, and the latter innervates the mucosa (O'Donohue et al. 1985).

2.1.3 NPY in the central nervous system (CNS)

NPY is one of the most abundant peptides of the brain. The highest concentrations in mammals are found in the hypothalamic nuclei (rat brain in Fig 2.4): the paraventricular hypothalamic nucleus (PVN) and the hypothalamic arcuate nucleus (ARC). These neurons send their axons towards the medial preoptic area, the lateral hypothalamic area (LHA), the anterior hypothalamic area, the periventricular nucleus, the ventromedial hypothalamus (VMH), the dorsomedial hypothalamus (DMH), the posterior hypothalamus and the amygdala (Chronwall et al. 1985). NPY is co-localized in neurons with several other neuropeptides and neurotransmitters, such as agouti-related peptide (AgRP) (Mihaly et al. 2000), somatostatin (Chronwall et al. 1984; McDonald 1989) and enkephalin (Hunt et al. 1981; Lin et al. 2006). The hypothalamus is the key structure and NPY one of the key players in regulating feeding and energy expenditure (Beck 2006).

NPY is present and co-localized with NA but not DA in the brain (Hunt et al. 1981; Hokfelt et al. 1983; Hokfelt et al. 1983; Everitt et al. 1984). The main noradrenergic sites containing NPY in the brain are located in the brainstem: the pons and the medulla oblongata (rat brain in Fig 2.4). These include the locus coeruleus (LC), Al/Cl cell groups in the ventrolateral medulla oblongata, and the nucleus tractus solitarius (NTS), which project to the PVN in the hypothalamus (Everitt et al. 1984; Chronwall et al. 1985).

Relatively high numbers of NPY-immunoreactive cell bodies are dispersed through the cortex, the CA1-CA4 molecular layer of the hippocampus and the dentate gyrus (Allen et al. 1983). NPY cells in the supramamillary nucleus give rise to fibres in the hippocampus, the subiculum, the lateral septum, the cingulate gyrus, the amygdala and LC in the brainstem, thus, being part of the limbic circuits (Wahlestedt et al. 1989). The limbic system originates emotions and feelings, and allows mammals to distinguish between the agreeable and disagreeable. Further, NPY immunoreactive fibres innervating the hypothalamic suprachiasmatic nucleus (SCN) arise from perikarya of the thalamus. This innervation may be particularly important for the regulation of circadian rhythms and associated functions (Albers and Ferris 1984; Albers et al. 1984). In the spinal cord, NPY levels appear to be highest in the dorsal horn region (Allen et al. 1984; Gibson et al. 1984), where it is co-localized with gamma-aminobutyric acid (GABA) (Rowan et al. 1993).

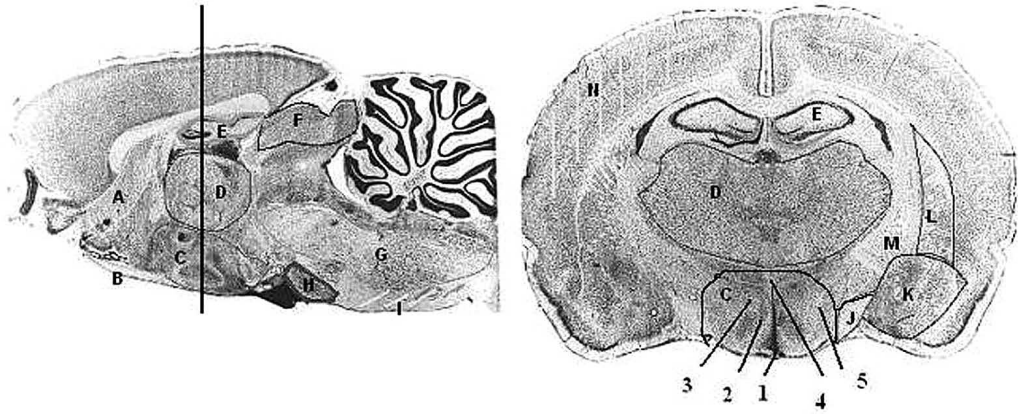

Figure 2.4. Sagittal and coronal view of the rat brain. The figure on the left shows a midsagittal view and the figure on the right displays the coronal view of the rat brain. The vertical line in the sagittal section marks the level at which the coronal section is taken. A = the basal forebrain; B = the optic chiasm; C = the hypothalamus (1 = ARC; 2 = VMH; 3 = DMH; 4 = PVN and 5 = LHA); D = the thalamus; E = the hippocampus; F = the superior and inferior colliculi; G = the reticular formation; H = the pons; I = the medulla oblongata; J = the optic tract; K = the amygdala; L = the caudate/putamen; M = the internal capsule; N = the cortex. In the coronal section, the structures are marked on one side of the brain, although they are identical on both sides. All of the hypothalamus and the thalamus are circled for easier comprehension of the structures. The brain sections are modified from rat brain atlas (www.brainmaps.org).

2.2 NPY receptors

NPY mediates its effects through the activation of at least four G_i/G_o-protein-coupled receptor subtypes named Y_1, Y_2, Y_4 and Y_5 confirmed by the International Union of Basic and Clinical Pharmacology. Additionally, y_3 and y_6 have been proposed to exist. All other five receptors, except y_3, have been cloned in rodents and/or humans (Eva et al. 1990; Bard et al. 1995; Rose et al. 1995; Weinberg et al. 1996). The family of NPY

receptors can be sorted into three subfamilies: the Y_1 subfamily consisting of subtypes Y_1, Y_4 and y_6 , the Y_2 subfamily consisting of subtype Y_2 and the Y_5 subtype remaining alone in a subfamily (Larhammar and Salaneck 2004).

Numerous endogenous and synthesized C-terminal fragments of NPY are known, e.g. NPY_{2-36}, NPY_{3-36}, NPY_{13-36} and NPY_{18-36}. None of these has been shown to confer any relative benefit, other than N-terminal cleaving being useful to discriminate agonism for Y_1 and Y_2 receptors. C-terminal fragments are not totally selective for Y_2 receptors since they can also activate Y_5 receptors at similar concentrations (Silva et al. 2002). On the other hand, leucine in position 31 and/or proline in C-terminal position 34 of the NPY or PYY molecule, i.e. [Leu^{31}, Pro^{34}]NPY, [Pro^{34}]NPY, and [Pro^{34}]PYY are more selective for Y_1 than for Y_2 receptors (Fuhlendorff et al. 1990; Krause et al. 1992; Grandt et al. 1994). However, [Pro^{34}]-substituted peptide analogues also potently activate Y_5 receptors (Haynes et al. 1998). [Phe^{7}, Pro^{34}]NPY has shown to be a selective Y_1 agonist with 3000-fold preference for Y_1 over Y_2 and Y_5 reseptors (Soll et al. 2001).

Opposed to unselective agonists, several selective NPY receptor antagonists have been described. The first small molecule competitive antagonist was BIBP3226, which is selective for Y_1 receptors (Doods et al. 1995). BIIE0246 is the first selective Y_2 receptor antagonist (Doods et al. 1999), and CGP71683A is a selective Y_5 receptor antagonist (Criscione et al. 1998). The development of a Y4 selective antagonist has not been reported until very recently, when UR-AK49 was discovered (Ziemek et al. 2007). The potencies, and known agonists and antagonists of the four confirmed NPY receptors are summarized in Table 2.1.

Table 2.1. Pharmacological properties of NPY receptors.

Receptor	Endogenous agonists in order of potency	Antagonists
Y_1	$NPY = PYY > NPY_{2-36} = NPY_{3-36}$	BIBP3226; GW1229; SR120819A; BIBO3304; LY357897; J-115814; H394/84
Y_2	$NPY = PYY = NPY_{2-36} = NPY_{3-36} > PP$	BIIE0246; T_4-$[NPY_{33-36}]_4$
Y_4	$PP > PYY = NPY$	UR-AK49
Y_5	$NPY = PYY = NPY_{2-36} = NPY_{3-36} > PP$	CGP71683A; GW438014A†; L-152,804††

†(Daniels et al. 2002) and ††(Kanatani et al. 2000). Table is modified from Silva et al. 2002.

The Y_1 receptor was the first NPY receptor to be cloned (Eva et al. 1990). It is expressed in various peripheral organs, blood vessels and neurons innervating the tissues (Mihara et al. 1990; Larhammar et al. 1992; Peaire et al. 1997; Hokfelt et al. 1998; Matsuda et al. 2002). In CNS, Y_1 receptors are found in the hypothalamus, the hippocampus, the cortex, the thalamus, the amygdala, the brainstem and the dorsal horn in the spinal cord (Migita et al. 2001; Kopp et al. 2002). Y_1 has equally good

affinity to NPY_{1-36} and PYY, but the affinity weakens as the N-terminal part of the peptide is cleaved by peptidases (Larhammar et al. 1992).

The Y_2 receptor was first cloned in 1995 (Rose et al. 1995). Localization of the Y_2 receptors suggests that it is mostly presynaptic (Chen et al. 1997), and it regulates the release of NPY and other transmitters (Wahlestedt et al. 1986). Y_2 receptors are found with Y_1 receptors in the vasculature (Zukowska-Grojec et al. 1998) and in the intestine (Rettenbacher and Reubi 2001). In the brain, Y_2 receptors are widely expressed in the hypothalamus, the hippocampus, the cortex, the amygdala, the brainstem and the substantia nigra compacta (Stanic et al. 2006). The affinity to the Y_2 receptor does not suffer from further cleaving of the N-terminal ligand; NPY_{2-36}, NPY_{3-36}, NPY_{13-36} and PYY_{3-36} bind to the receptor well (Eberlein et al. 1989; Rose et al. 1995; Chen et al. 1997).

Existence of the y_3 receptor has been proposed on the basis of pharmacological studies, but it has not yet been cloned or well characterized (Herzog et al. 1993; Lee and Miller 1998). The most striking feature of this receptor is that PYY has an affinity at least ten times lower than NPY (Lee and Miller 1998). Y_3 is presumably expressed in the NTS of the brain (Lee and Miller 1998), vascular endothelial cells (Nan et al. 2004) and cardiac myocytes (Balasubramaniam et al. 1990).

The cloning of the Y_4 receptor was first described in 1995 (Bard et al. 1995). It is widely present in peripheral tissues (Barrios et al. 1999; Pheng et al. 1999). Y_4 binding sites are also distributed in the mammalian brain, being most concentrated in the hypothalamus (Dumont et al. 1998). Y_4 binds PP with high affinity, while NPY and PYY are less potent agonists (Gehlert et al. 1996). Sequence identity of *NPY4R* gene between species is low; rat and mouse share only 74–78% amino acid identity with the other orders of mammals which share 83–86% identity.

The Y_5 receptor was cloned in 1996 (Weinberg et al. 1996). In the periphery, the Y_5 receptors are present in the gastrointestinal tract (Goumain et al. 1998), testis, pancreas and spleen (Statnick et al. 1998). The receptor can also be found in CNS: the hypothalamus (Weinberg et al. 1996), the cortex, the hippocampus and the brainstem (Grove et al. 2000). The Y_5 receptor shares 60% amino acid identity with the Y_1 receptor, and the ligand binding affinity resembles that of Y_1 (Weinberg et al. 1996). It is distinct from that described for the Y_2, y_3 and Y_4 receptors.

The y_6 receptor has been cloned only in mice so far, and it appears it is not functional in other species (Gregor et al. 1996; Weinberg et al. 1996; Widdowson et al. 1997). The distribution of the y_6 receptors is not very widespread. It is synthesized in the hypothalamus of the mouse brain (Weinberg et al. 1996). The binding properties resemble that of Y_4, and PP shows higher affinity to y_6 than PYY and NPY (Gregor et al. 1996). There is evidence to suggest that the murine y_6 receptor may be a pseudogene and is not fully expressed in rat or human brain (Gregor et al. 1996). This is perhaps due to a single base insertion in the human, and possibly rat, y_6 gene leading to a truncation in the third extracellular loop (Widdowson et al. 1997).

2.3 Physiological functions of NPY

2.3.1 NPY in the regulation of energy homeostasis and food intake

NPY affects energy homeostasis mainly by regulating the hypothalamic feeding behaviour and WAT lipid storage. Peripheral orexigenic signals, such as starvation, insulin-dependent diabetes mellitus and exercise increase hypothalamic NPY signalling and promote the need to eat (Beck et al. 1992; Inui 2000). NPY and AgRP are localized together with high concentrations in the hypothalamus, especially in ARC from where neurons project to the primary feeding centre PVN. The ARC-PVN neuronal tract is known as "the axis of hunger" (Fig. 2.4). NPY/AgRP neurons also project to LHA, where orexins and melanin-concentrating hormone (MCH) are released to further increase the feeding. ARC also contains neurons with pre-opiomelanocortin (POMC)-derived alpha-melanocyte stimulating hormone (α-MSH), and cocaine and amphetamine-regulated transcript (CART) peptide that are considered as anorexigenic peptides. The neuroendocrine control of feeding has been extensively studied and multiple review articles have been published in recent years (Morton and Schwartz 2001; Konturek et al. 2005; Valassi et al. 2008).

Long-term regulators of energy balance are leptin and insulin that serve as adiposity signals. Leptin derives from adipocytes, and the levels are increased in obesity. Leptin binds to its receptors on the α-MSH/CART neurons in ARC and stimulates a specific signalling cascade that results in the inhibition of orexigenic neuropeptides, while stimulating anorexigenic peptides (Jequier 2002). NPY and leptin also form a neuro-hormonal feedback loop: leptin acts on leptin receptors in NPYergic neurons (Schwartz et al. 1996; Houseknecht and Portocarrero 1998) and inhibits NPY expression in ARC to suppress further feeding (Schwartz et al. 1996; Wang et al. 1997). Obese humans have high plasma levels of leptin due to the greater size of adipose tissue, but this elevated leptin signalling does not induce expected reductions in food intake. This suggests that obese subjects are resistant to the effects of endogenous leptin (Considine et al. 1996). Ghrelin is a hormone with several effects opposite to those of leptin. Ghrelin is released from an empty stomach before feeding. Ghrelin reaches the brain via vagus nerve, but its primary hypothalamic targets are the NPY/AgRP neurons in ARC (Asakawa et al. 2001). Thus, ghrelin promotes feeding via NPY-mediated mechanisms. Ghrelin may also directly inhibit the anorexigenic α-MSH/CART neurons in the hypothalamus (Nakazato et al. 2001). Ghrelin levels are high in human anorexia (Tolle et al. 2003); in obesity they are low, but fail to decline after a meal as they do with lean subjects (English et al. 2002). Furthermore, an additive effect of high leptin and low ghrelin levels on human metabolic disturbances has recently been found (Ukkola et al. 2008). Hence, subjects resistant to leptin and with low ghrelin levels may present a higher risk for developing type 2 diabetes and atherosclerosis.

Peripheral short-term satiety signals, such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), PYY and PP, and the hunger signal ghrelin originate from the gastrointestinal tract in response to the current energy status. They reach NTS in the caudal brainstem through the vagus nerve. From NTS, afferent fibres project to ARC

and vice versa. Peripheral signals are integrated with the long-term signals leptin and insulin, and with additional central inputs in ARC, where the complex response modulating food intake is finally processed (Valassi et al. 2008). Experimental evidence also suggests that cleaved PYY_{3-36} mediates direct anorexigenic signalling via ARC Y_2 receptors, which results in inhibition of NPY/AgRP neurons and activation of POMC neurons in the hypothalamus (Balasubramaniam et al. 2007).

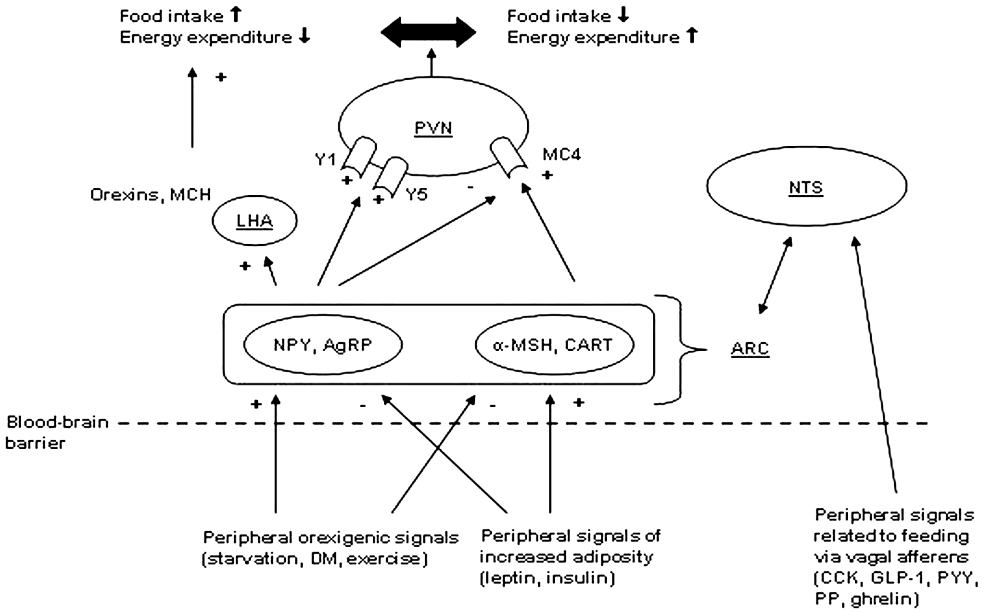

Figure 2.4. The ARC-PVN axis of hunger. Peripheral orexigenic signals (starvation, DM and exercise) and long-term energy regulators (leptin and insulin) cross the blood–brain barrier, and act on neurons within ARC. Orexigenic signals increase NPY/AgRP mediated mechanisms in the hypothalamus. Projections from ARC extend to LHA, where orexins and MCH are released to further increase feeding. NPY acts via Y_1 and Y_5 receptors, which are expressed on neurons in PVN. Leptin and insulin directly stimulate the α-MSH/CART neurons in ARC, or decrease the activity of the orexigenic NPY/AgRP pathway, and thus increase the signalling in anorectic neurons. α-MSH is an agonist and AgRP is an antagonist for the melanocortin receptor MC4, which is also expressed in PVN. In addition, NTS in the brainstem serves as a gateway for neural signals (CCK, GLP-1, PYY, PP and ghrelin) from the gastrointestinal tract to the central feeding centres. Projections between ARC and NTS act, in response to starvation or satiety signals, to increase or decrease energy intake and energy expenditure. AgRP = agouti-related peptide; α-MSH = alpha-melanocyte-stimulating hormone; ARC = the arcuate nucleus; CART = cocaine and amphetamine-regulated transcript; CCK = cholecystokinin; DM = diabetes mellitus; GLP-1 = glucagon-like peptide-1; LHA = lateral hypothalamic area; MC4 = melanocortin 4 receptor; MCH = melanin-concentrating hormone; NPY = neuropeptide Y; NTS = the nucleus tractus solitarius; PP = pancreatic polypeptide PVN = the paraventricular nucleus; PYY = peptide YY; Y1 = NPY Y_1 receptor; Y5 = NPY Y_5 receptor. + = effect is stimulatory; - = effect is inhibitory.

NPY is one of the most powerful stimuli of feeding when administered centrally to animals (Levine et al. 2004). It produces a robust and sustained increased food intake even in satiated animals (Levine and Morley 1984; Kalra et al. 1988). Peripheral NPY

has not been reported to induce energy intake or weight gain. NPY up-regulation in the hypothalamus after starvation is reversed by re-feeding (Beck et al. 1992). NPY concentrations are significantly increased in the hypothalamus of obese Zucker (fa/fa) rats (Beck et al. 1990), leptin null (ob/ob) and leptin receptor deficient (db/db) mice (Chua et al. 1991; Wilding et al. 1993) and Akita male mice (Toyoshima et al. 2007) suggesting NPY to be a major component at the origin of hyperphagia in obese animals. Administration of leptin to ob/ob but not db/db mice results in near normalization of obesity, hyperglycemia, and hypothalamic *NPY* mRNA levels (Stephens et al. 1995; Schwartz et al. 1996), whereas leptin-deficient obesity is attenuated by genetic depletion of NPY in mice (Erickson et al. 1996). In humans, a significant linkage between obesity and *NPY* has been found within non-diabetic obese Mexican-American families (Bray et al. 1999). However, in another study with South-American Pima indians, it was reported that no such linkage between obesity and *NPY* could be detected (Norman et al. 1997).

NPY has also been shown to modulate dietary preferences. When rats have the choice between three sources of pure macronutrient (casein, lard and mixture of sucrose-cornstarch-dextrose), they preferentially increase their intake of carbohydrates after an acute i.c.v. injection of NPY (Stanley et al. 1985). When rats can choose between high-carbohydrate and high-fat diet preceded by NPY administration, they also prefer carbohydrates over fat, which is similar to what is seen after food deprivation (Welch et al. 1994). The element of preference may be due to the sweetness of sucrose, and orosensory mechanisms could play a role in the orexigenic effects of NPY (Lynch et al. 1993). Interestingly, NPY-related feeding behaviour seems to be modulated in two different sites in the brain. The hypothalamus modulates consummatory effects, whereas the brainstem plays a role in the appetite-stimulating behaviour (Ammar et al. 2005; Baird et al. 2008).

The receptors mediating NPY's feeding effects in rodents are Y_1 and/or Y_5. Y_1-deficient or mice treated with selective Y_1 antagonist have shown that Y_1 receptor is needed in NPY-induced feeding (Kanatani et al. 2000; Polidori et al. 2000). The role of Y_5 in energy homeostasis has mostly been demonstrated by the use of selective Y_5 antagonists or antisense oligodeoxynucleotides (Kanatani et al. 2000; 2000; Levens and Della-Zuana 2003; Mashiko et al. 2003; Ishihara et al. 2006). Central administration of Y_5 agonist increased food intake in both lean and obese Zucker rats (Beck et al. 2007), but failed to increase NPY-induced food ingestion in Siberian hamsters (Pelz and Dark 2007). Within the hypothalamus, presynaptic Y_2 receptors act to inhibit NPY release and NPY-induced feeding (Feletou and Levens 2005). It has also been known since the 1970s that PP has an inhibitory control over food intake and body weight via Y_4 receptors (Malaisse-Lagae et al. 1977). Efforts to find novel appetite suppressant drugs that inhibit the interaction of NPY with either the Y_1 or Y_5 receptor subtypes have proven disappointing so far. Attempts have now been made to identify a Y_2 and/or Y_4 stimulator that would suppress appetite in the hypothalamus (Balasubramaniam et al. 2007), but administration of both endogenous and exogenous agonists of the receptor is likely to cause side effects, particularly regarding pituitary

hormone release, as well as on the cardiovascular and gastrointestinal systems (Feletou and Levens 2005).

2.3.2 NPY in the regulation of lipid metabolism

Hypothalamic NPY administration promotes lipogenesis by stimulating lipoprotein lipase (LPL) activity in WAT that leads to adipocyte hypertrophy in rats (Fig. 2.5) (Billington et al. 1991; 1994). *In vitro*, it has been shown that NPY increases adipocyte size via Y_1-mediated inhibition of lipolysis (Labelle et al. 1997). Central NPY decreases SNS activity assessed by reductions in brown adipose tissue (BAT) thermogenesis in rats (Billington et al. 1991). It also contributes to enhanced fat accumulation by increasing food ingestion and reducing metabolic expenditure via central Y_1 receptors (Pelz and Dark 2007). Hence, hypothalamic NPY decreases sympathetic tone, which leads to increased energy storage. Additionally, chronic i.c.v. administration of NPY stimulates *de novo* lipogenesis in the liver by increasing the hepatic acetyl coenzyme-A carboxylase activity in rats (Zarjevski et al. 1993). Central administration of NPY_{1-36} or a selective Y_5 agonist has also been shown to increase circulating levels of triglycerides. The secretion is inhibited by giving a selective Y_1 antagonist (Zarjevski et al. 1993; Stafford et al. 2008). It has been hypothesized that NPY mobilizes stored hepatic triglycerides, which are then transfered to apoB, the primary apolipoprotein component of low density lipoprotein (LDL), stabilizing apoB for secretion (Stafford et al. 2008). Thus, it can be speculated whether NPY is involved in cholesterol synthesis by increasing triglyceride accumulation in the liver to provide starting material for LDL.

The direct peripheral effects of NPY on adipose tissue have been addressed in a paper by Kuo et al. (Kuo et al. 2007). In their work, they used chronic stress to stimulate NPY release from mouse sympathetic nerves, and showed that increased NPY augmented diet-induced obesity in mice, but it had no effect on body weight or food intake. The mice showed gross abdominal obesity, liver and skeletal muscle steatosis, and impaired glucose tolerance, which were attenuated by a local administration of Y_2 antagonist, or regional fat-targeted *NPY2r* knockdown procedure. These data suggest that NPY increases adipose tissue mass through local, Y_2 receptor-mediated mechanisms. The local effect was further supported by an *in vitro* study, where NPY stimulated adipogenesis, i.e. proliferation and differentiation of preadipocytes.

2.3.3 NPY in the regulation of glucose metabolism

Chronic central NPY administration has been shown to increase peripheral plasma insulin in the absence of hyperphagia in rats (Zarjevski et al. 1993). Central Y_1 receptor-induced hyperinsulinemia is independent of food ingestion, whereas the Y_5-induced hyperinsulinemia is dependent on food ingestion. This suggests that NPY-regulated hyperinsulinemia is controlled via distinct mechanisms (Gao et al. 2004). It has been observed that low plasma insulin levels correlate with increased hypothalamic NPY levels in rats (Sahu et al. 1988; Calza et al. 1989). When plasma insulin levels increase upon feeding (Schwartz et al. 1992) or central administration of insulin

(Schwartz et al. 1991), brain NPY levels decrease. Thus, there is a functional relationship between NPY and insulin. Central NPY stimulates insulin release whereas insulin inhibits hypothalamic NPY release by negative feedback reaction in normal animals (Fig. 2.5). Obesity causes changes in glucose metabolism, and insulin seems to fail to down-regulate NPY levels which remain elevated (Schwartz et al. 1991).

Central (i.c.v.) NPY administration results in a pronounced increase in the insulin-stimulated glucose uptake by adipose tissue, but in a marked decrease in uptake by the skeletal muscle (Zarjevski et al. 1994). The peripheral effects of NPY on glucose homeostasis have not been widely studied. Vettori et al. have shown that an acute intravenous infusion of NPY inhibits glucose-stimulated insulin release possibly by a direct effect of NPY on β-cells in rats (Vettor et al. 1998). In addition, *in vitro* studies have shown that NPY inhibits insulin secretion via Y_1 receptors (Moltz and McDonald 1985; Morgan et al. 1998). Since feeding induces NPY release from the gut nerve terminals (Morris et al. 1997), peripheral NPY can possibly inhibit insulin secretion via pancreatic NPY receptors upon energy intake (Fig. 2.5).

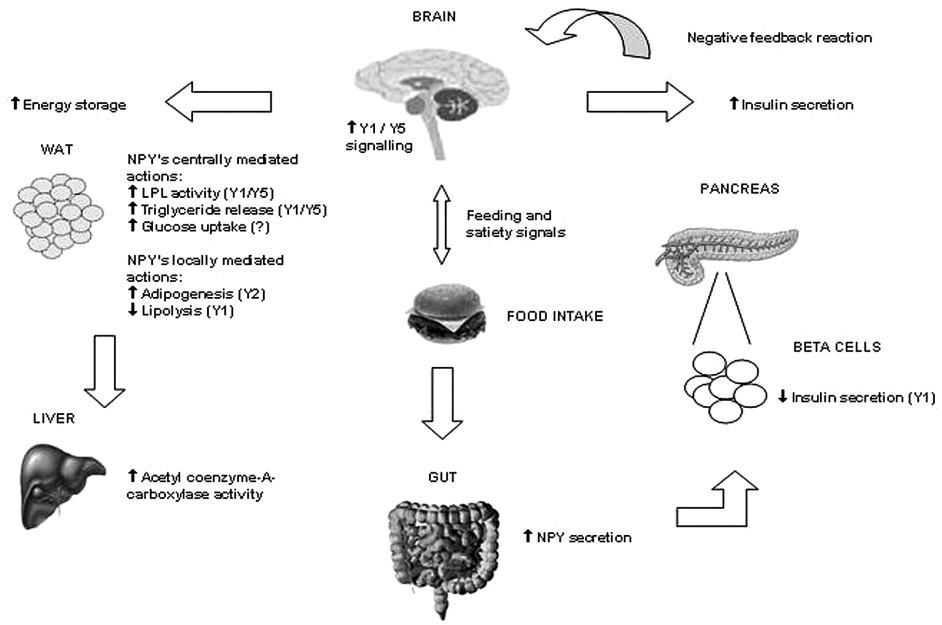

Fig. 2.5. Schematic presentation of the NPY's possible effects on energy intake, and lipid and glucose metabolism. Feeding increases hypothalamic Y_1 and Y_5 signalling and this promotes energy storage in WAT by increasing WAT LPL activity. Central NPY activity also activates triglyceride release into circulation and enhanced WAT glucose uptake. Local NPY in WAT promotes adipogenesis by acting on Y_2 receptors and inhibits lipolysis via Y_1 receptors. Hence, adipocyte number and size are increased. NPY is able to increase *de novo* lipogenesis in the liver, which may lead to enhanced cholesterol synthesis. Central Y_1 and/or Y_5 activation leads to increased insulin secretion from the pancreas, which in normal situation autoregulates its own release via negative feedback. Feeding-stimulated NPY release from the gut can possibly inhibit insulin secretion by directly activating the pancreatic Y_1 receptors on β-cells. Y_1, Y_2, Y_5 = NPY receptor subtypes 1, 2 and 5, respectively; WAT = white adipose tissue; LPL = lipoprotein lipase; ↑ = effect is stimulatory; ↓ = effect is inhibitory.

2.3.4 NPY in the regulation of the cardiovascular system

2.3.4.1 NPY regulating blood pressure and heart rate

NPY can act either as a vasoconstrictor or a vasodilatator depending on the site of action. Central NPY is hypotensive and this effect is mediated via Y_1 receptors in NTS of the brainstem (Yang et al. 1993). More detailed studies have shown that activation of Y_1 receptors in NTS also inhibit adrenergic α_2-induced hypotension (Yang et al. 1994). NTS Y_1 receptors are under the influence of Y_2 receptors, which antagonize the cardiovascular responses of Y_1 activation (Yang et al. 1993). Hence, Y_2 receptors inhibit the hypotensive effect of Y_1 receptors, and at the same time the Y_1 subtype antagonizes the cardiovascular actions induced by α_2-adrenoreceptors. It has been proposed that in a situation of high release of catecholamines, more NPY is also co-released, and to balance the increased brainstem α_2-activation, NPY serves as a homeostatic factor via Y_1 receptors to avoid too strong central adrenergic vasodepressor responses (Diaz-Cabiale et al. 2007).

Peripherally administered NPY exerts both hypertensive and vasodilative effects (Lundberg et al. 1989; Pernow and Lundberg 1989; Malmstrom 2000; You et al. 2001). Constriction is elicited by stimulating Y_1 receptors on vascular smooth muscle cells (VSMC) and dilation by stimulating Y_2 receptors on the vascular endothelium (Kobari et al. 1993; You et al. 2001). Vasoconstriction is most pronounced in small arteries independent of adrenergic mechanisms, but requires higher plasma NPY levels than those observed at baseline (Lundberg et al. 1987). The contraction response via Y_1 receptors leads to enhanced cellular Ca^{2+} influx, which can be attenuated by calcium channel blockers, such as diltiazem (Uneyama et al. 1995). NPY-induced vasodilatation is seen as enhanced endothelial release of nitric oxide and it is completely abolished by the removal of endothelium (You et al. 2001). In humans, intra-arterial NPY infusion has been shown to increase vascular resistance in hyper- and normotensive patients (Nilsson et al. 2000). In veins, NPY infusion-induced vasoconstriction has been shown to last several hours and to be unchanged by simultaneous administration of α-adrenergic antagonists (Peduzzi et al. 1995; Lambert et al. 1999). Vasoconstriction could, however, be reversed by nitroglycerin or bradykinin (Peduzzi et al. 1995).

Spontaneously hypertensive rats and non-obese diabetic mice are established animal models of hypertension. Both models show increased levels of NPY in small arteries, and more pronounced contractions of mesenteric arteries in response to NPY in a microvascular myograph compared with control animals (Gradin et al. 2003; 2006). In addition, in these models, the enhanced contraction activity is inhibited by the Y_1 antagonist (Gradin et al. 2003; 2006). Thus, alterations in smooth muscle response to NPY seem to contribute to the enhanced arterial vasoconstriction in hypertensive but not normotensive animals.

In rats, exogenous NPY can prevent hypotension and stabilize body temperature in an experimental model of septic shock (Felies et al. 2004; Nave et al. 2004). In addition, a

septic shock increases endogenous NPY levels, which suggests that NPY may contribute to the compensatory mechanism, tending to bring arterial pressure back to normal levels in response to the shock (Wang et al. 1992).

Mammalian myocardium contains large quantities of NPY in neurons innervating the heart (Onuoha et al. 1999). NPY can increase the cardiomyocyte contraction force and frequency via Y_1 receptors and stimulate a negative contraction effect via Y_2 receptors (McDermott et al. 1997; Allen et al. 2006; Abdel-Samad et al. 2007). NPY also contributes to cardiomyocyte hypertrophy by increasing protein synthesis rate and/or inhibiting protein degradation in normotensive rats (Millar et al. 1994). A similar effect is also seen in cardiomyocytes isolated from spontaneously hypertensive rats (Bell et al. 2002). Under normal conditions when hypertension is absent, NPY's contraction effects are maintained at a constant level. This may be due to the fact that in the absence of pressure overload, there is no need for enhanced cardiomyocyte performance (Allen et al. 2006) However, the potency of NPY to elicit positive and negative contraction effects is significantly increased during cardiomyocyte hypertrophic growth (Allen et al. 2006). In an isolated guinea-pig sinoatrial preparation, NPY has been shown to reduce vagal bradycardia by inhibiting ACh release via presynaptic Y_2 receptors (Herring et al. 2008). The inhibitory effect was not observed at baseline heart rate but after vagal nerve stimulation. Therefore, NPY is at least partly responsible for reducing vagal activity. All these data combined suggest that NPY may increase the frequency and contractility force of the heart, i.e. it promotes positive chrono- and inotrophy during high sympathetic tone seen in cardiac failure, for example.

2.3.4.2 NPY and vascular remodelling

NPY has been shown to increase DNA synthesis and cell proliferation rate in a dose- and cell density-dependent manner with up to 20-fold potency over NA in rat, porcine and human aortic VSMC lines (Shigeri and Fujimoto 1993; Zukowska-Grojec et al. 1998; Nilsson et al. 2000). This mitogenic effect is dependent on the presence of inducible NPY receptors Y_1, Y_2 and possibly Y_5, and attenuated by incubation with calcium channel blockers (Zukowska-Grojec et al. 1993). NPY is also a trophic factor for rat cardiomyocytes (Millar et al. 1994). Hence, it is possible that NPY promotes arterial thickening and cardiac hypertrophy by increasing the number and size of smooth muscle cells in situations with high SNS activity. Endothelial dysfunction activates NPYergic system which, in turn, has been shown to promote the formation of thrombus and arterial neointima at the site of the injury *in vivo* in rats (Li et al. 2003; 2005). These data suggest that NPY may be a risk factor for accelerated development of atherosclerosis by increasing VSMC proliferation activity upon vascular injury and SNS activation.

Angiogenesis is the process of novel vessel formation. Under normal conditions, angiogenic activity is low, but during injuries and in many diseases (cancer, diabetic retinopathy, hypoxia, wound healing) the activity increases. At low physiological concentrations *in vitro*, NPY promotes arterial sprouting, and human endothelial cell

adhesion, migration, proliferation and capillary tube formation (Zukowska-Grojec et al. 1998). Angiogenesis, arterial sprouting and wound healing are impaired in Y_2 null mice (Ekstrand et al. 2003; Lee et al. 2003). Furthermore, neonatal hypoxia-induced retinal angiogenesis is blocked in Y_2 deficient mice or in rats treated with selective Y_2 antagonist (Koulu et al. 2004). Thus, Y_2 receptors seem to mediate NPY-regulated vascular formation.

2.3.5 NPY and hormone release

NPY released by human adrenal chromaffin cells has been shown to increase the secretion of adrenaline and NA *in vitro*, and consequently contribute to the sympathoadrenal tone *in vivo* (Cavadas et al. 2001). Additionally, NPY increases catecholamine release from mouse chromaffin cells *in vitro*, and the secretion has been reported to be mediated via Y_1 receptors (Cavadas et al. 2006; Rosmaninho-Salgado et al. 2007). However, conflicting results on the increased catecholamine release have been published depending on the species (Higuchi et al. 1988; Hexum and Russett 1989; Zheng et al. 1995).

The corticotrope axis (HPA axis), which includes the hypothalamus, the pituitary, and the adrenal cortex, secretes corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and corticosterone (cortisol in humans). In rats, a central infusion of NPY has been shown to increase the concentration of CRH in the hypothalamus, which leads to enhanced plasma levels of ACTH and corticosterone (Harfstrand et al. 1987; Wahlestedt et al. 1987; Haas and George 1989; Sainsbury et al. 1997). Similarly, ACTH and glucocorticoids regulate adrenal NPY gene expression and synthesis (Laborie et al. 1995; Hinson et al. 1998).

Central administration of NPY inhibits pulsatile growth hormone (GH) release in rats, and decreases growth hormone-releasing hormone (GHRH) mRNA levels in both rats and mice (Pierroz et al. 1996; Suzuki et al. 1996; Raposinho et al. 2001). Also, NPY decreases GHRH expression in primary rat hypothalamic cell cultures (Mano-Otagiri et al. 2006). The inhibitory effect may be a result of NPY-induced increase in hypothalamic CRH, which further suppresses the expression of GHRH and the release of GH from the pituitary (Luque et al. 2007).

NPY neurons in ARC participate in the modulation of gonadotropin releasing hormone/luteinizing hormone (GnRH/LH) secretion in rodents, primates and sheep (McDonald et al. 1985; Khorram et al. 1987; Kaynard et al. 1990; Malven et al. 1992). Central NPY suppresses the release of LH presumably through the inhibition of GnRH in ovariectomized animals, whereas LH release is stimulated in ovariectomized animals treated with ovarian steroids (Khorram et al. 1987). The mode of NPY action seems to depend on the state of energy balance; malnutrition causes an increase in NPY levels, which further causes a depression of gonadotropic axis activity and vice versa. These observations have been reviewed in detail elsewhere (Wojcik-Gladysz and Polkowska 2006).

2.3.6 NPY and bone

Bone tissue contains autonomic nerve fibres, and immunoreactivity to NPY has been found in the bone and bone marrow of rat tibia and femur (Bjurholm et al. 1988). NPY positive nerve fibres are predominantly found close to or within blood vessels in the epiphyses of the bone. Central regulation of bone metabolism is possibly due to the neuronal projections from the hypothalamus to sympathetic and parasympathetic nuclei in the brainstem. Hypothalamic Y_2 receptors regulate bone formation as shown in the conditional hypothalamic Y_2 receptor knock-out model, which displays increased osteoblast activity and rate of bone mineralization and formation (Baldock et al. 2002). On the contrary, Y_1 receptor activation inhibits bone production by nonhypothalamic pathways with potentially direct effects on bone tissue (Baldock et al. 2007).

In humans, the relationship between NPY and bone is not well known. Carriers of the human p.L7P allele show increased femoral neck bone mineral density (BMD) in postmenopausal women, which suggests that proline 7 substitution may be favourable in bone metabolism (Heikkinen et al. 2004). This could be explained by the increased exercise-stimulated GH release (Kallio et al. 2001) or by an altered autonomic nervous system activity (Jaakkola et al. 2005) in p.L7P subjects. It is also possible, that increased BMI in p.L7P subjects (Ding et al. 2005) contributes to the increased BMD, because it has been shown that higher BMI is a protective factor for BMD in Caucasian population (Castro et al. 2005).

2.3.7 NPY and behaviour

NPY levels have been shown to be decreased in depressed and suicidal patients, and in clinical subjects suffering from posttraumatic stress disorder (Rasmusson et al. 2000; Heilig 2004; Yehuda et al. 2006). In contrast, NPY levels are elevated in panic disorder patients (Boulenger et al. 1996). In experimental animal models, NPY has been shown to reduce anxiety (Thorsell et al. 2000; Redrobe et al. 2003; Tschenett et al. 2003; Karl et al. 2006). Several brain structures are involved in mediating anti-stress actions of NPY, with the most extensive evidence pointing to the amygdala and the hippocampus, and some evidence for regions within the septum and LC (Heilig 2004; de Lange et al. 2008).

The SCN drives physiological and behavioural circadian rhythms controlled by light. In rodents, responses to light/dark rotation can be measured as locomotor activity. Injection of NPY into the SCN region during middle of the active phase of the individual's day advances the circadian clock, while having little effect during the subjective night in Syrian hamsters (Albers and Ferris 1984; Kallingal and Mintz 2007). NPY knock-out mice show a significantly delayed onset of physical activity to the new time of light offset when exposed to gradual changes in photoperiod (Kim and Harrington 2008). In another study, NPY-deficient mice consistently demonstrated suppressed levels of locomotion (Karl et al. 2008). Hence, light and NPY appear to exert opposing influences on the circadian clock in SCN.

NPY plays a role in ethanol consumption and resistance. The preference for alcohol is inversely related to NPY levels in the brain (Thiele et al. 1998), and self-administration of ethanol in alcohol-dependent rats can be reduced by central administration of Y_2 antagonist to increase NPY levels (Rimondini et al. 2005). Furthermore, retroviral overexpression of NPY in the amygdala suppresses ethanol self-administration in rats with high basal levels of anxiety, whereas the NPY antisense treatment has an opposite effect on the alcohol consumption (Primeaux et al. 2006). In humans, the evidence for NPY in the regulation of alcohol dependence is mainly based on functional polymorphisms in the *NPY* gene (Lappalainen et al. 2002; Zhu et al. 2003; Mottagui-Tabar et al. 2005).

2.4 Gene modified animal models in NPY research

Genetically modified animal models provide a wealth of information about the physiological roles of different peptides and receptors. To date, there are several methods available for creating genetically modified animal models. The conventional method utilizes fertilized oocytes, in which the transgene construct is injected, and the cells are implanted into pseudopregnant females. Another method makes use of blastocyst embryonic stem cells that are grown in cultures and transfected with the desired transgene. Stem cells that have received the DNA of interest can be characterized and transferred into mouse embryos. Transgenic models can also be generated by using viral gene transfer, which is based on a virus carrying the transgene and infecting the targeted cells. Viral vectors may be problematic because of their immunogenicity, but newer vectors that are safer to use are becoming more and more available.

The first NPY overexpressing (OE) mouse model was created by Thiele et al., but surprisingly it was soon abandoned due to the lack of a clear phenotype (Thiele et al. 1998). NPY-OE rat has shown an anxiolytic and seizure-resistant phenotype, but it, too, failed to show any of the expected effect on feeding behaviour. Furthermore, the first NPY knock-out mouse created by Erickson et al. did not differ from wildtype mouse in fed-state food intake or related parameters (Erickson et al. 1996). Many other mouse models with modifications in *NPY* or its receptor gene expression levels have been generated since then, and they have allowed us to draw a more precise picture for the physiological role of NPY. A summary of the known phenotypes of the genetically modified animals for *NPY* or its receptors are shown in tables 2.2 - 2.4.

2.4.1 NPY overexpressing animal models

The first NPY-OE mouse model on FVB/n background failed to demonstrate a clear phenotype. In the mouse, NPY was overexpressed under its natural promoter. The NPY-OE mice had a lower preference for ethanol, and they were more sensitive to the sedative and hypnotic effects of alcohol than wildtype mice (Thiele et al. 1998). Unfortunately, this is the only study available on that particular model, and the line no longer exists for further studies.

Another NPY-OE mouse was created by using the *Thy1* promoter, which restricted NPY overexpression strictly within neurons in the CNS (Inui et al. 1998). The Thy1-NPY hemizygous mice on BDF1 background showed no effect on energy homeostasis. The homozygous model led to an obese phenotype, but only after appropriate dietary exposure (Kaga et al. 2001). The Thy1-NPY transgenic mice also displayed behavioural signs of anxiety, and the anxiety-like behaviour was reversed, at least in part, by administration of corticotropin-releasing factor (CRF) antagonists into the CNS (Inui et al. 1998). These results were somewhat surprising, because NPY is associated with increased feeding and anxiolysis.

In the conditional OE-model, NPY is overexpressed until given doxycycline, which blocks the transcription of NPY (Ste Marie et al. 2005). The main conclusion of the study with these mice was that changing NPY levels from high to very low have little effect on body weight regulation. No effect on food intake was observed at any stage.

The NPY-OE rat model was generated on Sprague-Dawley background by introducing a 14.5 kbp fragment of the rat NPY genomic sequence into oocytes (Michalkiewicz and Michalkiewicz 2000). No differences in food intake or body weights were found between the NPY-OE and wildtype rats (Michalkiewicz et al. 2001). This is most likely due to the increased levels of NPY in the hippocampus but not in the hypothalamus (Thorsell et al. 2000; Michalkiewicz et al. 2003). The NPY-OE rats are insensitive to normal anxiogenic-like effect of restraint stress, and they display absent fear suppression of behaviour in a punished drinking test (Thorsell et al. 2000). These results fit with the expected anxiolytic phenotype. In addition, NPY-OE rats show increased total vascular resistance, and increased arterial pressure in response to NA injection measured by the tail-cuff method (Michalkiewicz et al. 2001). In a long-term telemetric study, the hemodynamic parameters showed an opposite phenotype: decreased catecholamine release and blood pressure at baseline and during stress (Michalkiewicz et al. 2003). This suggests that the NPY-OE rats are more responsive to the effects of stress, as the tail-cuff method is rather stressful for rats and most likely increases SNS activity during tests.

PVN- and LHA-specific overexpression of NPY in rat generated by retroviral transgene delivery showed that the NPY system regulates energy intake and energy expenditure in a site-specific manner: PVN controls the initiation of food intake and activation of the HPA-axis, and NPY in LHA regulates meal size and core temperature (Tiesjema et al. 2007). Amygdala-specific NPY-OE rats showed blunted increase in alcohol intake following repeated withdrawal in combination with a history of ethanol consumption, or in rats with high basal levels of anxiety (Primeaux et al. 2006; Thorsell et al. 2007). Amygdala NPY-OE rats were also anxiolytic in the open field test (Thorsell et al. 2007). Thus, amygdala seems to be the major area regulating anxiolytic behaviour.

Table 2.2. Major phenotypes observed in NPY-OE transgenic animal models.

Species	Examined behaviour/parameter	Effect	References
Mouse	Body weight	↔/↑	(Inui et al. 1998; Kaga et al. 2001; Ste Marie et al. 2005)
	Food intake	↔/↑	
	Impaired glucose tolerance	↔/↑	
	Anxiety	↑	(Inui et al. 1998)
	Voluntary ethanol intake	↓	(Thiele et al. 1998)
	Ethanol-induced sedation	↑	
Rat	Body weight	↔	(Michalkiewicz et al. 2001)
	Food intake	↔	
	Anxiety	↓	(Thorsell et al. 2000; 2007)
	Blood pressure	↑/↓	(Michalkiewicz et al. 2001; 2003)
	Voluntary ethanol intake	↓	(Primeaux et al. 2006)

↑ = effect is stimulatory; ↓ = effect is inhibitory, ↔ no effect.

2.4.2 NPY knock-out mouse models

NPY-deficient mice ($NPY^{-/-}$) on 129/Sv background show unchanged food intake and body weights under normal conditions, but hyperphagia after food deprivation (Erickson et al. 1996). Baseline endocrine parameters also appear normal (Erickson et al. 1997). The $NPY^{-/-}$ mouse crossed to the leptin deficient ob/ob mouse proved that functional NPY is required for the full manifestation of the obese phenotype (Erickson et al. 1996). Next $NPY^{-/-}$ 129/Sv mice were backcrossed to C57BL/6 background to produce 129/Sv - C57BL/6 hybrids (Bannon et al. 2000). Even so, NPY deficiency has no significant feeding effect in mice fed with a normal rodent diet. However, energy expenditure is elevated during fasting, and hyperphagia and weight gain are blunted during re-feeding (Patel et al. 2006). Finally, $NPY^{-/-}$ mice on the obesity-prone C57BL/6 background were produced by backcrossing the 129/Sv mice to C57BL/6 strain for seven generations (Segal-Lieberman et al. 2003). These mice show unexpectedly increased body weights and adiposity together with impaired feeding response after a fast (Segal-Lieberman et al. 2003). Backcrossing of ten generations to C57BL/6 attenuates the hyperphagic and thermoregulatory responses to fasting and diet-induced obesity (DIO) in $NPY^{-/-}$ mice (Patel et al. 2006). Resistance to DIO is associated with increased expression of anorexigenic peptides namely POMC and CRH in the hypothalamus (Patel et al. 2006). $NPY^{-/-}$ mice on C57BL/6 background show only subtle changes in glucose homeostasis, i.e. elevated serum insulin levels while being insulin tolerant, but display increased pancreatic islet mass, and enhanced basal and glucose-stimulated insulin secretion (Imai et al. 2007).

Hybrid 129/Sv - C57BL/6 $NPY^{-/-}$ mice are anxiogenic in open field locomotor monitors and hypoalgesic to an acute thermal stimulus in comparison with wildtype controls (Bannon et al. 2000). They also show increased alcohol consumption, and they are less sensitive to the sedative/hypnotic effects of ethanol, as shown by more

rapid recovery from ethanol-induced sleep (Thiele et al. 2000). These results fit well with the expected phenotypes that NPY regulates anxiolysis and possibly sedation.

Table 2.3. Major phenotypes observed in *NPY* single knock-out mouse models.

Mouse strain	Examined behaviour/parameter	Effect	References
129/Sv	Body weight	↔	(Erickson et al. 1996)
	Food intake	↔	
	Ethanol-induced sedation	↔	(Thiele et al. 2000)
	Ethanol-induced activity	↔	
129/Sv - C57BL/6	Body weight	↔	(Patel et al. 2006)
	Fast-induced re-feeding	↓	(Bannon et al. 2000)
	Anxiety	↑	
	Algesia	↓	
	Ethanol-induced sedation	↓	(Thiele et al. 2000)
	Endocrinological parameters	↔	(Erickson et al. 1997)
C57BL/6	Body weight	↑	(Segal-Lieberman et al. 2003)
	Adiposity	↑	
	Insulin secretion	↑	(Imai et al. 2007)

↑ = effect is stimulatory; ↓ = effect is inhibitory, ↔ no effect.

In summary, negative regulation of feeding at baseline is not seen in any of the $NPY^{-/-}$ mice. However, reductions in fasting-induced re-feeding were observed. Only $NPY^{-/-}$ ob/ob mice succeeded to show that lack of NPY reduces body weight. Even destruction of NPY in adulthood did not manage to downregulate feeding from baseline (Ste Marie et al. 2005). Thus, compensatory mechanisms must substitute the missing hypothalamic NPY. It is also possible that the background strain effect overcomes the NPY-regulated effects. This is supported by the fact that changes, although contrary to the expected, in body weights and adiposity were seen in the C57BL/6 $NPY^{-/-}$ mice. Parameters measuring anxiogenesis and ethanol consumption showed that NPY is indeed an important factor regulating these behaviours.

2.4.3 NPY receptor knock-out mouse models

To date there are four cloned and verified NPY receptors in humans. Knock-out mice for all these receptors have been generated. Some receptors have been studied in many laboratories resulting in several mouse models. The data from different laboratories are not consistent, which suggests that compensatory mechanisms replace the missing receptor possibly by increasing the expression levels of other NPY receptors.

2.4.3.1 Y_1 deficient mice

Homozygous Y_1 deficient ($Y_1^{-/-}$) mice on a mixed 129/Sv - C57BL/6 background show moderate obesity and mild hyperinsulinemia without hyperphagia caused by impaired control of insulin secretion and/or low energy expenditure (Kushi et al. 1998; Baldock et al. 2007). Leptin infusion normalizes hyperinsulinemia and glucose turnover in $Y_1^{-/-}$ mice (Burcelin et al. 2001). The increased weight gain suggests enhanced Y_5 activation, for example, to compensate the loss of Y_1 receptors. The behavioural phenotype is strongly circadian rhythm-dependent, and it can be modified by restraint stress, which increases the frequency of the explorative-like behaviour (Karl et al. 2006). Furthermore, $Y_1^{-/-}$ mice have been reported to be aggressive in spontaneous/territorial aggression and resident-intruder tests (Karl et al. 2004). This behaviour pattern is reduced by treatment with a 5-HT-1A agonist, which suggests that NPY regulates the 5-HT system through Y_1 receptors, and therefore it integrates aggressive behaviour with other survival-related physiological functions, such as food intake.

In a different strain (C57BL/6), $Y_1^{-/-}$ mice demonstrate a marked reduction in NPY-induced food intake in comparison with wildtype controls (Kanatani et al. 2000). $Y_1^{-/-}$ mouse crossed to the ob/ob mouse show significantly reduced body weights compared with obese ob/ob mice that is due to a reduction in food intake (Pralong et al. 2002). This suggests that hyperphagia in leptin deficiency is partially dependent on chronic stimulation of Y_1 receptors.

$Y_1^{-/-}$ C57BL/6 mice also display an absent blood pressure response to NPY, whereas they retain normal responses to other vasoconstrictors such as NA and angiotensin II (Pedrazzini et al. 1998). Thus, this data strengthens the hypothesis that NPY's vasoconstrictive effects are mediated via $Y_1^{-/-}$ receptors. Plasma levels of catecholamines are increased in $Y_1^{-/-}$ mouse possibly by enhancement of TH activity (Cavadas et al. 2006). Furthermore, Y_1 receptors seem to mediate ethanol's hypnotic effects, because $Y_1^{-/-}$ mice have been reported to be less sensitive to the sedative effects of ethanol in comparison with wildtype mice (Thiele et al. 2002).

$Y_1^{-/-}$ mice (mixed 129/Sv – Balb/c) develop hyperalgesia to acute, thermal cutaneous and visceral pain and exhibit mechanical hypersensitivity (Naveilhan et al. 2001) possibly via increased synthesis of calcitonin gene-related peptide and substance P in dorsal root ganglion neurons (Shi et al. 2006). $Y_1^{-/-}$ mice also show a marked resistance to barbiturates and they are less sensitive to sodium pentobarbital-induced sedation (Naveilhan et al. 2001), which is in line with the decreased ethanol sedation in Y_1 null mice (Thiele et al. 2002).

Germline $Y_1^{-/-}$ mice display significantly greater cancellous bone volume and bone formation rate, whereas hypothalamic-specific Y_1 deletion does not alter cancellous bone volume (Baldock et al. 2007). This suggests a peripheral and direct mechanism of bone formation via Y_1 receptors at tissue level.

2.4.3.2 Y_2 deficient mice

Y_2 deficient ($Y_2^{-/-}$) mice on a mixed 129/Sv - C57BL/6 background show sustained reduction in body weight and adiposity (Sainsbury et al. 2002). However, in another model of Y_2 deficiency (hybrid 129/Sv – Balb/c), the mice develop increased body weight, food intake and fat deposition with decreased energy expenditure (Naveilhan et al. 1999). These disparities can perhaps be explained by the use of different inbred mouse strains within the studies. However, Y_2 deficiency in ob/ob mice attenuates the increased adiposity, hyperinsulinemia and hyperglycemia, and reduces serum cholesterol levels, but no effect on food intake was observed when both these $Y_2^{-/-}$ mice were crossed to the ob/ob mouse (Naveilhan et al. 2002; Sainsbury et al. 2002). Hence, these data, and the data obtained from $NPY^{-/-}$ob/ob double knock-outs suggest that increased adiposity and diabetic phenotype in the absence of leptin is in part mediated via NPY acting on Y_2 receptors.

Deletion of Y_2 receptors results in a potent suppression of anxiogenic and stress-related behaviours, some of which may be due to the inhibition of CRF pathways (Redrobe et al. 2003; Tschenett et al. 2003). $Y_2^{-/-}$ mice also display increased sensitivity to sodium pentobarbital-induced sedation, an effect opposite to that observed in Y_1 knock-outs (Naveilhan et al. 2001).

NPY-induced aortic sprouting and in vivo matrigel capillary formation are decreased in $Y_2^{-/-}$ mice (Lee et al. 2003). In addition, Y_2 null mice display delayed skin wound healing with reduced neovascularization (Ekstrand et al. 2003), and reduced hypoxia-induced retinal angiogenesis (Koulu et al. 2004). These results suggest that NPY plays a role in angiogenesis via peripheral Y_2 receptors.

Hypothalamus-specific $Y_2^{-/-}$ mice show a significant decrease in body weight and an increase in food intake (Sainsbury et al. 2002). They also show three to five times higher levels of PP and corticosterone in plasma, which could explain the hyperphagia not observed in germline $Y_2^{-/-}$ mice (Sainsbury et al. 2002). $Y_2^{-/-}$ mice also display increased trabecular bone volume and thickness compared with control mice (Baldock et al. 2002). Conditional hypothalamic $Y_2^{-/-}$ mice show an identical increase in trabecular bone volume, which suggests that central Y_2 receptors are crucial for the regulation of bone formation (Baldock et al. 2002) as opposed to local Y_1-mediated actions.

2.4.3.3 Y_4 deficient mice

Loss of Y_4 receptor function ($Y_4^{-/-}$ mice) contributes to reduced body weight gain and decreased adiposity due to inefficient nutrient uptake during digestion (Sainsbury et al. 2002). A notable increase in plasma PP levels is also observed, which is the most likely explanation for the reduced food intake and body weight seen in these mice. $Y_4^{-/-}$ mice display a slower heart rate associated with a higher pulse interval and lower blood pressure compared with control mice (Smith-White et al. 2002). The response to NPY-induced increase in blood pressure is reduced. The authors suggest that the reduced

vasoconstriction and vagal inhibitory activity evoked by NPY in $Y_4^{-/-}$ mice is due to a reduction in sympathetic activity, possibly resulting from altered NPY activity, which secondarily affects the adrenergic transmission. Furthermore, Y_4 receptors seem to be involved in reproductive function and fertility as deletion of Y_4 receptors rescues fertility in sterile ob/ob mice and improves their sex hormone levels (Sainsbury et al. 2002).

Deletion of both receptors in $Y_2Y_4^{-/-}$ double knockout mice results in greater reductions in adiposity and greater increases in cancellous bone volume than in mice with deficiency of either the Y_2 or Y_4 receptor alone (Sainsbury et al. 2003). Furthermore, breeding $Y_2Y_4^{-/-}$ double knock-outs with ob/ob mice, results in highly significant reductions in body weight and WAT mass compared with wildtype ob/ob mice (Lee et al. 2008). $Y_4^{-/-}$ob/ob mice show no improvement in adiposity compared with ob/ob mice (Sainsbury et al. 2002). Therefore, deletion of Y_2 and Y_4 receptors synergistically protects against weight gain, and the antiobesity effect of $Y_2Y_4^{-/-}$ deficiency is stronger than the obesity effect of Y_1 deficiency, since $Y_1Y_2Y_4^{-/-}$ triple knockouts do not develop obesity on a high-fat diet (Sainsbury et al. 2006).

2.4.3.4 Y_5 deficient mice

Y_5 null ($Y_5^{-/-}$) mice on a hybrid 129/Sv - C57BL/6 background develop mild late-onset obesity characterized by increased body weight, food intake and adiposity (Marsh et al. 1998). $Y_5^{-/-}$ob/ob mice are just as obese as wildtype ob/ob mice pointing out that Y_5 receptor is not a critical physiological feeding receptor in mice (Marsh et al. 1998). Elsewhere, $Y_5^{-/-}$ mice on C57BL/6 background show reduced but not completely abolished responses to Y_5 agonist-stimulated feeding, but the body weights are similar with wildtype controls (Kanatani et al. 2000). The authors do not show evidence to indicate that the agonist is strictly specific for Y_5 receptors. Thus, it is possible that the stimulated feeding is controlled via Y_1 receptors, for example. The lack of either Y_1 or Y_5 receptors do not diminish the central NPY-induced feeding responses, and the knock-out mice develop obesity syndrome similar to that in wildtype animals (Raposinho et al. 2004). Thus, it seems that the Y_5 receptor does not play a dominant role in NPY-induced feeding.

Table 2.4. Major phenotypes observed in NPY receptor single knock-out mouse models.

Targeted gene	Mouse strain	Examined behaviour/parameter	Effect	References
NPY1r	129/Sv - C57BL/6	Body weight, adiposity	↑	(Kushi et al. 1998; Baldock et al. 2007)
		Insulin secretion	↑	
		Energy expenditure	↓	
		Bone mass	↑	(Baldock et al. 2007)
		Behavioural aggression	↑	(Karl et al. 2004)
	C57BL/6	NPY-induced food intake	↓	(Kanatani et al. 2000)
		NPY-induced blood pressure	↓	(Pedrazzini et al. 1998)
		Basal blood pressure and heart rate	↔	
		Catecholamine secretion	↑	(Cavadas et al. 2006)
		Ethanol-induced sedation	↓	(Thiele et al. 2002)
	129/Sv – Balb/c	Algesia	↑	(Naveilhan et al. 2001)
NPY2r	129/Sv – Balb/c and 129/Sv - C57BL	Body weight	↑/↓	(Naveilhan et al. 1999; Sainsbury et al. 2002)
	129/Sv - C57BL	Anxiety	↓	(Redrobe et al. 2003; Tschenett et al. 2003)
		Bone volume	↑	(Baldock et al. 2002)
	129/Sv – Balb/c and 129/Sv	Angiogenesis	↓	(Naveilhan et al. 2002; Lee et al. 2003)
NPY4r	129/Sv - C57BL/6	Body weight, adiposity	↓	(Sainsbury et al. 2002; 2003; 2006)
		Bone mass	↔	(Sainsbury et al. 2003)
		Blood pressure	↓	(Smith-White et al. 2002)
NPY5r	129/Sv - C57BL/6	Body weight, adiposity, food intake	↑	(Marsh et al. 1998)
	C57BL/6	Food intake	↔	(Kanatani et al. 2000)

↑ = effect is stimulatory; ↓ = effect is inhibitory, ↔ no effect.

In summary, all four NPY receptors studied in gene-modified animal models seem to contribute to the body weight and feeding, although the results in all the models were not what expected. Loss of Y_1 and/or Y_5 receptors caused increased weight gain and adiposity, although it was originally thought that ligands for Y_1 and Y_5 control positive energy balance in the hypothalamus and deletion of these receptors would eventually stop this signalling chain. Therefore, deletion of NPY feeding receptors alone is not sufficient to prevent obesity, because there might be mechanisms to compensate the

loss of Y_1 and Y_5 signalling. Y_2 knock-outs, on the other hand, should have shown increased food intake and adiposity, since central presynaptic Y_2 receptors inhibit NPY release and further impact on feeding. The results from two different mouse strains are controversial, but the other showed sustained weight gain (Naveilhan et al. 1999), and hypothalamus-specific deletion of Y_2 receptors produced an increase in food intake, although a decrease in body weights was observed (Sainsbury et al. 2002). Thus, it is impossible to say whether the different phenotypes are due to the different mouse strains used or to the compensatory mechanisms in other genes as a result of the knock-down procedure. Based on the animal models, it may be reasonable to conclude that Y_2 and Y_4 receptors mediate the anorexigenic effects of their ligands. It has also been shown rather clearly that NPY via Y_2 receptors is involved in angiogenesis, i.e. sprouting of new blood vessels. Furthermore, NPY's behavioural effects are most likely controlled via Y_1 receptors in the brain.

2.5 The Leucine 7 to Proline 7 polymorphism (p.L7P) in the human *NPY* gene

The human *NPY* gene was cloned in 1984 (Minth et al. 1984). The gene is located in chromosome 7 p15.1 (Baker et al. 1995). As mentioned in chapter 2.1.1, the *NPY* gene consists of four exons and the amino acid sequence homology among mammals is high. Most variation between species is in the signal peptide sequence and it displays one amino acid variation in length between different species (Hoyle 1999). The signal peptide is needed for proper folding and packaging of the mature peptide into the endoplasmic reticulum where posttranslational processing takes place. Once the peptide has reached this cell organelle, the signal peptide is cleaved off by a specific peptidase, degraded and the peptide is subjected to processing.

Healthy humans display NPY plasma levels of 31-69 pmol/l with a mean (SD) value of 52 (10) pmol/l (reference data for EURIA-NPY RIA kit, Euro-Diagnostica, Malmö, Sweden). However, lower plasma levels have also been reported elsewhere (Theodorsson-Norheim et al. 1985). Increased NPY levels have been found in patients with neuroblastoma (Kogner et al. 1993) and phaeochromocytoma tumors (Adrian et al. 1983; Emson et al. 1984).

2.5.1 p.L7P polymorphism in general

The p.L7P (NM_000905.2:c.20T>C; rs16139) allele was first described by Karvonen et al. (Karvonen et al. 1998). The single amino acid polymorphism (leucine to proline) is located in the exon 2 of the *NPY* gene. It is in the signal peptide, which is cleaved off from the mature protein. However, the polymorphism is functional or it is in linkage disequilibrium with another functional polymorphism (Zhou et al. 2008). *In vitro* and *in vivo* studies have shown that carriers of the p.L7P allele display altered cellular and plasma NPY concentrations (Kallio et al. 2001; 2003). Suggested hypothesis is that the p.L7P increases the processing and release of mature NPY from cells, whereas the wildtype L7L subjects produce more of the carboxy terminal containing form of the

gene (Kallio et al. 2001; Wang et al. 2008). Interestingly, carriers of the p.L7P allele do not show increased levels of NPY in circulation at rest (Kallio et al. 2001). A physical treadmill exercise, or similar, is required to observe a significant increase in plasma NPY levels between the L7P and L7L genotypes, which suggests that NPY is released into circulation from the sympathetic neurons innervating the vessels (Kallio et al. 2001).

The p.L7P genotype is most common in Nordic/Eastern European populations, and less common in Central or Southern Europe with prevalence of 6-14% in different study regions (Karvonen et al. 1998; Ding 2003; Pesonen 2008). The p.L7P prevalence in Asian and African American populations is very low or close to 0% (Ding et al. 2002; Lappalainen et al. 2002; Ding 2003; Jia et al. 2005).

2.5.2 p.L7P association studies with obesity, lipids and type 2 diabetes

The p.L7P allele was first associated with increased total and LDL cholesterol levels in non-diabetic obese and non-obese Finnish and Dutch study subjects (Karvonen et al. 1998; Karvonen et al. 2001). p.L7P carriers show decreased serum levels of free fatty acids during and after exercise (Kallio et al. 2001), but the levels normalize at rest (Kallio et al. 2003). In children, the p.L7P allele is associated with higher birth weights and increased serum levels of triglycerides (Karvonen et al. 2000). p.L7P genotype has also been associated with increased BMI in two large Swedish populations (Ding et al. 2005), and with impaired glucose tolerance and type 2 diabetes in Swedish men, which indicates that the polymorphism contributes to the development of metabolic syndrome (Nordman et al. 2005). Patients with type 2 diabetes and p.L7P genotype develop diabetic retinopathy more frequently than wildtype L7L diabetic patients (Niskanen and Voutilainen-Kaunisto 2000). The association of p.L7P allele with various metabolic and cardiovascular diseases has recently been reviewed in detail (Pesonen 2008).

2.5.3 p.L7P association studies with atherosclerosis and coronary heart disease (CHD)

The p.L7P allele is associated with increased arterial intima media thickness in both non-diabetic subjects and type 2 diabetic patients, independent of serum lipids (Niskanen et al. 2000; Karvonen et al. 2001). Carriers of the Pro7 substitution show increased systolic and diastolic blood pressure (Karvonen et al. 2001) and heart rate (Kallio et al. 2001; Kallio et al. 2003). Furthermore, the p.L7P polymorphism is an independent predictor for myocardial infarction and stroke in hypertensive patients (Wallerstedt et al. 2004). The p.L7P was not associated with angiographic restenosis or adverse clinical events after stent placement in coronary arteries (Pesonen et al. 2003). Type 1 diabetic patients with the Pro7 substitution have more CHD than their healthy counterparts (Pettersson-Fernholm et al. 2004). The frequency of the p.L7P allele in non-diabetic CHD patients, however, does not differ from that of the L7L genotype based on a cross-sectional EUROASPIRE study (European Action on Secondary Prevention by Intervention to Reduce Events) (Erkkila et al. 2002).

2.5.4 p.L7P association studies with depression and related diseases

In Swedish men, the p.L7P has been associated with depression (Heilig et al. 2004). It has also been connected with alcohol drinking and alcohol dependence in several Caucasian study populations (Kauhanen et al. 2000; Ilveskoski et al. 2001; Lappalainen et al. 2002), although the results are not supported by all (Zhu et al. 2003). In a recent study, a novel polymorphism in the *NPY* promoter was associated with traits of anxiety in Finnish men (Zhou et al. 2008). Furthermore, the promoter haplotype contained also the p.L7P variant, suggesting a possible linkage between the two polymorphisms (Zhou et al. 2008).

3 AIMS OF THE STUDY

The current studies were undertaken to investigate the role of NPY released from the SNS and brain noradrenergic neurons. This research is a continuation of the clinical p.L7P studies, where it has been speculated that increased SNS NPY may be responsible for the earlier development of metabolic syndrome-related symptoms. The new NPY overexpressing mouse model to be created should help to determine the differences between the well-known hypothalamic NPY and the less-characterized SNS NPY in controlling energy metabolism, hormone release and cardiovascular function

The specific aims of this study were:

1. To create a noradrenergic neuron-specific NPY overexpressing mouse line.

2. To study how NPY overexpression in SNS and brain noradrenergic neurons affects the metabolic phenotype of the OE-NPY^{DBH} mice.

3. To study how NPY overexpression in SNS and brain noradrenergic neurons affects the autonomic regulation of the cardiovascular system, and how the OE-NPY^{DBH} mice respond to an acute immobilization stress or cold exposure.

4. To study the effect of NPY transgene on the behaviour of the mice assessed with series of behavioural tests.

5. To study the effect of NPY overexpression in SNS on the development of arterial neointima induced by femoral artery angioplasty surgery in mice.

4 MATERIALS AND METHODS

4.1 Animals (I-III)

OE-NPY^{DBH} and their littermate wildtype mice of C57BL/6 strain were used unless stated otherwise. The animals were produced and housed at the local laboratory animal facility (University of Turku). The mice were kept in an animal room maintained at 21±1 ºC with a fixed 12:12 h light-dark cycle. Standard rodent chow (SDS, Essex, UK) and water were available *ad libitum*. Experimental procedures were approved by the local animal ethics committee (The Lab-Animal Care & Use Committee at the University of Turku).

In study I, groups (n = 7-15 per group) of 3- and 6-month-old male and 6-month-old female mice were used unless stated otherwise. Plasma was used from 3-month-old mice, and serum from 6-month-old mice. In study II, groups of 3-month-old male and female mice (n = 7-9 per group) were used. In stress studies, mice were habituated to handling before the experiments. Radiotelemetric catheter was implanted into weight-matched 3-month-old males (n = 5-7 per group). In study III, 3-month-old male OE-NPY^{DBH} mice of both FVB/n and C57BL/6 background (n = 8-9 per group) were used. Wildtype littermates were always used as control mice.

4.1.1 Ethical aspects

All the experiments were planned with care to minimize the number of animals to be used. The procedures were carried out in accordance with the European Communities Council Directive 86/609/EEC. Buprenorfin (Temgesic® Schering-Plough, 0.1 mg/kg i.p. or subcutaneously) was given for analgesia when needed. The mice were anaesthetized before sacrifice with approved anaesthetics medetomidine and ketamine. In study I, also CO_2 anaesthesia before decapitation was used.

4.1.2 Anaesthesia and sample collection

Terminal blood samples were obtained after a four-hour-fast between 10:00 and 14:00 h. For plasma, blood was collected from vena cava with a 23G needle and 1 ml syringe containing 100 µl of heparin (50 U/ml) into a heparin-coated tube. The samples were centrifuged (4000 rpm for 10 min), and plasma was stored at -70°C until analyzed. To access vena cava, the mice were quickly anaesthetized with ketamine (Ketalar® Pfizer, 75 mg/kg i.p.) and medetomidine (Domitor® Orion, 1 mg/kg i.p.). Serum was collected from trunk blood after decapitation under CO_2 anaesthesia into non-heparin-coated tubes, centrifuged, and stored as described above.

4.1.3 OE-NPY^{DBH} mouse model

Total RNA from a mouse hypothalamus was purified by GenElute Mammalian Total RNA kit (Sigma Diagnostics, St. Louis, MO) and synthesized into cDNA (M-MLV RT Rnase kit, Promega, Madison WI). To isolate the 300 basepair (bp) *NPY* coding cDNA, exons 2, 3, and part of exon 4 were amplified by PCR using two primer pairs: forward-1 with reverse-1 and forward-2 with reverse-2 (Table 4.1, Fig. 4.1). A silent mutation was created 120 bp from the beginning of exon 2 to distinguish the transgenic and endogenous NPY. The mutation created a unique NaeI restriction site into the NPY transgene without changing the protein-coding amino acid chain. Mutated nucleotide was included in primers reverse-1 and forward-2 (Table 4.1). Additionally, NsiI restriction sites were created before and after the start and stop codons to facilitate the ligation of NPY cDNA into a cloning plasmid (Table 4.1, Fig. 4.1). In the third PCR run, the complete NPY cDNA was combined by using the NPY cDNA strands obtained from two previous PCR runs as template DNA, and primers forward-1 and reverse-2. Resulting cDNA was purified from 2% agarose gel and cleaved with NsiI. A pIRES-CMVb plasmid, containing a viral 580 bp internal ribosomal entry site (IRES) followed by a 3.7 kbp reporter gene *LacZ* encoding β-galactosidase and a poly-A tail, was linearized with NsiI, and the *NPY* cDNA was ligated upstream of the IRES fragment at NsiI site. The bicistronic NPY-IRES-LacZ construct was excised with NotI and blunted with T4 DNA polymerase (NEB, Ipswich, MA). The construct was then inserted in a cloning vector downstream of the 5.8 kbp promoter of the human *DBH* gene (Mercer et al. 1991), and the final 10.6 kbp transgene construct was excised from the vector with NotI (Fig. 4.1). The construct was sequenced gene by gene to verify the correct alignment of base pairs before it was microinjected into fertilized FVB/n eggs. The oocytes were transferred into pseudopregnant FVB/n females to generate hemizygous offspring.

Table 4.1. PCR primers used in this study.

Primer	Sequence	Location	Purpose
Forward-1	5’-CATGTTATGCATATGCTAGGTAACAAGCGA-3’	Exon 2	Transgene construct
Reverse-1	5’-TCCTCTGCC**G**GCGCGTC-3’	Exon 2	Transgene construct
Forward-2	5’-GACGCGCC**G**GCAGAGGA-3’	Exon 2	Transgene construct
Reverse-2	5’-CAAGTAATGCATTCACCACATGGAAGGGTC-3’	Exon 4	Transgene construct
Forward-3	5’- AACAAACGAATGGGGCTGTGT-3’	Exon 2	Genotyping
Reverse-3	5’- GTGATGAGATTGATGTAGTGTCG-3’	Exon 2	Genotyping

NsiI restriction site is underlined in forward-1 and reverse-2 primers. NaeI site is underlined in reverse-1 and forward-2 primers. Mutated nucleotide is in **bolded** in reverse-1 and forward-2 primers (guanine is adenosine in the endogenous sequence).

Eight transgene DNA-positive founder mice (F1) were born and bred with FVB/n mice to produce F2 hemizygous transgenic animals. Based on primary phenotyping studies, line number 32 was chosen for further studies and backcrossed to C57BL/6 for six generations to produce a line of mice with a predominantly C57BL/6 genetic background.

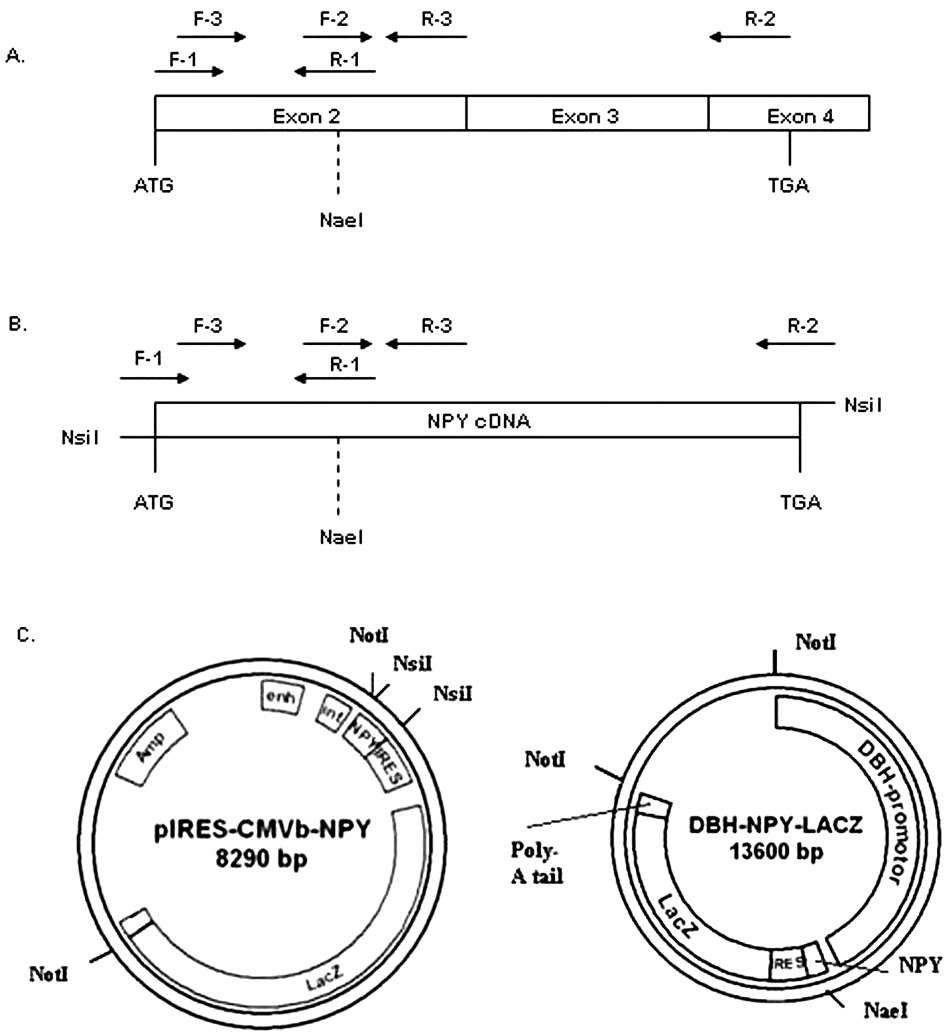

Figure 4.1 The transgene construct. (A) A schematic drawing of the NPY cDNA with exons 2, 3 and 4, and the locations of the three primer pairs used in the study. The genetic start (ATG) and stop (TGA) codons are also depicted. The exons are not proportionally correct. (B) The final NPY cDNA to be inserted into the pIRES-CMVb plasmid showing NsiI recognition sites created with PCR at both ends. (C) Plasmid on the left shows NPY cDNA inserted at the NsiI cloning site in pIRES-CMVb vector. The NPY-IRES-LacZ construct was removed with NotI. The construct was then ligated into the cloning vector on the right containing human DBH promoter. Finally, the whole transgene construct was excised with NotI.

4.1.4 Genotyping of OE-NPY^{DBH} mice

Genomic DNA was isolated from tail biopsies from weaned pups with a commercial kit (Gentra, Minneapolis, MN). Genotypes were determined by PCR using forward-3 and reverse-3 primers located in exon 2 of the *NPY* gene (Table 4.1). The PCR products were digested with NaeI and run on 2% agarose gel. Wildtype DNA showed a single 190 bp band and transgenic DNA 190, 120 and 70 bp bands.

4.2 Transgene expression analyses

4.2.1 Bacterial β-galactosidase expression analysis (I, III)

Mice of both genotypes were killed by decapitation (I) or by blood removal under anaesthesia (III). Brains, adrenal glands and sympathetic ganglia (superior cervical ganglia and superior mesenteric ganglia) were removed, quickly frozen in liquid nitrogen, and stored at -70°C. Coronary brain (80 µm), adrenal (35 µm) and ganglia (20 µm) frozen sections were mounted on gelatin-coated slides and stained for *E.* coli β-galactosidase as previously described (Harvey et al. 2005). The slides were fixed for 10 minutes in fixing solution containig 2% formalin and 0.2% glutaraldehyde in 0.1 M sodiumphosphate buffer (pH 7.3), 5 mM EGTA, and 2 mM $MgCl_2$. The slides were washed 3 times in rinsing buffer (0.1 M sodiumphosphate buffer pH 7.3, 0.1% sodiumdeoxycholate, 0.2% Nonidet P40, and 2 mM $MgCl_2$) after which they were stained in a solution containing 4% Xgal (v/v, stock 40 mg/ml X-gal in dimethyl sulfoxide), 0.165% potassium ferricyanide (w/v) and 0.211% potassium ferrocyanide (w/v) in rinsing buffer at 30°C overnight. The slides were washed 2-3 times in phosphate-buffered saline (PBS), counterstained with Nuclear Fast Red (sympathetic ganglia), dried, coverslipped, and analyzed under a light microscope.

4.2.2 NPY immunohistochemistry (I)

Mice of both genotypes were killed by decapitation. Brains and adrenal glands were removed and immersed in 4% paraformaldehyde fixative overnight and cryoprotected with 30% sucrose o/n before freezing. Frozen sections of coronary brain and adrenal glands (35 µm thick) were cut with a cryostat and placed in PBS with 0.01% Triton-X. Free-floating sections were washed in PBS, and preincubated with blocking buffer (5% goat normal serum, 0.01% Triton-X in PBS) for 30 minutes at room temperature. The sections were incubated o/n at 4°C with rabbit polyclonal anti-NPY antibody (Affiniti Research Products Ltd., Exeter, UK) diluted 1:1500, followed by incubation with biotinylated goat anti-rabbit antibody (1:3000) (Molecular Probes Inc., Eugene, OR) for 1 hour at room temperature. Standard Vectastain Elite ABC-kit (Vector Laboratories, Burlingame, CA) was used in conjunction with the biotinylated antibody, using light and subsequent horseradish peroxidase activity was developed using 3,3'-diaminobenzidene as a substrate (Sigma Fast DAB tablets, Sigma Diagnostics). The immunostaining was visualized microscopy.

4.3 Experimental protocols

4.3.1 Body weights and food consumption (I)

Body weights were recorded before sacrificing the animals. Additionally, a set of adult males and females were separated into single cages and allowed to adjust to the change for one week. The mice were weighed weekly for 3-8 weeks. Food was freely available, but carefully weighed every two days to calculate the average consumption and spillage of chow per day per mouse.

4.3.2 Locomotor activity (I)

Five-month-old male mice (n = 8-11 per group) were placed individually into transparent polypropylene locomotor activity cages housed in a photo-beam recording system (San Diego Instruments, San Diego, CA). Fine movements, ambulations and rearings were recorded over 10 minute intervals for 24 h. The measurements started at 12:00 h after a 24 h habituation.

4.3.3 Core body temperature (I, II)

Rectal body temperatures were measured with a digital thermometer (Ellab, Roedovre, Denmark) from 3-month-old male and female mice (n = 7-10 per group) between 10:00 and 12:00 h.

4.3.4 Trabecular BMD (I)

The nose-anal and femur lengths of male mice (n = 6 per group) were measured. After sacrifice, the femurs were collected and immediately frozen in liquid nitrogen and stored at -70°C until imaged. Micro-CT (Skyscan 1072 MicroCT, Skyscan n.v. Aartselaar, Belgium) was used to image the femurs. The Micro-CT system was calibrated by scanning two hydroxyapatite phantoms with known densities of 250 and 750 mg/cm^3. For each specimen a volume of interest that contained trabecular bone and bone marrow was defined in the stack of the images. The trabecular bone (high brightness voxels) was separated from the bone marrow (low brightness voxels) lying inside the volume of interest by using a global thresholding technique. Thereafter a histogram of grayscale values was calculated for all the voxels representing cancellous bone. The grayscale values of the voxels were converted to BMD using the relation established by calibration. Two types of baseline corrections were used (Lorenz and Boltzmann). Each type of baseline correction was followed by a Gaussian fit, which resulted in two different Gaussian curves that were analyzed separately. Position of the peak in a Gaussian curve was considered to represent the mean BMD for the sample. Thus, three different BMD values in total were obtained for each specimen and finally an average BMD value was calculated from these three values.

4.3.5 White adipose tissue weight and fat cell size (I)

WAT weight was determined by collecting the subcutaneous, epididymal/gonadal, and retroperitoneal fat pads at sacrifice. The fat cell sizes were determined from a total of eight gonadal fat pads from 3-month-old female mice as previously described (Di Girolamo et al. 1971). Briefly, WAT was collected into tubes containing Krebs-Ringer HEPES medium supplemented with defatted BSA (40 mg/ml), L-glutamine (300 µl) and collagenaseA (10 mg per one gram WAT). The tissue was incubated for 50-60 minutes at 37°C with vigorous shaking. Cells were immediately imaged under a light microscope and diameters were calculated using Cell*A imaging software (Soft Imaging System GmbH, Münster, Germany).

4.3.6 Liver and skeletal muscle morphology and enzyme histochemistry (I)

The left lateral lobe and *m*. gastrocnemius from 3- and 6-month old male mice were dissected, placed in liquid nitrogen and stored at -70°C. Frozen sections (5 µm) were cut, and a pilot series of samples were stained for standard histological stains including hematoxylin and eosin (H&E), Van Gieson, oil-red-O, sudan black (liver) and H&E, COX-SDH, ATP 3.4, ATP 10.4 and NADH (muscle). Based on the results from the pilot study, the study samples were stained for H&E, oil-red-O, sudan black (liver), and H&E and ATP 3.4 (muscle). The slides were analyzed under a light microscope.

4.3.7 Intraperitoneal glucose tolerance test (IGTT) (I)

Mice were fasted from 6:00 h to 10:00 h and administered intraperitoneally with glucose (5 % w/v, 1 g/kg body weight). Tail vein blood glucose was measured at 0, 20, 40, 60 and 90 minutes with Precision Xtra™ Glucose Monitoring Device (Abbott Diabetes Care, IL). Areas under the resultant glucose curves (AUC) were calculated with the trapezoidal method in GraphPad Prism 4.3 software.

4.3.8 Implantation of telemetric device and data acquisition (II)

Male mice (n = 5-7 per group) weighing 31.9 ± 1.4 g (wildtype) and 31.2 ± 1.3 g (OE-NPY^{DBH}) were used in the study. Mouse blood pressure transmitters (TA11PA-C10, Data Sciences International, St. Paul, MN) were used to directly measure arterial pressure and heart rate. The mice were anaesthetized during the induction phase with 4-4.5 % isoflurane and maintained on 2-2.5 % isoflurane. The mice were kept on a heated table throughout the surgery to maintain body temperature, and they received one dose (0.1 mg/kg subcutaneously) of Temgesic® (Schering-Plough) before the surgery to alleviate the pain. The mice were shaved, and a ventral midline incision was made to access the left carotid artery. The catheter was inserted into the artery and the transmitter probe was positioned subcutaneously on the right flank of the animal. The catheter was sealed in place and the incision was closed with sutures. During recovery from anaesthesia and surgery, the mice were placed on a heating pad. Buprenorfin was given for postoperative analgesia twice daily for three days including the day of the

operation. Using the acquisition program (Dataquest A.R.T. Version 3.1, Data Sciences International, St. Paul, MN), the data were recorded every five minutes for ten seconds with a sampling rate of 1000 Hz. Hemodynamic changes during recovery period (days 3-7 after surgery) were monitored. Baseline measurements were started at day ten from surgery and continued for five consecutive days.

4.3.9 Immobilization stress test (II)

The mice were brought into the experimental room at 6:00 h. Water but no food was freely available. The experiment was performed between 9:00 h and 14:00 h. Prior to the test, the mouse was immobilized for 1 hour by placing it inside a plastic bag with several airholes. The bag was taped to the table so that the mouse was prevented from moving freely. After an hour, the mouse was anaesthetized, the blood (terminal sample) was immediately drawn and plasma was separated by centrifuging the samples. Adrenal glands were removed and processed as described in biochemical analyses (NPY protein concentrations in tissues and plasma). The animals for baseline measurements were all anaesthetized avoiding as much stress as possible when handling the mice.

4.3.10 Cold exposure test (II)

Mice were brought to the experimental room the day before and fasted from 8:00 h on the day of the experiment. Water was freely available during the whole experiment. Rectal body temperatures were measured with a digital thermometer before placing the mice in 4°C for 2 h after which the rectal body temperature was immediately measured again. The animals were quickly anasthetized, the blood was removed (terminal sample) and plasma was separated by centrifuging the samples. Control mice were kept at room temperature, the body temperatures were measured, and the mice were sacrificed as described above.

4.3.11 Behavioural tests (II)

The OE-NPY^{DBH} and wildtype control mice could not be distinguished by their appearance. The tester was blind to the genotype in all experiments.

4.3.11.1 Primary behavioural screen (SHIRPA)

Male mice (n = 10 per group) of ten weeks of age were used in this test. The complete test battery can be found in an article by D.C. Rogers (Rogers et al. 1997) and this study was performed according to the modified description published earlier (Lahdesmaki et al. 2002). The animals were brought to the experimental room at 6:00 h, and the experiment took place between 10:00 h and 14:00 h. Each animal was first placed in a transparent cylindrical viewing jar, and the behaviour was recorded for five minutes (5 observations). The mouse was then transferred to an arena to observe its

immediate reactions to a new environment (8 observations). Following the arena the animal was subjected to a series of manipulations with tail suspension and using a grid across the width of the arena (10 observations). Finally, the animal was restrained in a supine position using tail-neck grip to record autonomic behaviours (5 observations). After this, the body temperature was measured using a rectal probe and a digital thermometer. Throughout the whole test vocalization, urination, irritability, and aggression, if any, were recorded.

4.3.11.2 Elevated plus-maze test

Elevated plus-maze test is a method for assessment of anxiety-like behaviour in rodents (Lister 1987), and the animals cannot be habituated to earlier handling. The complete description of the test can be found elsewhere (Lahdesmaki et al. 2002). Twelve OE-NPY^{DBH} and ten wildtype male mice of 18-22 weeks of age were used in this test. The apparatus was comprised of two open arms facing each other and two closed arms, and the entire device was elevated to a height of 50 cm above the floor. The mice were placed individually in the center of the maze facing the open arm, and the behaviour was recorded for 5 minutes. The number of entries into each arm, the time spent in different parts of the maze, and the latency to enter an open arm was measured. An entry into an arm was defined as a mouse having entered the arm with all four legs.

4.3.11.3 Open field test

Immediately after the elevated plus-maze test, the mouse was transferred to the open field arena (55 x 33 cm). The arena was the same one as used in the SHIRPA protocol. The animal was observed for 5 minutes, and the number of crossed squares (measure of locomotor activity), and the number of rearings were counted.

4.3.11.4 Light-dark exploration test

Light-dark exploration test is also an anxiety-like behaviour screening test for laboratory rodents (Costall et al. 1989). The experiment was performed seven days after the elevated plus-maze test and open field tests on the same group of mice. The device consisted of two plexiglass boxes (30 x 30 x 30 cm), one white and brightly illuminated and the other black and covered by a lid. In between, there was a grey "semi-dark" box (10 x 30 x 30 cm). The test was performed according to methods already published (Voikar et al. 2001). The mice were individually placed in the middle of the brightly-lit box facing the opening to the darker compartment. The number of entries into each box and the latency to re-enter a light compartment, and the time spent in different compartments were measured over five minutes.

4.3.12 Femoral artery angioplasty surgery (III)

The angioplasty surgery was performed as described earlier (Sata et al. 2000). Under ketamine-medetomidine anaesthesia, the right femoral artery was exposed by a dissection, and the accompanying femoral nerve was separated. The femoral artery and vein were ligated proximally and distally with 6-0 silk suture to temporarily prevent blood flow. A small artery branch was isolated in a similar manner, and dilated with a few drops of 1% lidocaine hydrochloride (Xylocard® AstraZeneca). A transverse arteriotomy was performed in the side branch, and a straight spring wire (0.38 mm in diameter, Cat. no. C-SF-15-15, Cook, Bloomington, IN) was carefully inserted through the arterioctomy into the femoral artery. The wire was retracted inside the artery for three times and then left in place for one minute to dilate the artery. On catheter removal, the branch artery was secured by a ligation with 6-0 silk suture, and the blood flow in femoral artery and vein was restored by releasing the proximal and distal ligations. The skin incision was closed with 6-0 absorbable suture, and the animal was given atipamezole (Antisedan® Orion, 1 mg/kg i.p.) to attain faster recovery from anaesthesia.

4.3.12.1 Morphometric analyses

The mice were sacrificed exactly four weeks from surgery. Blood was removed, and the operated femoral artery was carefully excised, perfused with 0.9% saline, and fixed in phosphate-buffered formalin overnight at 4°C. Unoperated control vessels were obtained from the left femoral arteries at the same level as the injured arteries. Vessels were placed in 70% EtOH and stored at 4°C until embedded in paraffin. Cross-sections of 5 μm were stained with H&E and Masson's trichrome, and imaged with an Olympus DP70 digital camera (Olympus America Inc., Center Valley, PA) on a Leica DMR microscope (Leica Microsystems GmbH, Wetzlar, Germany). Neointimal hyperplasia was measured from the luminal side of the internal elastic lamina, and the media as an area between the external and the internal lamina. The areas were calculated by using a Cell*A imaging software (Soft Imaging System GmbH, Münster, Germany). The results were averaged from 3-6 arterial sections per mouse.

4.3.12.2 NPY immunohistochemistry

Paraffin-embedded sections of 5 μm were deparaffinized and blocked with a blocking solution (methanol + 2% H_2O_2). The sections were washed and blocked with a 5% goat normal serum. Arteries from FVB/n mice were stained for NPY as described above (NPY immunohistochemistry). Slides were analyzed under the light microscope, and imaged with the digital camera. Staining intensity and NPY positive particles per media-intima area were calculated with ImageJ 1.33 software (NIH image downloaded from http://rsb.info.nih.gov/ij/download.html) by using a global thresholding technique. Slides from three mice per group were stained, and the results were averaged from 2-3 arterial sections per mouse.

4.4 Biochemical analyses

4.4.1 NPY protein concentrations in tissues and plasma (I, II)

Blood was collected with vena cava puncture under ketamine-medetomidine anaesthesia. Brain and both adrenal glands were removed, and washed with cold saline. Medial basal hypothalamus was isolated with a mouse brain block using a 2 mm section caudal to the optic nerve chiasma and excluding hippocampal and cortical areas. The brainstem section extended 3 mm caudal from the hypothalamic section including LC and A5 noradrenergic nuclei but cerebellum and cerebral cortex were discarded. Tissues were homogenized with 500 µl of 0.1 N HCl and placed immediately on ice. The tissue homogenates were sonicated, and NPY concentrations were determined with EURIA-NPY radioimmunoassay (RIA) kit (Euro-Diagnostica, Malmö, Sweden) with some modifications to the kit's instructions: tissues were diluted with 0.1 M Na_2HPO_4, buffer pH 8.3, and the standards with the manufacturer's diluent. Plasma NPY concentrations were determined with the RIA kit by diluting plasma 1:4 to the assay buffer with 5% bovine serum albumin. The radioactivity of the antibody-bound ^{125}I-NPY was counted in a gamma counter (1282 CompuGamma Gamma Counter, Wallac Oy, Turku, Finland) for 2 min/sample and the NPY concentrations in samples and controls were calculated from the standard curve. The detection limit for EURIA-NPY was 16 pmol/l. Tissue NPY concentrations were adjusted to the protein levels determined with BCA Protein Assay Reagent kit (Pierce Biotechnology Inc., Milwaukee, WI) using phosphate buffer as a diluent: adrenal glands 1:5, brain stem 1:20 and hypothalamus 1:10. The absorbance was measured at 562 nm (Victor^2 1420 Multilabel Counter, Wallac Oy, Turku, Finland), and the unknown protein levels were calculated from the standard curve.

4.4.2 Liver and blood lipids (I)

Liver lipid contents were isolated and purified with the Folch method (Folch et al. 1957) from 3- and 6-month-old male mice. Chloroform:methanol 2:1 (v/v) was added to frozen tissues using 20 times the volume of the tissue, and the tissues were homogenized. Homogenates were lightly shaken for two hours and then centrifuged (3400 rpm, 5 min). Supernatants were removed into clean tubes, the volumes measured. 0.9% NaCl equivalent to one fifth of the supernatant volume was added before vortexing. Another centrifugation step followed (2000 rpm, 5 min), after which a biphasic system was obtained. The lower chloroform phase containing the lipids was removed into a clean tube and chloroform was evaporated under vacuum. The dried lipid pellets were re-suspended in distilled water, and the triglyceride contents from plasma, serum and tissue samples were quantified with Free Glycerol Reagent (F6428) and Triglyceride reagent (T2449, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Serum total cholesterol from 6-month-old females was measured with the BioVision Cholesterol Quantitation kit (Mountain View, CA).

4.4.3 Circulating hormones (insulin, corticosterone, ghrelin, leptin and resistin) (I, II)

Circulating concentrations of insulin (ELISA kit; Mercodia AB, Uppsala, Sweden), corticosterone (RIA kit; MP Biomedicals, LLC, Orangeburg, NY), ghrelin (RIA kit; Linco Research, St. Charles, MO), leptin and resistin (LINCO*plex* Adipokines kit; Linco Research) were measured according to the manufacturers' instructions.

4.4.4 Catecholamines in plasma and adrenal glands (II)

Plasma was centrifuged, immediately frozen with liquid nitrogen, and stored at -70°C until analyzed. Adrenal glands were removed and washed with cold saline. The fat pad was carefully removed under a dissecting microscope. One adrenal gland per mouse was placed in a tube containing 500 µl of 0.4 mM perchloric acid, in which the tissue was quickly frozen with liquid nitrogen and stored at -70°C. Tissues were sonicated, centrifuged and catecholamine contents (NA and adrenaline) were determined by HPLC with electrochemical detection as described earlier (Grouzmann et al. 1994; Cavadas et al. 2006). Plasma catecholamine measurements were also performed by HPLC with electrochemical detection with some modifications to methods published previously (Grouzmann et al. 2001). Plasma (210 µl) with dihydroxybenzylamine (Sigma Chemical Co., St Louis, MO) as an internal standard was extracted on an activated alumina at pH 8.6. The alumina was allowed to settle and the supernatant was aspirated followed by three washes with water. The catecholamines were then eluted with 120 µl of a mixture of 0.2 M acetic acid and 0.04 M phosphoric acid (v/v). A volume of 100 µl was injected into the chromatography system. The separation was achieved on a reversed-phase column Supershere 100RP-18 4 mm (Merck–Chemdat, Frankfurt, Germany) by using a 50 mM sodium acetate buffer mobile phase containing 50 mM sodium phosphate, 0.4 mM EDTA, 0.5 mM sodium octyl sulfonate (as an ion-pairing agent) and 12% (v/v) acetonitrile (pH 3.2) at a flow rate of 1.0 ml/min. The electrochemical detector (ESA Coulochem II) settings were +0.6 V for the conditioning cell, +0.3 V for the analytical cell's detector one and –0.3 V for detector two. The following order of elution was observed: NA, adrenaline and dihydroxybenzylamine. The recovery was 80% and the quantification limit was 0.25 pmol per injection.

4.4.5 [^{3}H]GDP binding to brown fat mitochondria (II)

Binding of [^{3}H]GDP to BAT mitochondria was measured according to the method described earlier (Nicholls 1976; Maffei et al. 1995; Savontaus et al. 1997). The scapular brown adipose fat pads were removed and placed in 250 mM ice-cold sucrose buffer. The weights of the pads were recorded before homogenizing the samples with a Potter S Homogenizer (Braun, Melsungen, Germany). The homogenate was used for immediate preparation of mitochondria with differential centrifugation. [^{3}H]GDP binding was determineed by incubating the mitochondria in a medium containing 100 mM sucrose, 20 mM TES, 1 mM EDTA, 10 mM choline chloride, 2 M rotenone, 0.125 mCi/l [^{14}C]sucrose, and 0.53 Ci/mmol of 10 µM [^{3}H]GDP at room temperature for 10

min. Nonspecific binding was assessed in the presence of excess unlabelled GDP (1 mM). After incubation, the reaction was terminated by filtering the mixture through glass fiber filters (Thomas Scientific, Swedesboro, NJ) with a Brandel Cell Harvester (Biomedical Research Devices, Gaithersburg, MD). The GDP binding from duplicate samples was assessed by measuring the radioactivity with Optic Phase 'High Safe' II scintillation cocktail (FSA Lab. Supplies, Loughborough, UK) and the liquid scintillation counter (1219 RackBeta Spectral, Wallac Oy, Turku, Finland). The amount of bound [^{3}H]GDP, corrected by the amount of [^{14}C]sucrose, represented the GDP binding. The protein content of the mitochondrial suspensions was determined with a BCA Protein Assay Reagent kit (Pierce Biotechnology Inc., Milwaukee, WI) according to manufacturer's instructions. The results are expressed as binding of GDP per milligram mitochondrial protein.

4.5 Statistical methods (I-III)

Statistical analyses were conducted using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA) and SAS Enterprise Guide 3.0 program (SAS Institute Inc, USA). Student's t-tests (parametric data) or Mann-Whitney's U-tests (non-parametric data) were used to compare groups of OE-NPY^{DBH} and wildtype mice. Genotype and stress or strain effects were analyzed using two-way analysis of variance (ANOVA) (studies II, III). The Bonferroni post hoc test was performed if ANOVA reached statistical significance. IGTT, locomotor activity and hemodynamic parameters (mean arterial pressure and heart rate) were analyzed with repeated measures two-way ANOVA (studies I, II). Linear regression analysis was used to compare body weight or WAT weight with other variables (AUC in IGTT, fasting insulin and leptin) and glucose (AUC) with ghrelin (study I). The categorical variables in the primary behavioural screen were analyzed using Pearson's χ^2 test (study II). A statistical significance was reached at $P < 0.05$ in all analyses.

5 RESULTS

5.1 Generation of the OE-NPY^{DBH} mouse model (I)

NPY and LacZ overexpressing mice under DBH promoter were generated resulting in eight founders from which two lines of transgenic mice were established based on the β-galactosidase staining results (line numbers 32 and 42). In later phenotyping experiments, only modest changes were observed in line 42, and it was abandoned. Genotype distributions in line 32 matched expected Mendelian ratios. NPY overexpression did not result in embryonic lethality, and the mice produce pups normally. Transgene expression was visualized with β-galactosidase analysis. It showed strong and specific LacZ staining in adrenal medulla, LC nuclei in the brainstem and sympathetic ganglia (Fig. 5.1), which are areas known to contain noradrenergic cell bodies. Staining was faint in the hypothalamus, the cortex, the medial habenular nuclei and the paraventricular thalamic nucleus. Blue colour was observed only in the OE-NPY^{DBH} mice and no ectopic staining was observed in any of the sections. Examination of coronal brain sections and adrenal glands using immunohistochemistry with a specific NPY antibody revealed that in the OE-NPYDBH mice, NPY was detected in the same areas as those observed in wildtype littermates. These areas included adrenal medulla, ARC, VMH, DMH, PVN, LHA, the hippocampus, the amygdala, the thalamus, and LC and A5 neurons of the brainstem. No ectopic NPY expression was observed in transgenic animals.

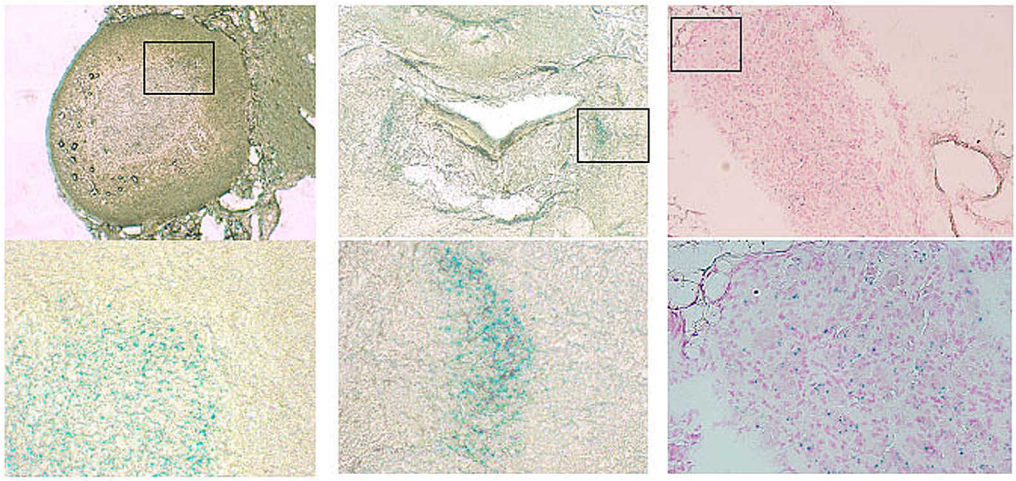

Figure 5.1. Expression of *DBH-LacZ* transgene in adrenal gland, brainstem and sympathetic superior mesenteric ganglion. The adrenal gland with 4x (top) and 20x (bottom) magnifications (left), the brainstem and the locus coeruleus nuclei around the 4th ventricle (middle) and the superior mesenteric ganglion with respective magnifications (right). The 20x area is cropped from 4x image as outlined.

5.2 NPY protein concentrations in tissues and plasma (I)

NPY concentrations in adrenal, brainstem and hypothalamus homogenates and plasma were measured with RIA. At baseline, the OE-NPY^{DBH} mice had a significantly higher NPY concentration in the adrenals and in the brainstem compared with wildtype mice (genotype: $P < 0.05$ and $P < 0.01$, respectively, sex: P = NS, genotype x sex interaction: P = NS, Fig. 5.2). NPY concentration in the hypothalamus did not differ between the genotypes, but was increased in females in comparison with males (genotype: P = NS, sex: $P < 0.01$, genotype x sex interaction: P = NS, Fig. 5.2). NPY concentration in plasma did not differ between the genotypes. However, NPY levels were significantly higher in male than in female mice. (genotype: P = NS, sex: $P < 0.001$, genotype x sex interaction: P = NS, Fig. 5.2).

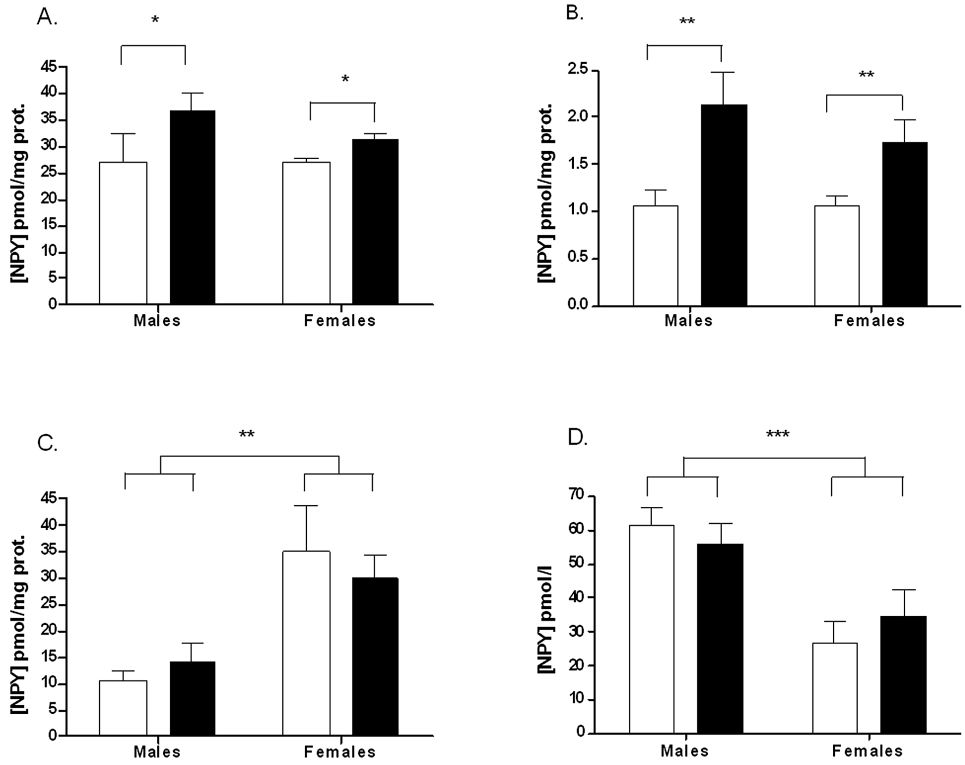

Figure 5.2. Tissue and plasma NPY levels. NPY concentrations in adrenal glands (A), brainstem (B) hypothalamus (C) and plasma (D) measured with RIA. Data is presented as mean ± SEM (n = 8 per group). White bar: wildtype mice; black bar: OE-NPY^{DBH} mice; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ by two-way ANOVA. Figure is from original communication I.

5.3 The effect of transgene on body composition, endocrinological parameters and lipid metabolism

5.3.1 Body composition, food intake and BMD (I)

Body weights were recorded from male and female mice at 3 and 6 months of age, and no difference between the genotypes was observed (Fig. 5.3). During a weekly follow-up, the mice retained the body weights steadily, and no difference in the amount of food consumed between the adult wildtype and transgenic mice was observed (Table 5.1). The mice also displayed similar body and femur lengths at 3 months (Table 5.1). BMD did not differ between the genotypes (Table 5.1). Basic morphology of the skeletal muscle was similar between genotypes (data not shown).

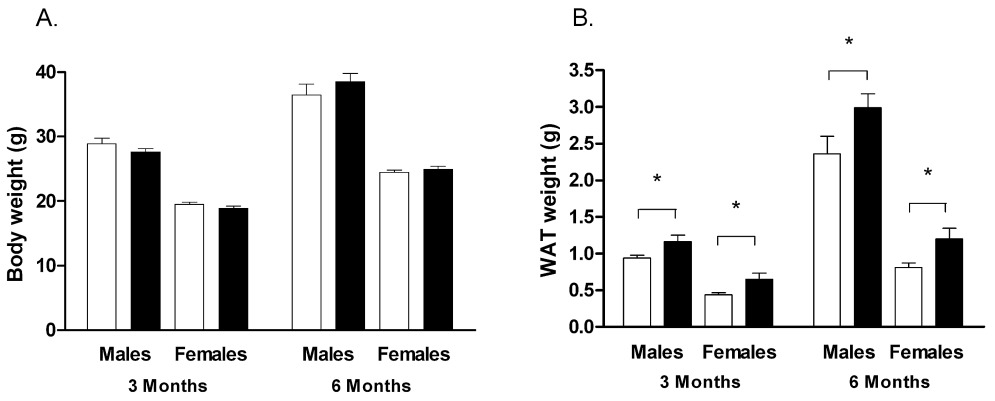

Figure 5.3. Body and white adipose tissue (WAT) mass at 3 and 6 months of age. Data is presented as mean ± SEM (n = 8-15). White bar: wildtype mice; black bar: OE-NPY^{DBH} mice. * = $P < 0.05$; ** = $P < 0.01$ by Student's t-test. Figure is modified from original communication I.

Table 5.1. Baseline body composition and SNS activity parameters in wildtype and OE-NPY^{DBH} mice at three months of age.

Measured parameter	Wildtype Males	OE-NPY^{DBH} Males	Wildtype Females	OE-NPY^{DBH} Females
Body temperature (°C)	37.16 ± 0.29	37.07 ± 0.46	37.47 ± 0.31	36.84 ± 0.23
Body length (cm)	9.51 ± 0.14	9.48 ± 0.15	9.13 ± 0.08	9.34 ± 0.11
Femur length (cm)	1.46 ± 0.04	1.46 ± 0.04	ND	ND
BMD (mg/cm^3)	1119 ± 13.7	1105 ± 27.5	ND	ND
Food consumption (g/day)	4.12 ± 0.27	4.19 ± 0.30	3.59 ± 0.53	3.64 ± 0.48
Mean fat cell diameter (μm)	ND	ND	40.34 ± 0.6	35.71 ± 0.4 *
BAT $^{[3]}$GDP binding (pmol/mg prot.)	32.6 ± 3.6	44.6 ± 2.9 *	43.0 ± 3.1	48.3 ± 2.2
Plasma noradrenaline (nmol/l)	1.38 ± 0.07	1.64 ± 0.12	ND	ND
Adrenaline (nmol/l)	0.78 ± 0.05	1.07 ± 0.07 **	ND	ND
Adrenal gland NA (nmol/μg prot.)	0.66 ± 0.11	0.35 ± 0.09	ND	ND
Adrenal gland adrenaline (nmol/μg prot.)	0.92 ± 0.13	0.47 ± 0.10 *	ND	ND

Values are presented as means ± SEM (n = 7-9 per group). ND = not determined. * = $P < 0.05$; ** = $P < 0.01$ compared with wildtype controls by Student's t-test or Mann-Whitney test.

5.3.2 Spontaneous locomotor activity and body temperature (I)

No differences in overall activity or number of rearings between the genotypes were observed. Also, no difference in rectal body temperature between wildtype and OE-NPY^{DBH} mice was observed (Table 5.1).

5.3.3 The effect of transgene on catecholamine levels (II)

As compared with the wildtype littermates, the OE-NPY^{DBH} mice had significantly higher levels of adrenaline in plasma (Table 5.1). A trend to increased NA levels was also observed (Table 5.1). In adrenal glands, the OE-NPY^{DBH} mice had significantly decreased levels of adrenaline in comparison with wildtype mice (Table 5.1), and the trend ($P = 0.06$) recorded for NA levels was similar (Table 5.1).

5.3.4 Body adiposity (I)

The OE-NPY^{DBH} mice had significantly more WAT relative to controls at 3 and 6 months of age (Fig. 5.3). The mean fat cell diameter was significantly smaller in the OE-NPY^{DBH} fat pads in comparison with wildtype controls (Table 5.1). The liver triglyceride levels in the OE-NPY^{DBH} mice were increased already at 3 months (Fig. 5.4), but the livers were not enlarged and did not show severe fatty liver morphology. No difference in circulating triglyceride and cholesterol between wildtype and OE-NPY^{DBH} mice was observed (Table 5.2).

5.3.5 Circulating leptin, resistin, and ghrelin (I)

Leptin levels did not differ between the genotypes, although a tendency ($P = 0.06$) to increased leptin in OE-NPY^{DBH} mice in comparison with wildtype controls was seen in 6-month-old males (Table 5.2). There was no difference in resistin levels between wildtype and OE-NPY^{DBH} mice (Table 5.2). Total ghrelin levels were significantly increased in the OE-NPY^{DBH} mice at 6 months over wildtype values (Table 5.2). In females, we did not observe any differences in circulating hormone levels (Table 5.2).

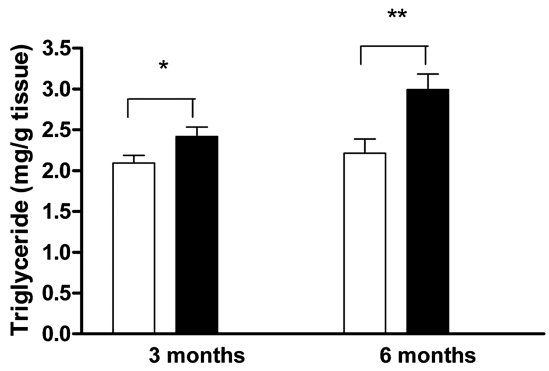

Figure 5.4. Liver triglycerides in 3- and 6-month-old males. Data is presented as mean ± SEM (n = 7-12 per group). White bar: wildtype mice; black bar: OE-NPY^{DBH} mice; * = $P < 0.05$; ** = $P < 0.01$ with Student's t-test.

Table 5.2. Endocrinological parameters in wildtype and OE-NPY^{DBH} mice.

	3-month-old males		6-month-old males		6-month-old females	
Measured parameter	**Wildtype**	**OE-NPY^{DBH}**	**Wildtype**	**OE-NPY^{DBH}**	**Wildtype**	**OE-NPY^{DBH}**
Triglyceride (mg/ml)	0.46 ± 0.06	0.47 ± 0.03	0.91 ±0.09	1.05 ± 0.07	ND	ND
Cholesterol (μg/μl)	ND	ND	ND	ND	1.59 ± 0.14	1.76 ± 0.08
Fasting insulin (μg/l)	< 0.025†	< 0.025†	1.60 ± 0.4	3.15 ± 0.6*	0.30 ± 0.02	0.43 ± 0.07
Leptin (pg/ml)	645.7 ± 126	967.7 ± 159	5596 ±935	8122 ± 897	1371 ± 211	1809 ± 312
Resistin (pg/ml)	1012 ± 130	722 ± 84	2163 ±133	2186 ± 194	3462 ± 252	3943 ± 191
Corticosterone (ng/ml)	30.3 ± 4.2	42.3 ± 8.8	98.8 ± 2.9	78.4 ± 14.9	136.9 ± 6.3††	135.6 ± 19††
Total ghrelin (pg/ml)	ND	ND	271.2 ± 82	368.8 ± 58*	ND	ND

Values are presented as means ± SEM (n = 7-15 per group). Plasma (at three months) and serum (at six months) were used except for insulin where plasma was used at all times. † = The detection limit in Mercodia Ultrasensitive Mouse Insulin ELISA kit was 0.025 μg/l. †† = Measured from 3-month-old females. ND = not determined. * = $P < 0.05$ with Student's t-test or Mann-Whitney test. Table is modified from original communication I.

5.4 The effect of transgene on glucose metabolism (I)

At 3 months, the glucose tolerance was normal in OE-NPY^{DBH} mice (Fig. 5.5). Six-month-old male but not female OE-NPY^{DBH} mice showed impaired glucose tolerance, although fasting glucose remained normal (Fig. 5.5). In 6-month-old transgenic mice, the AUC in IGTT was significantly elevated compared with wildtype mice (1552.0 ± 117.3 vs 1129.0 ± 53.1 mmol/l; $P < 0.01$), and it correlated positively with body weights (wildtype: r = 0.04, P = NS; OE-NPY^{DBH}: r = 0.64, $P < 0.05$). A strong correlation between glucose AUC and ghrelin was also observed in both genotypes (wildtype: r = 0.57, $P < 0.05$; OE-NPY^{DBH}: r = 0.63, $P < 0.05$).

Six-month-old males had increased plasma insulin in comparison with wildtype males (Table 5.2), and the insulin values correlated positively with body weights in both genotypes (wildtype: r = 0.60, $P < 0.05$; OE-NPY^{DBH}: r = 0.86, $P < 0.001$). Insulin levels of 3-month-old males remained under the insulin kit's detection level in both genotypes, and 6-month-old females showed no difference between genotypes in insulin concentration (Table 5.2).

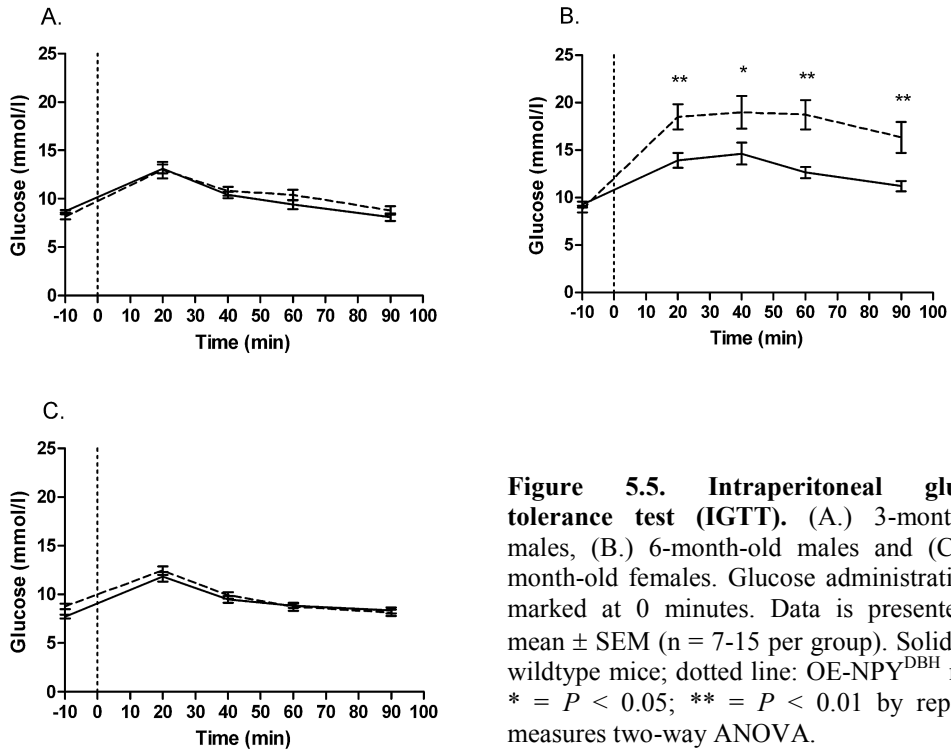

Figure 5.5. Intraperitoneal glucose tolerance test (IGTT). (A.) 3-month-old males, (B.) 6-month-old males and (C.) 6-month-old females. Glucose administration is marked at 0 minutes. Data is presented as mean ± SEM (n = 7-15 per group). Solid line: wildtype mice; dotted line: OE-NPY^{DBH} mice. * = $P < 0.05$; ** = $P < 0.01$ by repeated measures two-way ANOVA.

5.5 The effect of transgene on blood pressure (II)

Radiotelemetric transmitters were implanted in male mice at three months of age. In both transgenic and wildtype mice, the mean arterial pressure showed very regular diurnal changes with lower levels during the day and higher during the night (Fig. 5.6). During the recovery period from the implantation surgery, the OE-NPY^{DBH} mice showed significantly increased mean arterial pressure during nighttime in comparison with wildtype littermates ($P < 0.05$, Fig. 5.6). At baseline, no difference was observed in blood pressure between the genotypes (Fig. 5.6). No difference in heart rate was observed at any time (Fig. 5.6).

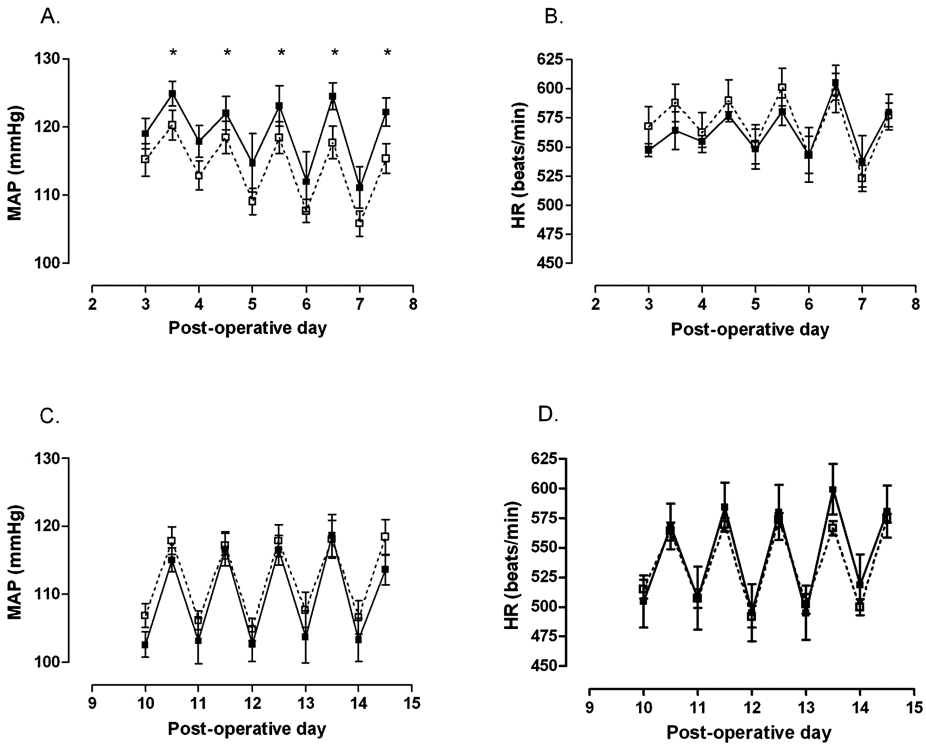

Figure 5.6. Mean arterial pressure (MAP) and heart rate (HR) measurements with radiotelemetry in conscious male wildtype and OE-NPY^{DBH} mice. Data was first recorded during recovery from surgery at days 3-7 (A, B), and baseline recordings were performed at days 10-14 after surgery (C, D). Data is presented as mean ± SEM (n = 5-7 per group). Open square and dotted line: wildtype mice; closed square and solid line: OE-NPY^{DBH} mice. * = $P <$ 0.05 by repeated measures two-way ANOVA. Figure is from original communication II.

5.6 The effect of acute immobilization and cold stress on NPY and corticosterone secretion

5.6.1 Plasma and adrenal gland NPY levels at baseline and after immobilization stress (II)

Immobilization stress significantly increased NPY levels from baseline in all the groups studied. In females, a significant increase in OE-NPY^{DBH} mice compared with wildtype controls was observed after stress (males: genotype: P = NS, stress: $P <$ 0.001, genotype x stress interaction: P = NS; females: genotype: $P < 0.01$, stress: $P <$ 0.001, genotype x stress interaction: $P = 0.06$, two-way ANOVA with Bonferroni post hoc test; Table 5.3). A significant decrease in adrenal NPY levels after stress was observed in OE-NPY^{DBH} but not in wildtype females, whereas in males, no difference between the genotypes after stress was observed (Fig. 5.8).

Table 5.3. The effect of acute immobilization on plasma NPY levels (pmol/l) in wildtype and OE-NPY^{DBH} mice.

Group	Wildtype males	OE-NPY^{DBH} males	Wildtype females	OE-NPY^{DBH} females
Control	63.8 ± 5.6	54.0 ± 7.2	26.6 ± 6.1	34.6 ± 9.1
Stressed	117.2 ± 12.4	101.9 ± 19.2	74.8 ± 6.6	140.3 ± 26.4 **

Values are presented as mean ± SEM (n = 7-9 per group). Stressed NPY levels are significantly ($P < 0.001$) increased in all the groups compared with their own baseline controls (asterisks not shown). ** = $P < 0.01$ compared with same sex wildtype controls by the Bonferroni post hoc test.

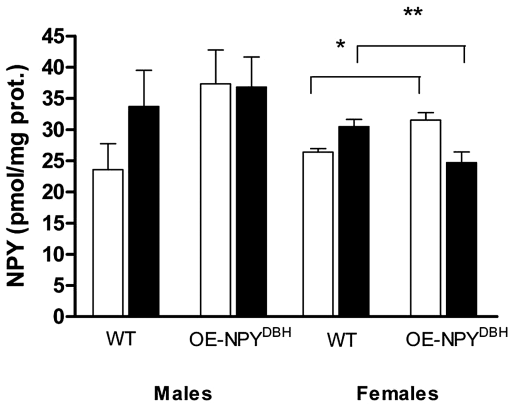

Figure 5.7. The effect of acute immobilization on NPY adrenal gland levels in wildtype and OE-NPY^{DBH} mice. Data is presented as mean ± SEM (n = 7-9 per group). White bar = control mice; black bar = stressed mice. * = $P < 0.05$, ** = P < 0.01 with the Bonferroni post hoc test.

5.6.2 Plasma NPY levels at baseline and after cold exposure (II)

Cold stress increased NPY levels from baseline, although statistically significant increase was reached only in females (females: genotype: P = NS, stress: $P < 0.001$, genotype x stress interaction: P = NS; males: genotype: P = NS, stress: $P = 0.06$, genotype x stress interaction: P = NS; two-way ANOVA, Table 5.4). No difference between genotypes was observed in any study groups.

Table 5.4. The effect of cold stress on plasma NPY levels (pmol/l) in wildtype and OE-NPY^{DBH} mice.

Group	Wildtype males	OE-NPY^{DBH} males	Wildtype females	OE-NPY^{DBH} females
Control	69.9 ± 12.4	75.4 ± 9.8	51.4± 6.1	48.6 ± 6.0
Stressed	113.8 ± 20.1	92.6 ± 17.0	77.3 ± 4.9 ***	88.3 ± 7.5 ***

Values are presented as mean ± SEM (n = 7-9 per group). Stressed NPY levels are significantly ($P < 0.001$) increased in females compared with their own baseline controls. In males, an increasing tendency was found between stressed and baseline groups ($P = 0.06$).

5.6.3 Plasma corticosterone levels at baseline, and after immobilization and cold stress (II)

Stressful experience increased corticosterone levels in both tests as expected, but no difference between the genotypes was observed (females: genotype: P = NS, stress: $P < 0.001$, genotype x stress interaction: P = NS; males: genotype: P = NS, stress: $P < 0.001$, genotype x stress interaction: P = NS; two-way ANOVA, Fig. 5.8). Females had significantly higher corticosterone levels than males (genotype: P = NS, sex: $P < 0.001$, genotype x sex interaction: P = NS; two-way ANOVA).

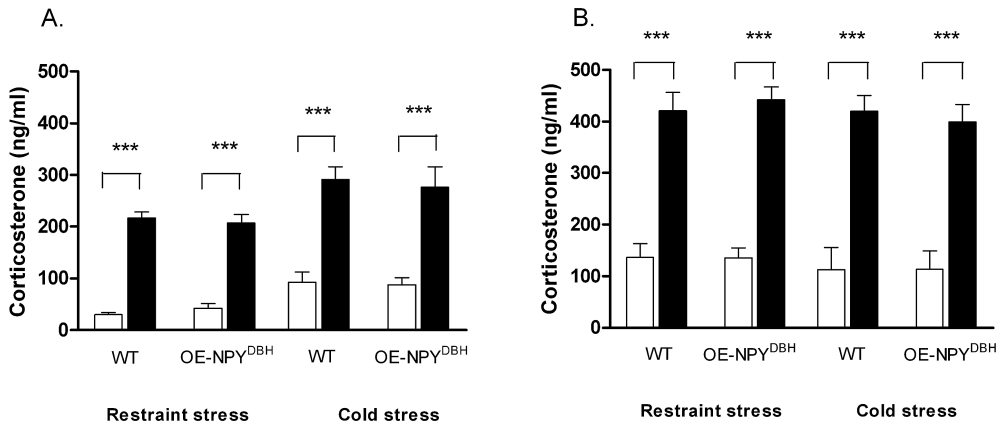

Figure 5.8. The effect of acute immobilization and cold stress on plasma corticosterone levels in wildtype and OE-NPY^{DBH} mice. (A.) males and (B.) females. Data is presented as means ± SEM (n = 7-9 per group). White bar = control mice; black bar = stressed mice. *** = $P < 0.001$ by two-way ANOVA. Figure is from original communication II.

5.6.4 The effect of cold on body temperatures (II)

Exposure to cold significantly decreased body temperatures from baseline in all the groups studied (males and females: genotype: P = NS, stress: $P < 0.001$, genotype x stress interaction: P = NS, two-way ANOVA). No difference in the change of body temperatures (ΔT) between the genotypes was observed in males (wildtype: 4.2 ± 0.6; OE-NPY^{DBH}: 3.5 ± 0.6 °C; P = NS) or females (wildtype: 2.6 ± 0.6; OE-NPY^{DBH}: 1.4 ± 0.4 °C; P = NS).

5.7 The effect of transgene on BAT mitochondrial $^{[3]}$GDP binding activity (II)

Binding of $^{[3]}$GDP to BAT mitochondria was significantly increased in OE-NPY^{DBH} males compared with wildtype males, and the difference was maintained after cold stress (genotype: $P < 0.05$, stress: P = NS, genotype x stress interaction: P = NS, two-way ANOVA, Fig. 5.9). In females, no difference between the genotypes was observed, but exposure to cold significantly increased BAT GDP binding in both

genotypes compared with respective baseline levels (genotype: P = NS, stress: P < 0.01, genotype x stress interaction: P = NS, two-way ANOVA, Fig. 5.9).

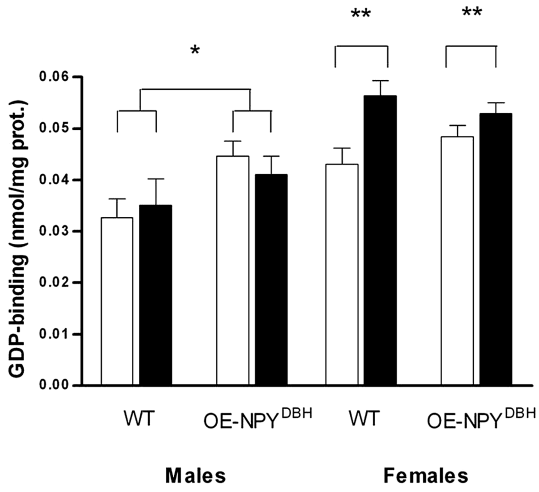

Figure 5.9. BAT mitochondrial $^{[3]}$GDP binding in wildtype and NPY^{DBH} mice. Binding of $^{[3]}$GDP to BAT mitochondria was measured at baseline and after cold exposure in males and females. Data is presented as means ± SEM. White bar = control mice; black bar = stressed mice. * = P < 0.05, ** = P < 0.01 with two-way ANOVA. Figure is modified from original communication II.

5.8 The effect of transgene on behaviour (II)

The Irwin's primary behavioural test (SHIRPA) showed no differences between the genotypes in any parameters measured with the present protocol. In the elevated plus-maze test, the OE-NPY^{DBH} mice showed a lower latency to enter an open arm (P = 0.05, Mann-Whitney test, Table 5.5), but there was no difference between the genotypes in time spent in different arms, or overall transitions from one arm to another. In the open field test, the OE-NPY^{DBH} mice showed a tendency to less locomotor acitivity as measured in crossed squares (P = 0.09, Mann-Whitney test, Table 5.5), but both genotypes showed equal number of rearings in the arena. The light-dark test revealed significantly lower latency for the OE-NPY^{DBH} mice to re-enter the illuminated box (P < 0.05, Mann-Whitney test, Table 5.5), and a tendency to more active movement between the different compartments. However, time spent in the illuminated box did not differ between the genotypes.

Table 5.5. Data from behavioral analyses measured with elevated plus-maze, open field and light-dark tests.

Elevated plus-maze test †	Wildtype	OE-NPY^{DBH}	*P* (M-W)
Latency enter open arm (sec)	86.90 ± 75.2	24.89 ± 26.6	0.05
Time in open arms (sec)	28.90 ± 33.2	35.70 ± 39.2	0.74
Time in closed arms (sec)	237.70 ± 43.5	233.6 ± 30.2	0.35
Entries into open arms (count)	3.70 ± 3.8	1.90 ± 1.1	0.39
Entries into closed arms (count)	7.90 ± 4.0	7.60 ± 3.4	0.79
Open field test			
Crossed squares (count)	102.40 ± 23.3	83.75 ± 23.3	0.09
Rearings (count)	30.11 ± 6.1	26.50 ± 8.5	0.18
Light-dark test ††			
Latency re-enter light box (sec)	99.14 ± 55.5	54.20 ± 23.8	0.04
Time in light box (sec)	67.89 ± 50.5	58.70 ± 21.5	0.66
Entries into light box (count)	3.11 ± 2.0	4.50 ± 1.7	0.09
Entries into dark box (count)	6.55 ± 1.7	8.50 ± 2.5	0.13

Data are presented as means ± SD (n = 8-10 per group). M-W = Mann-Whitney test. † = Three OE-NPY^{DBH} mice that did not produce active movement within one minute from the beginning of the test were discarded from the study. †† = Two OE-NPY^{DBH} and two wildtype mice that did not produce active movement within one minute from the beginning of the test were discarded from the study. Table is from original communication II.

5.9 The effect of transgene on vascular remodelling (III)

The mice underwent a femoral artery angioplasty surgery under ketamine - medetomidine anaesthesia. All the mice survived the surgery and the healing of the wounds occurred rapidly. One of the mice (C57BL/6 strain) suffered from an injury to the femoral nerve resulting in a self-amputation of the operated limb. This animal was eliminated from the study. Of all the vessels, 2 out of 16 and 4 out of 17 (FVB/n and C57BL/6 strains, respectively) were discarded from the calculation of results due to an unsuccessful cutting and mounting of the sections. A marked neointima formation in the femoral artery was seen four weeks after the surgery in all the vessels studied, whereas no changes were seen in the contralateral uninjured arteries.

5.9.1 Arterial neointima and media growth

The neointima formation calculated as a lesion area inside the internal elastic lamina (μm^2) was more pronounced in both strains of the OE-NPY^{DBH} mice than in wildtype control mice (genotype: $P < 0.01$, strain: P = NS, genotype x strain interaction: P = NS, two-way ANOVA, Fig. 5.10). The transgenic mice also appeared to experience more severe growth of the medial area after the injury than the wildtype mice (genotype: $P = 0.05$, strain: P = NS, genotype x strain interaction: P = NS, two-way

ANOVA, Fig. 5.10). In the OE-NPY^{DBH} mice (analyzed in C57BL/6), the injury significantly increased the arterial medial area from the uninjured contralateral side, but no such changes were observed in wildtype controls ($P < 0.05$, Student's t-test).

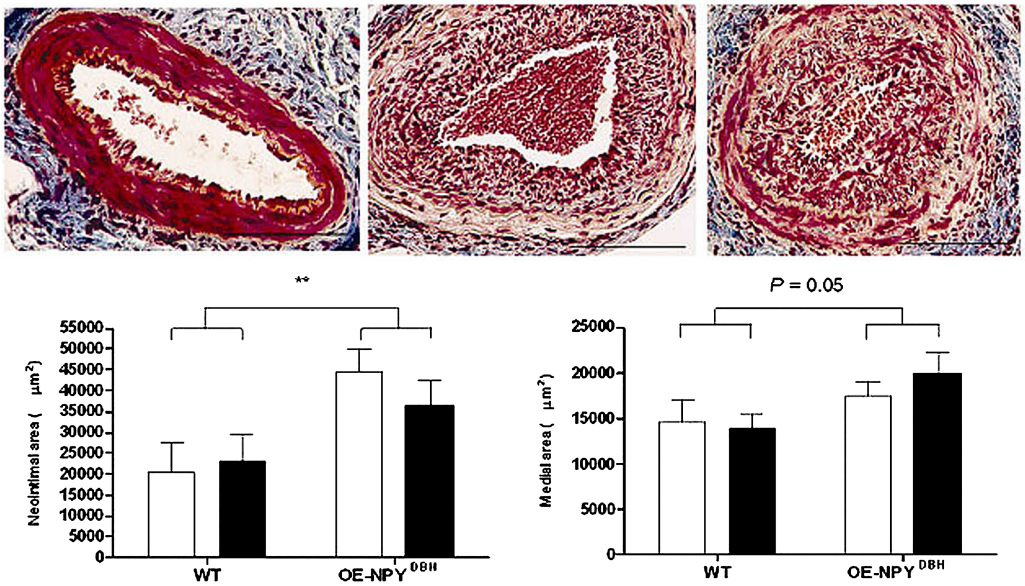

Figure 5.10. Arterial neointima and medial growth after vascular injury. Upper panel shows uninjured control (left), wildtype injured (middle) and OE-NPY^{DBH} injured (right) femoral arteries at four weeks from the surgery. Arteries were histologically stained with Masson's trichrome. Scale bar is 100 µm. Bottom panel shows calculated values (µm^2) for neointima and medial areas after injury for wildtype and OE-NPY^{DBH} arteries in two inbred mouse strains. Data is presented as mean ± SEM (n = 5-8 per group). White bar: FVB/n strain; black bar: C57BL/6 strain. ** = $P < 0.01$ genotype effect by two-way ANOVA. Figure is modified from original communication III.

6 DISCUSSION

6.1 Methodological aspects

6.1.1 Transgenic mouse model

With the help of previous NPY transgenic and knock-out models, important new insights have been revealed particularly in the regulation of food intake and energy homeostasis. The animal species most commonly used in genetic manipulations is the mouse. In NPY research, most pharmacological and behavioural data derives from rats, which can cause a problem, when comparing the results between the species. It should always be kept in mind, though, that an acute drug administration is very different from germline gene overexpression or knock-down systems. It is also important to understand that especially in constitutive overexpression and knock-out models compensatory mechanisms may confuse the results, and caution must be used in interpretation of the obtained data. For example, germline gene deletion of an NPY receptor can influence the expression and function of the remaining NPY receptors, which has been shown to occur in single, double and triple NPY receptor knock-out models (Lin et al. 2005).

Promoter is a gene region that drives the gene expression to a specific cell type. In transgenic animals, the promoters most used are the transgene's endogenous promoters or promoters of known housekeeping genes that are constitutively expressed in most cells. So far there have been no other germline NPY transgenic models with NPY overexpression completely outside of the hypothalamus than the OE-NPY rat (Thorsell et al. 2000; Michalkiewicz et al. 2003). The results from the rat model are quite surprising because NPY is overexpressed under its endogenous promoter and thus, hypothalamic overexpression would have been expected. Clinical data from p.L7P genotypes have suggested that peripheral NPY most likely deriving from SNS is important in the development of metabolic and vascular diseases. Therefore, we hypothesized that a novel gene-modified animal model should be created with an extrahypothalamic and more peripheral NPY overexpression to better model the situation with the human L7P polymorphism. In this thesis study, a mouse model with NPY under a well-known DBH promoter was created. The promoter region of the human *DBH* gene has been analyzed in detail elsewhere to identify the DNA sequences responsible for the tissue- and cell-specific expression of the gene. Sequences required for expression in adult and fetal noradrenergic neurons are located between 0.6 and 1.1 kbp pairs 5' to the DBH transcriptional start site (Hoyle et al. 1994). Therefore, the 5.8 kbp promoter sequence used in this study and by others (Mercer et al. 1991), includes all the regulatory elements needed for gene expression in DBH producing cells.

The hemizygous model, such as the one used in this study, requires a reliable genotyping technique. We chose the conventional PCR-based method together with a

restriction endonuclease treatment. When designing the transgene construct, a silent mutation was created in exon 2 of the *NPY* gene. This mutation introduced a new restriction site specific only to DNA of the transgenic mice. The PCR method allowed fast and reproducible screening of animals.

6.1.2 Measurement of transgene expression in mice

NPY is an abundant peptide in the brain and in SNS. When designing the transgene construct, there were two options to choose from: (A) to use the mouse *NPY* cDNA or (B) to use *NPY* cDNA of another species, e.g. human. By choosing the latter, the separation of the transgenic NPY from the endogenous could be done with species-specific antibodies. However, antibody cross-reactivity between species is common and it is highly unlikely that anti-human NPY antibody would not detect mouse NPY. By using the mouse endogenous *NPY*, it was more likely that the transgene is normally processed and translated in cells. The inconvenience of this method was to distinguish the transgenic and endogenous NPY, especially since the transgenic NPY was expressed in the same groups of cells as the endogenous peptide. Nevertheless, the first alternative was chosen: we amplified *NPY* cDNA from mouse brain in order to model the NPY overexpression as physiologically as possible.

Transgene construct was designed with an IRES sequence and a *LacZ* reporter gene. IRES enabled a cap-independent translation of *NPY* and *LacZ* into a single transgenic mRNA (Mountford and Smith 1995). Thus, we were able to produce co-expression of both genes from the same promoter. With this method, it was possible to screen transgenic tissues with β-galactosidase stain under a light microscope, and to confirm the expression of the transgene in expected areas or exclude individuals with ectopic transgene expression. To note, the expression pattern in OE-NPY^{DBH} mice fitted with the previously published DBH expression pattern (Mercer et al. 1991). We also stained the tissues for NPY, but immunohistochemistry was not sensitive enough to quantitate the differences between the genotypes. Immunohistochemistry confirmed that NPY expression was detected in the same areas in OE-NPY^{DBH} mice as in wildtype controls. Had NPY cDNA from another species been used, in situ hybridization or immunohistochemistry could have been used for transgenic NPY identification. In this study, NPY protein levels (endogenous + transgenic) were quantitated with RIA from plasma and tissue homogenates. The specific tissue areas where NPY levels were measured were chosen based on the results from β-galactosidase staining. It was shown that OE-NPY^{DBH} mice displayed on average 1.8- and 1.3-fold increases in brainstem and adrenal gland NPY levels, respectively, in comparison with wildtype mice.

6.1.3 Genetic background of OE-NPY^{DBH} mice

The transgene was originally transferred into FVB/n background. FVB/n mice are known to have large oocytes, they produce big litters, and the females do not easily kill the offspring, all of which are optimal qualities for transgenic mice. For our studies, however, C57BL/6 was a better suited background strain. First of all, C57BL/6 is an

obesity-prone strain unlike FVB/n. Secondly, behavioural analysis would be arguable to perform on mice that are visually challenged (Voikar et al. 2001). Therefore, the FVB/n transgenic mice were backcrossed to the C57BL/6 strain over six to eight generations. Five backcrossing generations is regarded as sufficient, although ten generations of backcrossing gives a congenic strain that is statistically 99.9% identical to the host inbred strain at all loci (http://jaxmice.jax.org/type/gemm/index.html). The advantage of using hemizygous transgenic mice over homozygous mice is that wildtype littermates can be used as control mice in all the experiments. When the animal is homozygous for the transgene, wildtype littermates are not produced in the same breedings, and thus, they have to be produced separately.

C57BL/6J mice have a naturally occurring in-frame five-exon deletion in *nicotinamide nucleotide transhydrogenase* (Nnt) gene, which results in a complete absence of Nnt protein in these mice (Freeman et al. 2006). Nnt deficiency results in glucose insensitivity and reduced insulin secretion. In this study, we have used C57BL/6N mice, but to be sure, we genotyped both transgenic and wildtype mice for the mutated *Nnt*. They were found to carry the wildtype form of the gene. Hence, impaired glucose tolerance in OE-NPYDBH mice cannot be explained by the mutation in the background strain.

6.1.4 Mouse model of the human p.L7P

The focus of this study was to create a novel noradrenergic neuron-specific NPY mouse model and to characterize the metabolic and vascular phenotype. Under the right circumstances, this new transgenic animal could be used as a tool to model the human p.L7P polymorphism in mouse as closely as possible. Human P7 carriers show normal plasma NPY levels at baseline, but increased secretion in response to exercise (Kallio et al. 2001). This is similar to what we have observed in OE-NPY^{DBH} female mice at baseline and after stress. The human p.L7P genotype is associated with alterations in metabolic, hormonal and sympathovagal balance as well as with faster progression of atherosclerosis and diabetic retinopathy. To model these human diseases in rodents is not straightforward. However, we were able to observe altered autonomic nervous system activity and metabolic profile in OE-NPY^{DBH} mice. Lipid metabolism in rodents is not regulated as in man, and mice are very resistant to e.g. atherosclerosis. LDL, which is the “bad” cholesterol in humans, is a minor lipoprotein in rodents. Rodent serum total cholesterol is mainly composed of high density lipoprotein (HDL). Therefore, unchanged levels of circulating cholesterol in rodents do not necessarily imitate the situation in humans. Our OE-NPY^{DBH} mouse, however, is not the ultimate model of the p.L7P polymorphism. We did not create a Leucine 7 to Proline 7 mutation in the NPY signal sequence. Such knock-in mouse would perhaps have been the closest model to the human situation. After careful deliberation, this approach was abandoned due to the variability in the *NPY* signal peptide sequence among species (Hoyle 1999).

6.2 Role of NPY on adiposity and glucose metabolism in OE-NPY^{DBH} mice

Clinical studies have suggested a relationship of the p.L7P polymorphism with lipid and glucose metabolism (Karvonen et al. 1998; Karvonen et al. 2000; Kallio et al. 2001; Karvonen et al. 2001; Ding et al. 2005; Jaakkola et al. 2005; Nordman et al. 2005; Jaakkola et al. 2006). In study I, the purpose was to investigate the metabolic phenotype and glucose metabolism in OE-NPY^{DBH} mice. We showed that overexpression of NPY in noradrenergic neurons of the central and peripheral nervous systems leads to increased WAT mass, increased triglyceride accumulation in the liver, weight-dependent hyperinsulinemia and impaired glucose tolerance. These changes occured without classic signs of hypothalamic NPY-induced obesity: hyperphagia, increased body weight, hypercorticosteronemia and weight-independent hyperinsulinemia. Furthermore, no difference in locomotor acticity was observed. The results indicate that NPY exerts metabolic effects not only in the hypothalamus but also in the brainstem, sympathetic ganglia and/or directly in the peripheral target tissues. In study II, we measured that the OE-NPY^{DBH} mice show increased adrenal release of catecholamines into circulation. Increased catecholamines do not fit with the present phenotype. It would be expected that increased SNS signalling results in weight loss and increased energy expenditure. It was observed that BAT GDP binding was significantly increased in OE-NPY^{DBH} males indicating increased non-shivering thermogenesis, which suggests alterations in energy regulation. With all aspects considered, it is perhaps possible that increased catecholamine release diminishes the adiposity phenotype of the OE-NPY^{DBH} model. There might be a subtle balance between NPY and noradrenaline/adrenaline at tissue level, and the phenotype is a mixture of these two systems.

The basic organization of an adipose depot shows mature adipocytes, stromal-vascular cells, blood vessels, lymph nodes and nerves (Hausman et al. 2001). Although blood flow is smaller than in other tissues, adipose tissue contains blood supply for delivery of metabolic substrates and removal of cellular metabolic products (Crandall et al. 1986). WAT is innervated by sensory and sympathetic nerves (Fishman and Dark 1987; Youngstrom and Bartness 1995). Hence, in our OE-NPY^{DBH} model there should be an increased NPY input in WAT via sympathetic neurons. At the cellular level, obesity is characterized by an increase in WAT mass, which occurs via increase in cell size (hypertrophy) and/or increase in cell number (hyperplasia). Central i.c.v. NPY administration promotes lipogenesis by stimulating lipoprotein lipase activity in WAT (Billington et al. 1991; 1994). Fat cells from the transgenic OE-NPY^{DBH} mice were significantly smaller and due to the heavier WAT weight probably more numerous. Thus, OE-NPY^{DBH} mice exhibit hyperplastic adipose tissue. This is opposite to hypothalamic NPY, which has been linked to fat cell hypertrophy. In humans, the development of hyperplastic adipose tissue is associated with the most severe forms of obesity and has the poorest prognosis for treatment (Hausman et al. 2001). WAT hypercellularity is also associated with leptin deficiency (Lofgren et al. 2005), which is likely to increase appetite and to lower energy expenditure. Hyperplastic adipose tissue could very well be the reason for regaining body weight after a significant weight loss;

fat is lost from the adipocytes, but the cell number stays the same. Fat loss decreases inhibitory leptin signalling to the brain and strengthens orexigenic signals in the hypothalamus.

Hyperplastic adipose tissue and increased adiposity without body weight gain in OE-NPY^{DBH} mice fit with the results of stress-induced NPY release on energy homeostasis (Kuo et al. 2007). The primary finding by Kuo et al. was that WAT mass was increased without changes in food intake or body weight after two weeks of daily stress and high fat diet. Prolonged stress and diet led to liver steatosis, impaired glucose tolerance and obesity, which were attenuated by local Y_2 receptor antagonist administration and fat-targeted *Y_2*-gene knockdown procedure. In addition, NPY directly stimulated adipogenesis *in vitro*. Thus, SNS NPY seems to increase WAT mass through local, Y_2-mediated mechanisms. In a recent clinical study, it was shown that the number of adipocytes for lean and obese individuals is set during childhood and adolescence showing very little variation during adulthood even in response to fasting, surgical removal of fat tissue or weight gain (Spalding et al. 2008). Since nearly 80% of overweight children go on to become obese in adults (Freedman et al. 2001), feedback mechanisms that control adipocyte turnover at molecular level in youth should be identified. This would offer an interesting novel pharmacological target to treat obesity already at an early age when the final number of fat cells in the body is being set. Based on our results and the data from others (Kuo et al. 2007), the NPY Y2R pathway is showing promising potential to be one of these targets.

In this study, it was shown that OE-NPY^{DBH} males develop age-related glucose intolerance and hyperinsulinemia as well as increased adiposity. In study I, we showed impaired glucose tolerance in males at six but not at three months of age. Later, we have observed that the glucose tolerance has worsened already in males at the age of four months (unpublished observations). In study I, females also developed increased adiposity, but less fat accumulation over age than males. This is most likely the reason why glucose metabolism remains normal in OE-NPY^{DBH} females at the age of six months. Hence, enhanced SNS NPY does not affect glucose tolerance *per se*. Hepatic triglycerinemia developed prior to glucose intolerance, which suggests that increased adiposity and altered lipid metabolism are crucial factors for impaired glucose metabolism in OE-NPY^{DBH} mice. In study I, we also showed that ghrelin levels were significantly increased and positively correlated with glucose AUC values in OE-NPY^{DBH} mice. Ghrelin has been shown to attenuate glucose-stimulated insulin release, and genetic removal of ghrelin improves the glucose tolerance in ob/ob mice (Dezaki et al. 2004; Sun et al. 2006). Therefore, it is possible that hyperghrelinemia also contributes to impaired glucose tolerance together with increased adiposity in OE-NPY^{DBH} mice. This remains to be studied further to allow conclusions.

Central NPY has been shown to increase insulin secretion (Zarjevski et al. 1993; Gao et al. 2004), but peripheral NPY inhibits insulin release from β-cells (Vettor et al. 1998). In study I, we observed that hyperinsulinemia developed with age and increased adiposity. It can be hypothesized that SNS NPY inhibits pancreatic insulin release, which would explain the normal fasting insulin values observed in young OE-NPY^{DBH}

males and females. However, it seems that modest overexpression in SNS is not enough to suppress insulin secretion below the levels observed in wildtype mice. Furthermore, it can be hypothesized that increased adiposity at six months overcomes the NPY's inhibitory effects and insulin levels rise beyond normal. Unfortunately, the insulin tolerance test was not performed on the 6-month-old group. Thus, we cannot state that the OE-NPY^{DBH} mice are insulin resistant.

6.3 Role of NPY on blood pressure in OE-NPY^{DBH} mice

NPY increases blood pressure in humans after peripheral administration (Ullman et al. 2002). Healthy p.L7P subjects have been shown to display increased basal heart rate without increases in blood pressure (Kallio et al. 2003). In study II, we investigated the blood pressure and heart rate levels in OE-NPY^{DBH} mice and compared the results with wildtype controls. Interestingly, the OE-NPY^{DBH} mice display increased mean arterial blood pressure only at night hours (active phase) during recovery from the surgery. At baseline or during the light phase of the day, no difference between the genotypes is observed. It is also noteworthy that no difference in heart rate is observed. This may be due to the increased blood pressure-induced baroreceptor reflex, which returns heart rate to normal. Our results correlate well with results from the NPY-OE rat model, in which higher tail blood pressure responses were obtained with a stressful and perhaps painful tail-cuff method, but the outcome at baseline was different with long-term radiotelemetric method (Michalkiewicz et al. 2001; 2003). Thus, these data together suggest that NPY does not significantly contribute to maintaining resting blood pressure. This may also imply that increased stress-induced blood pressure in NPY overexpressing animals reflect increased responsiveness to stress.

6.4 Role of NPY on catecholamine levels in OE-NPY^{DBH} mice

The OE-NPY^{DBH} mice show increased plasma levels of catecholamines, which most likely explains the enhanced BAT thermogenesis, although measured under anaesthesia. It is suggested that the overexpression of NPY in SNS modulates adrenal excitability. This theory is based on two findings: (A) an altered plasma/adrenal gland adrenaline ratio was found in OE-NPY^{DBH} males and (B) an increased stress-related NPY release from OE-NPY^{DBH} female adrenal glands was observed in study II. In our most recent studies, isolated adrenal glands from OE-NPY^{DBH} mice showed an increased baseline adrenal firing rate compared with wildtype controls in patch-clamp recordings (unpublished observations). These results agree with the previous studies by others. It has been shown that NPY enhances the secretion of catecholamines from human, mouse and rat adrenal chromaffin cells *in vitro* (Renshaw et al. 2000; Cavadas et al. 2001; 2006). The secretion is controlled via Y_1 receptors, because Y_1 null mice do not respond to the NPY-stimulated release of catecholamines similar to the wildtype controls (Cavadas et al. 2006). Furthermore, $Y_1^{-/-}$ mice show a higher catecholamine turnover rate and an enhanced TH activity, which suggest that NPY controls catecholamine synthesis and secretion in vivo (Cavadas et al. 2006).

The *in vivo* results with the OE-NPY^{DBH} mice fit with the clinical polymorphism data: NPY overexpression in noradrenergic neurons alters the regulation of the autonomic nervous system activity and neurotransmitter release. Healthy subjects with P7 allele of p.L7P have been shown to display decreased concentrations of sympathetic transmitters, similar blood pressure and increased heart rate under a non-stressful situation (Kallio et al. 2003; Jaakkola et al. 2005). However, cardiovascular autonomic regulation in P7 carriers is much more sensitive to changes in plasma NA concentrations than in subjects homozygous with L7 allele. This is seen as a strong negative association of NA concentrations with low-frequency variability of R-R intervals and with baroreflex sensitivity (Jaakkola et al. 2005). These clinical data put together suggest that P7 carriers are more prone to the undesired effects of NA when it is released during stress.

6.5 Stress effects in OE-NPY^{DBH} mice

The rapid activation of stress-responsive neuroendocrine system is a basic reaction of animals to distress in their environment. The sympatho-adrenomedullary system responds to stress by releasing sympathetic neurotransmitters NA, adrenaline and NPY into circulation (Pernow et al. 1986; Zukowska-Grojec et al. 1988; Zukowska-Grojec and Vaz 1988; Zukowska-Grojec et al. 1991; Kallio et al. 2001). Unlike NA, NPY is preferentially released by intense and prolonged sympathetic activation being a marker of a more severe stress (Zukowska-Grojec 1995). NPY levels are elevated in panic disorder patients (Boulenger et al. 1996), and decreased in subjects with posttraumatic stress disorder (Rasmusson et al. 2000; Yehuda et al. 2006). In addition, exposure to cold elevates plasma NPY levels in humans and rats (Morris et al. 1986; Zukowska-Grojec and Vaz 1988; Zukowska-Grojec et al. 1991), although not all studies support these findings (Mormede et al. 1990). In rodents, one of the best ways to evoke the sympathetic activity is an acute immobilization procedure. Very little data is available on the effects of immobilization stress on plasma NPY. Single restraint session in rats has been reported to increase plasma NPY levels at the end of the stress session (Zukowska-Grojec et al. 1988). Acute stress also suppresses the production of NPY mRNA in the amygdala (Thorsell et al. 1998), whereas repeated immobilization or stress-sensitization have an opposite effect on NPY expression levels (Thorsell et al. 1999; de Lange et al. 2008). These results suggest that NPY has a role in habituation to a stressful situation. Also glucocorticoids and CRH are key regulators of stress responses. CRH system is activated by different types of stress; in the hypothalamus, CRH expression and release are increased by physical or psychological stressors, while in the amygdala, preferentially by a psychological stress (Aguilar-Valles et al. 2005).

In study II, we exposed the OE-NPY^{DBH} and wildtype mice to stress by acutely restraining the mice and by placing them in cold (4°C). We tested both male and female mice, because in rodents, females display enhanced secretion of ACTH and corticosterone due to the estrogen-stimulated HPA-function (Handa et al. 1994). Both stressors increased plasma NPY levels from baseline, but a difference between the genotypes was observed only in females after restraint stress suggesting a sexually

dimorphic release of NPY in response to stress. Thus, there appears to be sex-related difference at the level of adrenal excitability in OE-NPY^{DBH} mice. This is similar to the enhanced catecholamine release after stress observed in female $Y_1^{-/-}$ mice (Cavadas et al. 2007). One possible explanation for the sexual dimorphism is that the adrenal medullary content of neurotransmitters in females is under the control of estrogen (de Miguel et al. 1989). Overexpressed sympathetic NPY does not modulate the basal or stress-induced HPA-axis activity, since we show that acute stressors stimulated the HPA-axis and increased the secretion of corticosterone similarly in all study groups.

The main physiological regulator of BAT non-shivering thermogenesis is NA released by sympathetic neurons densely innervating the BAT, which results in activation of β-adrenoceptors. Therefore, the increased plasma levels of catecholamines in association with reduced adrenal catecholamine content in OE-NPY^{DBH} mice fits well with the increased BAT activity in males. It seems that an increase in circulating NPY during stress is not enough to overcome the positive effects of increased circulating NA on BAT activity. The suppression of thermogenesis by NPY may need increased central hypothalamic expression of NPY as shown previously (Billington et al. 1991), and these NPY-neurons are not activated during the cold stress (Bing et al. 1998) or in the OE-NPY^{DBH} model.

6.6 Role of NPY on anxiety-related behaviour in OE-NPY^{DBH} mice

Several preclinical studies have shown that NPY is an anxiolytic agent (Thorsell et al. 2000; Redrobe et al. 2003; Tschenett et al. 2003; Karl et al. 2006). The amygdala is considered to be the key structure in the brain mediating anxiety (Schulkin 2006), and exogenous NPY (Heilig 1995) as well as overexpression of NPY in the amygdala (Primeaux et al. 2005) have been shown to reduce anxiety. Interestingly, mice overexpressing NPY in the amygdala did not differ from wildtype controls in anxiety measures (Thiele et al. 1998). However, that particular study was performed on the visually impaired FVB/n mouse strain. Additionally, brainstem is one of the important regions implicated in the regulation of anxiety (Abercrombie and Jacobs 1987; de Lange et al. 2008). Neurons in the nucleus of the solitary tract receive projections from the limbic structures, i.e. the medial prefrontal cortex and the central nucleus of the amygdala, which regulate behavioral responses to stress (Schwaber et al. 1982). NPY injected into the vicinity of LC in the brainstem has anxiolytic-like effects in rats, and this effect is mediated via Y_2 receptors (Kask et al. 1998). In study II, the primary behavioral screening did not reveal any differences between the genotypes, but in a series of anxiety measures, the OE-NPY^{DBH} mice showed a tendency towards anxiolytic behavior in comparison with wildtype mice. In addition, the spontaneous locomotor activity test in original study I did not show any differences between the genotypes. These results support the theory that NPY induces behavioral changes relevant to anxiety, but the overexpression of NPY only in the brainstem and not in the amygdala is perhaps not sufficient to clearly show the anxiolytic effects of NPY, as already suggested by others (Heilig et al. 1993).

6.7 Injury-induced neointima formation in OE-NPY^{DBH} mice

p.L7P allele is associated with vascular diseases, i.e. accelerated arterial neointima formation and earlier onset of atherosclerosis independent of other risk factors (Karvonen et al. 2001), and development of diabetic retinopathy in type 2 diabetic patients (Niskanen and Voutilainen-Kaunisto 2000). The polymorphism is also associated with metabolic factors increasing the risk of vascular disease such as increased serum LDL (Karvonen et al. 1998) and an earlier onset of type 2 diabetes in obese carriers of the L7P mutation (Jaakkola et al. 2006). As NPY has been shown to increase DNA synthesis and cell proliferation rate in rat, porcine and human aortic VSMC lines (Shigeri and Fujimoto 1993; Zukowska-Grojec et al. 1998; Nilsson et al. 2000), increased production and release of NPY may be in part responsible for these vascular diseases. To study vascular remodelling in mice often requires mechanical interventions. Problems with many of the models have raised questions about their reproducibility and physiological significance. In study III, we used a mouse model of vascular injury that resembles balloon-angioplasty (Sata et al. 2000). This model has been useful in studying arterial restenosis and most importantly, the method is reproducible in mice and rats (Sata et al. 2000; Li et al. 2003; Li et al. 2005). The data from the OE-NPY^{DBH} mouse in study III suggest that increased NPY in sympathetic nerves and brain noradrenergic neurons leads to increased susceptibility to arterial thickening after vascular injury possibly due to increased VSMC proliferation activity, and thus provides further support to the hypothesis that excess NPY in humans leads to vascular remodelling, as seen in carriers of the p.L7P polymorphism. Our results are also in line with previous work on the effects of exogenous NPY in the rat endovascular injury model (Li et al. 2003; 2005). A local periarterially inserted NPY pellet dose-dependently increased the formation of neointimal and medial areas, which was prevented by continuous infusion of NPY receptor Y_1 or Y_5 antagonists (Li et al. 2003). A similar effect was observed in rats exposed to mild chronic stress leading to increased endogenous NPY release from the SNS (Li et al. 2005).

6.8 Possible role of NPY in DBH-positive cells in metabolic syndrome

One of the main aims of this thesis study was to investigate the role of NPY in DBH-synthesizing cells in the development of the metabolic syndrome. The data obtained from the studies presented in this thesis combined with published results by others have made it feasible to draw a schematic picture of possible mechanisms of NPY released from SNS or brain noradrenergic neurons (Fig. 6.1). Chronic mild stress or overexpression of NPY in noradrenergic neurons elevates NPY production in noradrenergic neurons. Locally increased NPY results in increased WAT adipogenesis, possibly angiogenesis, WAT hyperplasia, and eventually obesity. Energy intake is not affected because the hypothalamic NPY pathway is normal. Increased adiposity together with enhanced ghrelin secretion and liver hyperlipidemia impair glucose metabolism, and further enhances the development of hyperinsulinemia. These are the very first steps in the development of type 2 diabetes. On the other hand, increased SNS NPY also adds VSMC proliferation activity after a local vascular inflammation

due to, e.g. denudation of endothelial cells. This leads to neointima growth towards the lumen resulting in artery stenosis, which is a hallmark of development of atherosclerosis. The fourth clinical indication of metabolic syndrome is hypertension. Increased SNS or brainstem NPY can increase blood pressure under stressful conditions directly or by enhancing the release of sympathetic transmitters, namely NA and adrenaline. Thus, NPY together with stress are potent factors in several pathways leading to a complex disorder called metabolic syndrome or syndrome X.

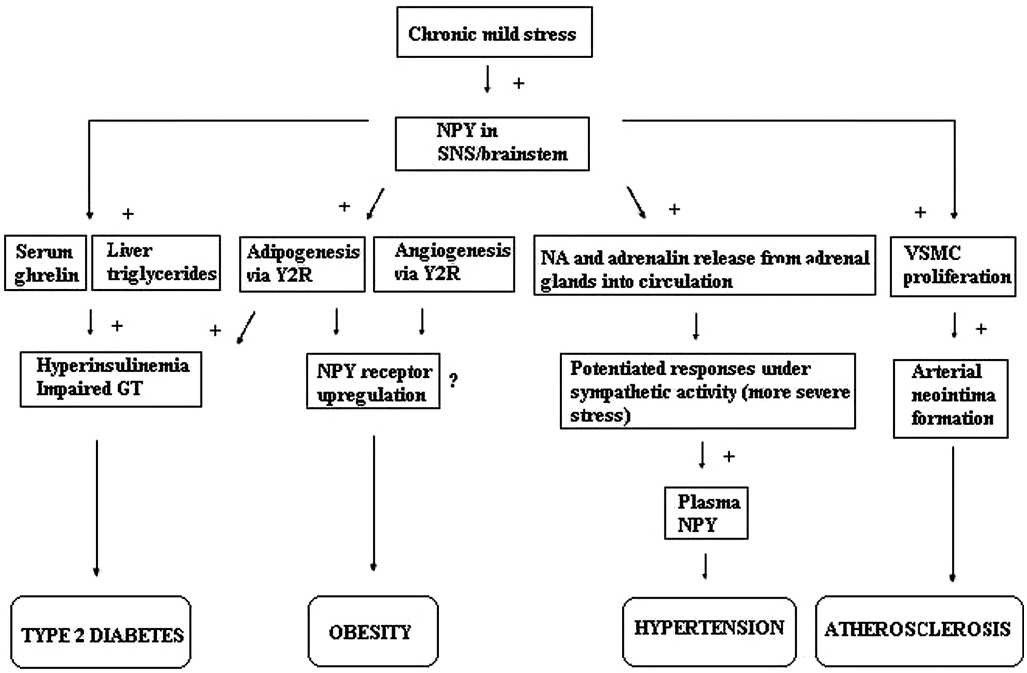

Figure 6.1. Possible roles of NPY in DBH-positive cells on the development of metabolic syndrome. Hypothetical mechanisms are based on the results from this study and data from Kuo et al., 2007. + = effect is stimulatory; ? = effect is open to doubt; Y2R = NPY Y_2 receptor; VSMC = vascular smooth muscle cell; GT = glucose tolerance

6.8.1 Comparison of the OE-NPY^{DBH} mouse with other mouse models of metabolic syndrome

Mouse models most used in the study of the human metabolic syndrome include the leptin deficient ob/ob and the leptin receptor null db/db mice. In addition, high-fat or high-calorie diets challenge mice to develop features associated with metabolic syndrome. The mouse strain most used in DIO research is the C57BL/6, because it develops obesity, hyperinsulinemia, hyperglycemia and hypertension on *ad libitum* high-fat diet (Surwit et al. 1988; Black et al. 1998). The characteristics of each of these models and the OE-NPY^{DBH} mouse in relation to obesity and glucose metabolism have been summarized in table 6.1.

Table 6.1. Comparison of obesity and diabetic phenotypes of mouse models.

Obesity or diabetes phenotype	C57BL/6 mouse	ob/ob mouse	db/db mouse	OE-NPY^{DBH} mouse
Induced or spontaneous	diet-induced	spontaneous	spontaneous	spontaneous
Phenotype onset	mature	young	young	mature
Hyperphagia	mild	yes	yes	no
Hyperinsulinemia	moderate	severe	severe	moderate
Impaired glucose tolerance	yes	yes	yes	yes
Hypercorticism	no	yes	yes	no
Non-shivering thermogenesis	decreased	decreased	decreased	increased
Liver steatosis	moderate	severe	severe	moderate

Data from the C57BL/6, ob/ob and db/db mice are collected from the available information on the Jackson Laboratory mouse models (www.jax.org).

Based on the collected data, it can be concluded that ob/ob and db/db mice are beyond the definition of metabolic syndrome (Fig. 1.1). They already are diabetic and show symptoms of cardiovascular disease. DIO model in C57BL/6 is closer to the OE-NPY^{DBH} mouse, but it takes several weeks on a special diet for the metabolic phenotype to develop (Collins et al. 2004). Despite the feeding, not all mice gain weight. Some mice are identified as diet resistant for unknown reasons. DIO C57BL/6 mice show hyperphagia, and therefore it is likely that they also show altered levels of food intake regulating peptides and hormones. Indeed, DIO mice have been shown to display increased serum leptin levels and arcuate nucleus-specific leptin resistance (Munzberg et al. 2004). DIO C57BL/6 mice also show thermoregulatory defects opposite to the OE-NPY^{DBH} model (Collins et al. 1997). Thus, the OE-NPY^{DBH} mouse is unique in that it shows not only increased SNS activity but also increased adiposity without hyperphagia, and no dietary interventions are required for the obesity to manifest in the OE-NPY^{DBH} mice. The phenotype is quite modest in the hemizygous OE-NPY^{DBH} model, which is why a homozygous mouse is to be developed. It is hypothesized to be a better model for intervention studies, such as for testing the efficacy of Y_2 antagonists on WAT reduction.

7 SUMMARY AND CONCLUSIONS

The studies using a novel OE-NPY^{DBH} mouse model have implicated that increased SNS and brainstem NPY alters the metabolic, hormonal and sympathovagal balance in mice. In summary, following conclusions were made according to the current thesis studies based on the aims:

1. A mouse model named OE-NPY^{DBH} mouse was successfully created. Two lines were studied at first, from which only one was selected for further phenotyping analyses. The model was backcrossed from FVB/n strain to the C57BL/6 strain for at least six generations, which produced enough strain congenity for the backcrossing to be sufficient.

2. Moderate overexpression of NPY in sympathetic nervous system and brain noradrenergic neurons causes increased adiposity and liver triglyceride accumulation, which leads to hyperinsulinemia and impaired glucose tolerance with age. The effect seems to be non-hypothalamic since no difference is seen in the hypothalamic NPY content between the OE-NPY^{DBH} mice and wildtype controls, or in feeding and other hypothalamically-induced NPY effects. These results suggest that NPY in the SNS and other DBH-synthesizing cells may play an important role in the pathogenesis of disturbances in glucose and lipid metabolism.

3. Transgenic overexpression of NPY in DBH-positive cells such as in the adrenal gland and in the brainstem result in increased levels of plasma catecholamines along with decreased adrenal gland catecholamine concentrations. This is reflected to an increased stress-related arterial blood pressure and BAT mitochondrial $^{[3]}$GDP binding activity suggesting increased non-shivering BAT thermogenesis. In addition, NPY shows a sexually dimorphic secretion pattern after restraint stress, which gives an important new insight into differential secretion of neurotransmitters between genders in response to stress.

4. Overexpression of NPY in noradrenergic neurons may act at the level of the brainstem to modulate anxiety-related behavior and help to cope with stressful situations.

5. Locally increased SNS NPY is responsible for the greater formation of arterial neointima and medial area after an arterial angioplasty surgery. Thus, increased sympatho-adrenomedullary-released NPY seems to be an important risk factor for vascular diseases related to the metabolic syndrome.

8 ACKNOWLEDGEMENTS

This work was carried out in the Department of Pharmacology, Drug Development and Therapeutics at the University of Turku between 2002 and 2009. I want to thank Professors Mika Scheinin, Markku Koulu, Erkka Syvälahti and Risto Huupponen for the new facilities of the department and for the support towards my work during these years. Drug Discovery Graduate School and especially Eeva Valve is also acknowledged.

My supervisor docent Eriika Savontaus has contributed enormously to this work. She has taught me almost everything I know about making science. Eriika has also accepted my own ideas in the laboratory and helped me to carry them through. I admire her amazing enthusiastic commitment to research work and teaching. I also thank my other supervisor docent Ullamari Pesonen. She has profound expertise in NPY research, and her experience in biochemistry has been important. I owe my gratitude to Professor Markku Koulu who despite teaching and administrative duties has still had time to throw in new ideas to this study. In addition, I want to thank Professor Zofia Zukowska from Georgetown University Medical Center for letting me come to work in her laboratory. Dr. Zukowska's lab is a model example of a work place where things get done.

I want to thank all the co-authors of this work: Katja Kaipio, Niko Moritz, Matias Röyttä, Petteri Rinne, Heikki Ruskoaho, Joana Rosmanino-Salgado, Claudia Cavadas, Ken Abe, Mia Kero and Laura Toukola. Your contribution means a lot to me, we all did this together!

I am very grateful to Professors Karl-Heinz Herzig and Amanda Sainsbury-Salis for the valuable comments and suggestions when reviewing this thesis. I also want to thank my mom for the reviewing of the language (the book and original publications).

A big thank to all the people in farmis over the years! Special thanks to Katja and Ulriikka for being such good friends during and after office hours! Raija Kaartosalmi, Helena Hakala, Elina Kahra, Ulla Uoti, Tuula Juhola and Maarit Nikula are acknowledged for the great technical support. Anja Similä, Hanna Tuominen and Ulla-Elina Hurme have been helpful in office assistance. Sanna Soini, Anna Huhtinen, Tuire Tirkkonen, Francisco Lopez-Picon, Milka Hauta-Aho, Mika Hirvonen, Jonne Laurila, Mikko Uusi-Oukari, Tomi Streng, Irma Holopainen, Tiina-Kaisa Kukko-Lukjanov, Hanna Laurén, Johanna Hilli, Susann Björk, Jussi Posti, Veronica Fagerholm, Sanna Palovaara, Tuire Olli-Lähdesmäki, Janne Lähdesmäki, Jori Ruuskanen, Amir Snapir, Ville-Veikko Hynninen, Kimmo Ingman, Sanna Tikka, Juha Järvelä and all the others who have worked at farmis during the past years have made the place an inspiring and friendly work environment.

Tanja, Minna, Kati and Elina, thanks for being the best “non-scientific” friends. I am grateful that I have become friends with Mirkka and Maija during lectures in Medisiina and Dentalia.

I warmly thank my family and Saku’s family for support. Especially vacations with Pilvi & Fabien and Sanna & Stefan have been the most fun.

Finally, and most importantly, I owe my deepest gratitude to my Saku. He has been my inspiration for this work and has guided me through at all times. I could not have finished this work without him and our dearest “girls”.

This work was financially supported by The Academy of Finland, Tekes (Finnish Funding Agency for Technology and Innovation), The Finnish Cultural Foundation (Central and Varsinais-Suomi funds) and The Turku University Foundation. These are all acknowledged.

Turku, January 2009

Suvi Ruohonen

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