NPY Family o
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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders: from Genes to Therapeutics

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Introduction

NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
from Genes to Therapeutics
Edited by Zofia Zukowska and Giora Z. Feuerstein

NPY family of peptides in neurobiology, cardiovascular system and metabolism: genes, diseases and therapeutics

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Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) have been the subject of intense research for over two decades. The genes for all three members of the family and for several receptors have been cloned, and multiple activities discovered. The high degree of conservation of structure of these peptides among species, and their ancient origin, appear to suggest they serve essential physiological roles in the animal kingdom. NPY emerged as a prominent neurotransmitter and neuromodulator in the central and peripheral nervous system, while PYY and PP, originally identified as gut peptides, emerged as powerful paracrine and endocrine regulators of not only the gastrointestinal system but also many neuroendocrine networks. The peptides have been heralded as potent vasoconstrictors and appetite stimulants, and implicated in diseases such as hypertension, ischemic heart disease and obesity. While in many instances specific receptors responsible for the peptides' activities have been identified and selective small molecule ligands developed – their therapeutic applications are still under investigations.

This book is the second of two volumes that comprehensively review and analyze the frontiers in research and drug development in the area of NPY peptides. The first book dealt with the newly discovered role of these peptides as autocrine and paracrine regulators of the body's host-defense responses, and tissues' adaptations to injury, ischemia or infection. The present book presents the state-of-the-art in more classical fields of research on NPY peptides, and reviews their role in the nervous, endocrine and cardiovascular systems, and metabolism. While many good reviews have been written on these topics in the past, a thorough analysis of evidence for the role of these peptides in diseases and potentials for clinical applications and therapeutics in this field has been missing. Therefore this perspective – from genes and structure-function, to diseases and therapeutics – has been interwoven into most of the chapters of this book.

Section II provides a thorough introduction to subsequent disease- and therapy-oriented chapters by reviewing current knowledge on the distribution and molecular pharmacology of NPY/PP peptides and their receptors, and their interactions with specific ligands. The core of the book presents a comprehensive overview of major systems and disease processes in which NPY, PYY and PP appear to play important roles. Special emphasis is placed on previously neglected areas of NPY science such as congestive heart failure, renal failure, and cerebrovascular diseases. A large portion of the book is devoted to well recognized activities of the peptides in regulation of obesity and metabolism, alcoholism, epilepsy, neurodegenerative disorders and anxiety. New insights into the mechanisms of action, specific receptors involved, and novel strategies for therapeutic interventions in these diseases are presented.

A special place in this book is also reserved for the newest frontiers of NPY peptide science such as their role in bone remodeling, neuroplasticity and neurodegeneration, as well as post-traumatic stress disorder. Closing this volume is a chapter reviewing epidemiology of NPY gene polymorphism, the first functional genetic mutation with potentially wide implications for many of the diseases reviewed in this book.

NPY family of peptides, receptors and processing enzymes

NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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An overview of neuropeptide Y: pharmacology to molecular biology and receptor localization

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Introduction

Neuropeptide Y (NPY) is a 36 amino acid residues peptide that was first isolated in 1982 from porcine brain [1]. The same group also isolated another peptide from porcine intestine that has high sequence homology with NPY and named it peptide YY (PYY) [2]. Furthermore, both peptides possess structural elements similar to those of the pancreatic polypeptides (PPs) [3]. They have thus been included in the same peptide family, called the Y family or NPY family [4] (Tab. 1). These peptides have 36 amino acid residues, contain several tyrosine residues, have a polyproline type II helix (amino acid residues 1 to 9), a beta-turn (amino acid residues 10–13), an amphiphilic alpha-helix (amino acid residues 14–30) and a C-terminal area that appears to adopt a flexible structure [5, 6]. Theses structural elements are likely to confer a hairpin like-structure, in which N- and C-terminal amino acid residues are in closed proximity as previously shown for avian PP [7, 8].

Soon thereafter, antibodies were raised against NPY and immunohistochemical techniques revealed the presence of NPY-like immunoreactive (ir) materials in the central nervous system of mammals, including human [9–12]. Interestingly, NPY-like ir was found to be widely distributed not only in the central nervous system (CNS), but also in peripheral nervous tissues, and represents one of the most abundant peptide in the CNS [13–15]. In contrast, PYY and PP are mostly found in endocrine cells of the intestine [16]. However, PYY-like ir was also shown to be present in the brainstem and various hypothalamic nuclei [17].

These peptides, especially NPY and PYY, are among the most highly conserved peptides during evolution (Tab. 1) [18–20], suggesting their significant roles in insuring basic physiological functions. In fact, this peptide family possesses a broad range of biological effects including increased food and water intake [21, 22], facilitated learning and memory processes [23], inhibition of glutamatergic excitatory synaptic transmission [24–26], modulated locomotor behaviours [27, 28], hypothermia [27, 29–31], decreased sexual behaviour

Table 1 Primary amino acid sequence of neuropeptide Y, peptide YY and pancreatic polypeptides

	1				5					10					15					20					25					30					35				Diff
NPY																																						
human	Y	P	S	K	P	D	N	P	G	E	D	A	P	A	E	D	M	A	R	Y	Y	S	A	L	R	H	Y	I	N	L	I	T	R	Q	R	Y-NH2	
rat	Y	P	S	K	P	D	N	P	G	E	D	A	P	A	E	D	M	A	R	Y	Y	S	A	L	R	H	Y	I	N	L	I	T	R	Q	R	Y-NH2	
porcine	Y	P	S	K	P	D	N	P	G	E	D	A	P	A	E	D	L	A	R	Y	Y	S	A	L	R	H	Y	I	N	L	I	T	R	Q	R	Y-NH2	1
guinea pig	Y	P	S	K	P	D	N	P	G	E	D	A	P	A	E	D	M	A	R	Y	Y	S	A	L	R	H	Y	I	N	L	I	T	R	Q	R	Y-NH2	
dogfish	Y	P	S	K	P	D	N	P	G	E	G	A	P	A	E	D	L	A	K	Y	Y	S	A	L	R	H	Y	I	N	L	I	T	R	Q	R	Y-NH2	3
lamprea	F	P	N	K	P	D	S	P	G	E	D	A	P	A	E	D	L	A	R	Y	L	S	A	V	R	H	Y	I	N	L	I	T	R	Q	R	Y-NH2	6
PYY																																						
human	Y	P	I	K	P	E	A	P	G	E	D	A	S	P	E	E	L	N	R	Y	Y	A	S	L	R	H	Y	L	N	L	V	T	R	Q	R	Y-NH2	
rat	Y	P	A	K	P	E	A	P	G	E	D	A	S	P	E	E	L	S	R	Y	Y	A	S	L	R	H	Y	L	N	L	V	T	R	Q	R	Y-NH2	2
porcine	Y	P	A	K	P	E	A	P	G	E	D	A	S	P	E	E	L	S	R	Y	Y	A	S	L	R	H	Y	L	N	L	V	T	R	Q	R	Y-NH2	2
guinea pig	Y	P	S	K	P	E	A	P	G	S	D	A	S	P	E	E	L	A	R	Y	Y	A	S	L	R	H	Y	L	N	L	V	T	R	Q	R	Y-NH2	3
dogfish	Y	P	P	K	P	E	N	P	G	E	D	A	P	P	E	E	L	A	K	Y	Y	S	A	L	R	H	Y	I	N	L	I	T	R	Q	R	Y-NH2	9
lamprea	F	P	P	K	P	D	N	P	G	D	N	A	S	P	E	Q	M	A	R	Y	K	A	A	V	R	H	Y	I	N	L	I	Y	T	Q	R	Y-NH2	14
PP																																						
human	A	P	L	E	P	V	Y	P	G	D	D	A	T	A	E	Q	M	A	Q	Y	A	A	D	L	R	R	Y	I	N	M	L	T	R	P	R	Y-NH2	
rat	A	P	L	E	P	M	Y	P	G	D	Y	A	T	H	E	Q	R	A	Q	Y	E	T	Q	L	R	R	Y	I	N	T	L	T	R	P	R	Y-NH2	8
porcine	A	P	L	E	P	V	Y	P	G	D	D	A	T	P	E	Q	M	A	Q	Y	A	A	E	L	R	R	Y	I	N	M	L	T	R	P	R	Y-NH2	2
guinea pig	A	P	L	E	P	V	Y	P	G	D	D	A	T	P	Q	Q	M	A	Q	Y	A	A	E	M	R	R	Y	I	N	M	L	T	R	P	R	Y-NH2	4
avian	G	P	S	Q	P	T	Y	P	G	D	D	A	P	V	E	D	L	I	R	F	Y	D	N	L	Q	Q	Y	L	N	V	V	T	R	H	R	Y-NH2	20

Diff represents the number of amino acid that is not identical to sequences found in human

[32], modulated cardiorespiratory parameters [33], shifted circadian rhythms [34], produced anxiolytic-like [35] and antidepressant-like [36] effects, modulation of the release of luteinizing hormone releasing hormone (LHRH) [37], and of corticotrophin releasing factor (CRF) [38] and implication in alcohol consumption [39]. In the periphery, NPY is a potent vasoconstrictor [40, 41]. Several of these effects appear to be physiologically relevant, based on data obtained using NPY antibodies, receptor antagonists, antisense oligonucleotides, knockout mice and transgenic animals (for reviews see [42–49]). Moreover, NPY, PYY and PPs as well as their receptors likely have direct implication in some pathological disorders including obesity, depression and anxiety-related behaviors, epilepsy, memory impairments, alcohol consumption and bone formation [42–52]. It has also been shown that depending on the cell type, the stimulation of NPY receptors induced various second messenger responses including the inhibition of adenylate cyclase [53, 54], synthesis of inositol phosphates [55], increased intracellular calcium [54], inhibition of calcium channels [56, 57] and inhibition of nicotinic cholinergic currents [58]. The various biological effects of NPY and its homologues are mediated by the activation of at least five classes of receptors known as the Y_1, Y_2, Y_4, Y_5 and y_6 subtypes [4, 59], all of which have been cloned and pharmacologically characterized using several agonists and antagonists (Tab. 2).

Molecular characterization of NPY receptors

The human gene coding for NPY is located on chromosome HSA7q15.1 in proximity to the HOXA cluster (HSA7q15–q14) [60], whereas PYY and PP genes are located only 10 kb apart from each other on chromosome HSA17q21.1 close to the HOXB cluster (HSA17q21–22) [61]. The human NPY gene is divided into four exons and three introns coding for a 97 amino acids precursor peptide [62]. The pre-pro NPY is then proteolytically processed to generate the C-terminal peptide of NPY (CPON) and NPY. Although, it has been shown that CPON and NPY are co-stored and co-released, no biological activity has been reported thus far for CPON [63]. The genomic organization of PYY and PP genes is highly similar to that of NPY [63] as well as the structural organization of the precursor protein [64, 65].

Y_1 receptors

The first NPY receptor to be cloned was initially reported as an orphan receptor isolated by screening a rat forebrain cDNA library [66]. Upon transfection into a cell line, this clone demonstrated a ligand selectivity profile typical of the Y_1 receptor [67] (Tab. 2). The human Y_1 receptor is located on chromosome HSA4q31.3–q32 and is coding for a 384 amino acid protein that has all the characteristics of the G-protein coupled receptor (GPCR) family including

Table 2: Comparative pharmacological profile of the neuropeptide Y, Y_1, Y_2, Y_4, Y_5 and y_6 receptor subtypes

Y_1		PYY ≥ NPY ≥ GR231118, [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY > NPY(2–36) >> PYY(3–36), PYY(13–36), NPY(13–36), hPP, rPP, bPP, C2–NPY
Y_2		PYY ≥ NPY ≥ NPY(2–36), PYY(3–36), PYY(13–36), NPY(13–36), C2–NPY >> [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY, hPP, bPP rPP, GR231118
Y_4		hPP, bPP, VR231118 ≥ rPP ≥ PYY, [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY > NPY >> NPY(2–36), PYY(3–36), PYY(13–36), NPY(13–36), C2–NPY
Y_5		hPP, PYY ≥ NPY, [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY, NPY(2–36), PYY(3–36) >> PYY(13–36), NPY(13–36), rPP, GR231118 > C2–NPY
y_6	A)	PYY, NPY, [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY, NPY(2–36), PYY(3–36), >> PYY(13–36), NPY(13–36), PPs
	B)	hPP, bPP, rPP >> PYY, NPY, [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY, NPY(2–36), PYY(3–36), PYY(13–36), NPY(13–36)
	C)	PYY ≥ NPY ≥ NPY(2–36), PYY(3–36), PYY(13–36), NPY(13–36), C2–NPY >> [Leu^{31}, Pro^{34}]NPY, [Leu^{31}, Pro^{34}]PYY, PPs

Ligand binding profile of the y_6 according to A) Weinberg et al., 1996, B) Gregor et al., 1996b and C) Matsumoto et al., 1996.
Most agonists at low micromolar concentrations will activate all NPY receptors.

potential glycosylation sites in the N-terminal portion and in the second extracellular loop, four extracellular cysteines in positions 33, 113, 198 and 296 which may form two disulfide bridge (Cys 33 and 296 and Cys 113 and 198), the presence of an intracellular cysteine in the C-terminal portion at position 338 that may be used for the attachment of palmitate residues into the cell membrane and possible phosphorylation sites in the intracellular domain. All mammalian Y_1 receptors cloned thus far display 90–95% homology with the human Y_1 receptor [20, 68]. Additionally, two variants of the mouse Y_1 receptors have been identified. Both isoforms bind NPY but the shorter one (307 amino acids) does not initiate second messenger responses [69].

As observed for many GPCR, the Y_1 receptor is rapidly internalized together with its ligand into endosomes and recycled to the cell surface within 60 min upon agonist stimulation [70–73]. Additionally, it has been shown that agonists induce rapid association of β-arrestin 2 with the Y_1 receptor protein [74]. Interestingly, the pseudopeptide Y_1 receptor antagonist, GR231118, can induce long-lasting disappearance of cell surface Y_1 receptors through a pathway distinct from the classical endocytotic/recycling pathway followed by stimulation with an agonist [73]. This suggests that chronic treatment with Y_1 antagonists such as GR231118 may induce cell surface Y_1 receptor losses, leading to apparent conditional knockout of Y_1 receptor activity and possibly being of clinical significance. Additionally, using fluorescence resonance energy transfer (FRET) and fluorescence microscopy, it has been shown that Y_1 receptors are able to form homodimers [75].

Y_2 receptors

Expression screening from cDNA libraries of neuroblastoma cells known as SMS-KAN [76], human hippocampus [77] or human brain [78] lead to the isolation of a human cDNA receptor clone which demonstrated a pharmacological binding profile similar to the Y_2 receptor (Tab. 2). This receptor has been cloned from various species and revealed 90–95% homology between species [20, 68]. However, the overall homology between the Y_1 and Y_2 subtypes is only 31%, which represents one of the lowest homology for the same receptor family. The human Y_2 receptor gene is located in close proximity to the Y_1 and Y_5 genes on chromosome HSA4q31 and has a single intron of approximately 4.5 kb located in the 5'-untranslated region [79]. The human Y_2 receptor gene codes for a 381 amino acid protein and possesses the typical seven transmembrane helix receptor structure, including a single glycosylation site in the N-terminal region, two extracellular cysteines in positions 122 and 203 that may form a disulphide bridge, a single cysteine in the C-terminal region at position 342 that could serve as attachment site for palmitate and phosphorylation sites in the intracellular domain.

In contrast to the Y_1 receptor, the Y_2 subtype does not appear to be internalized following agonist stimulation [70] or if it does so, it is according to a very

slow process. These data have been confirmed using bioluminescence resonance energy transfer 2 (BRET2) technique by measuring interaction between Y_2 receptors and β-arrestin 2. In fact, after agonist stimulation, very slow association rate was observed between the Y_2 receptor protein and β-arrestin 2 [74]. Additionally, as also seen for the Y_1 subtype using FRET techniques, Y_2 receptors can form homodimers [75].

Y_4 receptors

Sequence homology screening with the Y_1 receptor probe lead to the isolation of a new human NPY receptor cDNA [80–82]. After transfection in cell lines, the expressed protein demonstrated very high affinity for PP-related peptides (Tab. 2). Sequence homology between human and other species Y_4 receptors is one of the lowest (less than 75%) reported for orthologous GPCR of different mammalian species [20]. Additionally, the human Y_4 receptor protein has higher homology with the human Y_1 (43%) than Y_2 (34%) receptor [83]. The human Y_4 receptor is located on chromosome HSA10q11.2–q21 [84] and is coding for a 375 amino acid residue protein. As for all members of this peptide family, the Y_4 receptor protein has all the characteristics of a GPCR including four glycosylation sites, a seven transmembrane helix structure, four extracellular cysteines in positions 34, 114, 201 and 298, an intracellular cysteine in position 340 and the presence of several serines and threonines in the C-terminal and the intracellular loops which may serve as phosphorylation sites for various protein kinases.

The Y_4 subtype has been shown to be internalized following agonist stimulation [70]. However, another group failed to observe Y_4 receptor internalization [85]. On the other hand, using BRET2 technique, it was shown that after agonist stimulation, the Y_4 receptor interacts with β-arrestin 2, but at a slower rate than observed for Y_1 and Y_5 receptors in the presence of agonists [74]. Additionally, using the same technique and Western blot with antibodies directed against GFP, it have been shown that Y_4 receptors constitutively form homodimers that dissociate after agonist stimulation [86]. However, no heterodimers were observed when the Y_4 receptor cDNA is co-transfected with other NPY receptor cDNAs [86].

Y_5 receptors

NPY is one of the most potent orexigenic agent and this effect was proposed to be mediated by an atypical Y_1 or 'feeding receptor' [87, 88]. The pharmacological profile of a NPY receptor cloned from human and rat tissues was found to be similar to that of this atypical feeding receptor (Tab. 2) and classified as the Y_5 subtype [89, 90]. The human Y_5 receptor gene is transcribed in opposite direction to the Y_1 gene from a common promoter region on chromosome

HSA4q31–q32, suggesting at least partial coordinated transcriptional regulation [91]. This gene is coding for a protein of 446 amino acids, and has the usual characteristics of a GPCR including glycosylation sites in the N-terminal region and possible cysteine disulphide bridge in the extracellular domain (Cys 114 and 198), a cysteine residue at the C-terminal segment (which could serve as attachment for palmitate) and phosphorylation sites in the intracellular domain. In comparison with other NPY receptor subtypes, the Y_5 receptor protein has a very long third intracellular loop (more than 130 amino acids) and an unusually short C-terminal tail. Additionally, sequence homology between the Y_5 and other NPY receptor subtypes is very low (30%) [20, 68].

Using FRET techniques, it was shown that human Y_5 receptor subtypes are able to form homodimers [75]. When Y_1 and Y_5 receptors are co-transfected, they appear to form heterodimers, and the ligand selectivity profile of Y_1–Y_5 heterodimers is highly similar to results obtained in homogenates containing a mix population of Y_1 and Y_5 receptors [92]. Additionally, agonist stimulation of the rhesus Y_5 receptor induced a rapid association of β-arrestin 2 with the receptor protein, suggesting that this subtype is also internalized [74].

y_6 receptors

Three groups have reported the cloning of another NPY receptor in different species [93–95], and has been designated as y_6 [4]. Rather surprisingly, upon transfection of the mouse and rabbit y_6 receptor clone into cell lines, distinct pharmacological profiles have been reported, either Y_2-like [94], Y_4-like [93] or Y_5-like [95] (Tab. 2). Furthermore, transfection of the human y_6 receptor cDNA failed to be fully translated and to generate a functional receptor. In fact, the y_6 receptor is not expressed in rat [96], while in human and primates the cDNA contains a single base deletion resulting in the expression of a non-functional NPY receptor protein which is truncated from the sixth transmembrane domain [93, 94].

Site directed mutagenesis of Y_1 receptors

Several groups have generated mutants of the Y_1 receptor in order to identify key amino acids responsible for the interaction of NPY with this receptor [97–103]. Overall, 68 positions of the human Y_1 receptor have been mutated, in most cases by alanine. For example, it was shown that replacing Asp residues in positions 104 (first extracellular loop), 194 (second extracellular loop), 200 (second extracellular loop) and 287 (Top TM7) of the Y_1 receptor protein resulted in the complete loss of detectable [^{125}I]NPY binding [97]. These results suggested that bridges formation between positively and negatively charged amino acid residues of NPY and the Y_1 receptor, respectively. Additionally, computer-assisted modeling suggested that the C-terminal tyro-

sine amide moiety of NPY might dock at a pocket formed by hydrophobic amino acids of transmembrane domains 1, 2, 6 and 7 of the Y_1 receptor protein [97]. Further studies revealed that Tyr100 in TM2, Phe286 in TM 6 and His298 at the top of TM7 are also critical for the interaction of NPY with the Y_1 receptor protein [98]. However, in order to potentially distinguish between direct and indirect effects of mutants (conformational changes that will affect the three-dimensional structure of the receptor), several ligands must be used. In that regard, it was shown that alanine mutants in position Tyr100, Asp104, Trp288 and His 298 of the human Y_1 receptor had no effect on specific antagonist binding using either [^{3}H]BIBP3226, [^{125}I]GR231118 or [^{3}H]J-104870, while no or low levels of specific [^{125}I]NPY or [^{125}I]PYY binding were detected [99, 103]. On the other hand, alanine substitution of Tyr211 in TM5 markedly reduced specific [^{3}H]BIBP3226 binding, but had no effect on specific [^{125}I]NPY, [^{125}I]PYY, [^{125}I]GR231118 and [^{3}H]J-104870 binding [99, 101, 103]. Furthermore, mutation of Phe173 in TM4 by Ala resulted in the loss of specific [^{3}H]J-104870 binding whereas an Ala mutant of Lys303 in TM7 decreased specific [^{125}I]GR231118 binding without affecting [^{3}H[BIBP3226, [^{125}I]NPY or [^{125}I]PYY binding [99, 103]. Contradictory results have also been reported. For example, while Du et al. have reported that mutation of Phe173 in TM4, Phe286 in TM6 and His 298 at the top of TM7 resulted in a decrease in [^{3}H]BIBP3226 binding while [^{125}I]PYY was not affected [101], Sautel and collaborators found that both [^{125}I]NPY and [^{3}H]BIBP3226 bindings were affected by mutation of Phe286, while mutation of His298 resulted in a decrease in [^{125}I]NPY but not [^{3}H] BIBP3226 binding [99]. Overlaps between different radioligands have also been reported, revealing that different radioligands interact with the same amino acid residues of the Y_1 receptor, especially Trp163 in TM4 and Asp 287 at the top of TM6 [99, 103]. Additionally, it has been shown that glycosylation sites in the N-terminus are crucial for correct expression of the Y_1 receptor at the cell surface, especially Asn11 [102], while having no effect on its binding properties [100], suggesting that glycosylation sites are required for receptor trafficking in mammalian cells. Deletion of the C-terminal tail of the Y_1 receptor had no effect on its binding property [97]. Finally, since it has been suggested that NPY preferentially binds at the surface of the transmembrane segment [98], it is surprising that substitution of Trp163, which is located deep in the fourth transmembrane domain abolished binding of all radioligands [103]. It may be that this substitution induces a change in the tridimensional conformation of the Y_1 receptor. This may also apply for mutation of Asp85 located in deep TM2 by either Asn, Ala or Glu abolished [^{3}H]NPY binding [102]. Taken together, these data indicate that Y_1 receptor agonists and antagonists share overlapping binding sites, but each ligand also interacts with specific amino acid residues of the Y_1 receptor.

As to other subtypes, considering that the Y_2 receptor antagonist, BIIE0246 has been found to be inactive on the cloned chicken Y_2 receptor [104], comparative sequence between the chicken and human Y_2 receptor lead to the identification of three amino acid residues that could be responsible for BIIE0246

binding to the Y_2 receptor subtype namely Gln 135, Leu227 and Leu284 [105]. Of these three amino acids, Leu227 in the TM5 appears to be the most important in the direct interaction between BIIE0246 and the Y_2 receptor protein [105]. Additionally, no data is currently available regarding the interaction of NPY and its homologues with the Y_4 and Y_5 receptor proteins.

Neuropeptide Y receptor ligand profile

Endogenous ligands

NPY and PYY have low nanomolar affinities for Y_1, Y_2, Y_5 and y_6 receptors while PPs are much less potent (over 100 nM; except human PP for the Y_5 subtype) [4] (Tab. 3). In contrast, PPs are more potent on the Y_4 subtype than NPY

Table 3. List of agonists and antagonists of the NPY family that display nanomolar affinities for a given NPY receptor subtype

Subtype	Agonists	Antagonists
Y_1	NPY [67] PYY [212] [Leu^{31}, Pro^{34}]NPY [67] [Leu^{31}, Pro^{34}]PYY [213] [Arg^{6}, Pro^{34}]NPY [116] [Phe^{7}, Pro^{34}]NPY [116]	BIBP3226 [125] BIBO3304 [126] GR231118 [138] GR231118-OMe [146] GI264879A [131] LY357897 [127]
Y_2	NPY [76, 77] PYY [77] NPY(2–36) [77] NPY(3–36) [77] PYY(3–36) [77] C2–NPY [77] CycloS-S [Cys^{20}, Cys^{24}]NPY [116] Truncated NPY [119, 120]	BIIE0246 [147] JNJ-5207787 [151]
Y_4	rPP [81, 106] hPP [81, 106] GR231118 [142] [Leu^{31}, Pro^{34}]PYY [175]	
Y_5	NPY [89, 90] PYY [89, 90] NPY(3–36) [89, 90] PYY(3–36) [89, 90] [Leu^{31}, Pro^{34}]NPY [89, 90] [Leu^{31}, Pro^{34}]PYY [89, 90] hPP [188, 189] [hPP(1–17), Ala^{31}, Aib^{32}]NPY [122] [cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP [122]	CGP71683A [152] JCF 109 [153] NPY5RA [154] GW438014A [156] L-152,804 [157] CP732925 [155]

and PYY [80–82, 106] (Tab. 3). These endogenous peptides are processed at their N-termini by a dipeptidyl peptidase IV enzyme which remove the first two amino acid residues to generate C-terminal fragments, 3–36 [107–109]. These fragments, especially NPY(3–36) and PYY(3–36) demonstrate marked decreases in affinity for the Y_1 subtype while being as potent as the native peptide on the Y_2 and Y_5 receptors [4] (Tab. 3). Interestingly, it has recently been shown that circulating PYY(3–36) and PPs that are released after a meal can inhibit food intake [110, 111]. In addition, the NPY fragment NPY(1–30) has been isolated from the rat brain [112] and that both NPY [113] and PYY [114] can be hydrolyzed by neutral endopeptidase-24.11 to generate this fragment. The physiological relevance of this fragment is unknown but could be related to the termination of NPY and PYY effects, since N-terminal fragments have very poor affinities for a variety of NPY receptors [115]. On the other hand, it has been reported that NPY(1–30) could decrease spontaneous locomotor activity and induce hypothermia, suggesting limited activities in some paradigms [30].

Synthetic agonists

Recently, more selective Y_1 receptor agonists have been developed. In contrast to [Leu^{31}, Pro^{34}]PYY or [Leu^{31}, Pro^{34}]NPY which bind to Y_1, Y_4 and Y_5 receptor subtypes with similar affinities, [Arg^{6}, Pro^{34}]NPY and [Phe^{7}, Pro^{34}]NPY were shown to be 100 to 1000 times more potent on Y_1 than Y_2 and Y_5 receptors [116, 117]. However, their affinity for the Y_4 subtype has not been investigated yet. Interestingly, substituted analogues of [Pro^{34}]PYY in position 6 or 7 (by Arg and Phe, respectively) may represent highly selective radioligands for the Y_1 subtype. Truncated analogues of NPY [118–121] revealed to be potent and selective Y_2 receptor agonists. Recently, a new highly selective Y_2 receptor agonist, cycloS-S[Cys^{20}, Cys^{24}]NPY was shown to interact with Y_2 receptors in low nanomolar affinity while being almost inactive in Y_1 and Y_5 (Ki of 1500 and 6250, respectively) assays [116]. This peptide represents a major advantage over other truncated NPY analogues. Additionally, the development of highly selective Y_5 receptor agonists have been reported including [Ala^{31}, Aib^{32}]NPY, [hPP(1–17), Ala^{31}, Aib^{32}]NPY and [cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP [122, 123].

Synthetic antagonists

A number of non-peptide receptor antagonists for the Y_1 receptor (Tab. 3) have been reported thus far including SR120819A [124], BIBP3226 [125], BIBO3304 [126], LY357897 [127], J-104870 [128], diaminoalkyl substituted benzimidazole [129], 1-substituted-4-methylbenzimidazole [130], GI264879A [131], N-[3-(4-methylphenyl)-3-(2-pyridyl)propyl]-N'-[3-(1*H*-imidazole-4-

yl)propyl]guanidine [132], J-115814 [128], H409/22 [133] and benzazepines derivatives [134, 135]. Some of these antagonists have been used either as radioligands [136] or blockers to investigate the autoradiographic distribution of the Y_1 subtype and to establish in greater details their ligand selectivity profiles. Most of these molecules demonstrated better selectivity and specificity for the Y_1 *versus* other NPY receptor subtypes as compare to most Y_1-preferential agonists [49, 59, 137].

The peptidergic Y_1 antagonist, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$ known as GR231118 or 1229U91 [138, 139] has been radiolabeled and use as radioligand to target Y_1 receptor sites [140, 141]. Most specific [^{125}I]GR231118 labelling is sensitive to Y_1 receptor antagonist [140, 141]. However, low levels of labelling to the Y_4 subtype cannot be excluded, since GR231118 has potent agonistic properties on Y_4 receptors [142, 143] and its radiolabeled form binds with low nM affinities to cells transfected with the Y_4 receptor cDNA [140].

The difference between [^{125}I]GR231118 and [^{125}I][Leu^{31}, Pro^{34}]PYY to label Y_1 sites is that [^{125}I]GR231118 can target Y_1 binding sites in their high and low affinity states while [^{125}I][Leu^{31}, Pro^{34}]PYY recognizes only the high affinity state. On the other hand, using both agonist ([Leu^{31}, Pro^{34}]PYY) and antagonist (GR231118) as radiolabeled probes may provide valuable information as it is well known that antagonists bind to GPCR in low and high affinity states with similar affinities whereas agonists are able to discriminate between affinity states. Interestingly, competition binding experiments using human brain tissues revealed that most Y_1 receptors are in the low affinity state in this preparation [144]. Under these conditions, [^{125}I]GR231118 could prove more useful as it can target all affinity states of the receptor. Moreover, comparison between specific [^{125}I][Leu^{31}, Pro^{34}]PYY/BIBO3304-sensitive and [^{125}I]GR231118/BIBO3304-sensitive sites following drug treatment or under pathological conditions could provide more detailed information on Y_1 receptor dynamics as we recently demonstrated in p-chlorophenylalanine (PCPA) treated animals [145]. Interestingly, replacing the amide by a O-methyl ester residue (OMe) in GR231118 resulted in an analogue that conserved its affinity for the Y_1 receptor subtype but has markedly reduced affinity for the Y_4 receptor [146]. This could represent a good radioligand for the Y_1 receptor.

A Y_2 receptor antagonist, BIIE0246 has been developed [147] and revealed to be highly selective for the Y_2 *versus* Y_1, Y_4 and Y_5 subtypes in various assays [148–150]. Recently, the characterization of a second Y_2 receptor antagonist has been reported, JNJ-5207787 [151]. This new antagonist is apparently ten times less potent than BIIE0246, but has the main advantage to be able to cross the blood brain barrier [151].

The characterization of several Y_5 receptor antagonists have been reported thus far (Tab. 3) including CGP71683A [152], JCF109 [153], NPY5RA-972 [154], 3-[2-[6-(2-tert-butoxyethoxy)pyridin-3-yl]-1H-imidazol-4-yl]benzonitrile hydrochloride salt [155], GW438014A [156], L-152,804 [157], pyrrolo [3, 2-d] pyrimidine derivatives [158], 2-substituted 4-amino-quinazolin deriv-

atives [159], alpha-substituted N-(sulfonamino)alkyl-beta aminotetralins [160–162]. With their wide distribution to academic laboratories, it should help to establish better the role of the Y_5 receptor in the organism.

On the possible existence of other NPY receptor subtypes

Various groups have proposed the existence of a receptor that possesses high affinity for NPY, but not PYY, in several assays including the rat brain [163], rat colon [164], rat lung [165] and rat and bovine adrenals [166]. However, evidence for the existence of this subtype is still circumstantial and this receptor has not been cloned yet. High levels of Y_1 receptors were found to be expressed in bovine chromaffin cells [167]. Additionally, Y_2 and Y_4 mRNA are known to be expressed in the rat colon [168] and brainstem nuclei [169]. It thus appears that the atypical pharmacological profile "(Y_3)" observed in these tissues may in fact result from the expression of multiple known subtypes of NPY receptors. Accordingly, a more detailed study using NPY receptor antagonists have demonstrated that the contractile effects induced by NPY and related molecules in the rat colon was due to the activation of both Y_2 and Y_4 receptors [170].

Using either [^{125}I]hPP [171], [^{125}I]rPP [42] or [^{125}I]bPP [172–174], moderate to very high levels of specific binding were seen in the medial preoptic area, paraventricular hypothalamic nucleus, interpeduncular nuclei, nucleus tractus solitarius, area postrema and dorso vagal nucleus of the rat brain. The detailed ligand selectivity profile of those sites has not been clearly established and may represent more than a single population of sites. In agreement with this hypothesis, quantitative receptor autoradiography demonstrated that the labelling seen with [^{125}I]rPP (a Y_4 ligand with low affinity for the Y_5 receptor) was similar albeit not identical to that of specific [^{125}I]hPP sites. Indeed, some rat brain nuclei are enriched with specific [^{125}I]hPP binding but not [^{125}I]rPP binding (Tab. 4) [42]. Furthermore, considering that GR231118 [142, 143] and [Leu^{31}, Pro^{34}]PYY [175] as well as their iodinated counterparts [140, 172] possess high affinities for the Y_4 receptor subtype, it was surprising that only very low specific [^{125}I]GR231118 [140, 141] and [^{125}I][Leu^{31}, Pro^{34}]PYY [172, 176–178] binding was detected in rat brain areas such as the medial preoptic area, paraventricular hypothalamic nucleus and interpeduncular nuclei. Taken together, those results suggest that [^{125}I]hPP could recognize additional population of NPY-related binding sites. These specific sites may represent the Y_5 subtype as hPP is known to possess high affinity for this receptor [89, 178]. On the other hand, if they were of the Y_5 subtype, it should have been possible to detect them using [^{125}I][Leu^{31}, Pro^{34}]PYY in the presence of Y_1 receptor antagonist, but this was not the case [178]. Furthermore, binding sites characterized as Y_5 receptors in the rat brain [178, 179] may in fact include more than one population of sites. Accordingly, further studies using highly selective Y_4 and Y_5 receptor agonists and antagonists as well as molecular approaches will be

Table 4 Quantitative autoradiographic analysis of [^{125}I]hPP and [^{125}I]rPP in various regions of the rat brain

Brain regions	[^{125}I]hPP	[^{125}I]rPP
External plexiform of the olfactory bulb	15 ± 2*	5 ± 1
Anterior olfactory nucleus	27 ± 2*	13 ± 1
Frontal cortex	8 ± 2	11 ± 2
Parietal cortex	9 ± 1	8 ± 2
Caudate putamen	19 ± 2	23 ± 2
Ventral hippocampus	24 ± 2*	16 ± 1
Medial preoptic area	11 ± 1	10 ± 1
Paraventricular hypothalamic nucleus	10 ± 1	9 ± 1
Thalamus	32 ± 2	31 ± 3
Interpeduncular nucleus	100	100
Cerebellum	3 ± 1	3 ± 1
Nucleus tractus solitarius	34 ± 3*	22 ± 2
Area postrema	205 ± 15*	150 ± 13
Dorsal vagal nuclei	109 ± 9	98 ± 8

Comparative quantitative autoradiographic data represented as percentage of specific binding (total binding being substracted from the nonspecific binding obtained on adjacent coronal sections) quantified in the interpeduncular nucleus (100%) for both [^{125}I]hPP and [^{125}I]rPP. Values are the mean ± SEM of 3 to 5 determinations. * $p < 0.01$

required to resolve this issue. In that regard, we have recently demonstrated the existence of specific [^{125}I][Leu^{31}, Pro^{34}]PYY sites resistant to blocking concentrations of Y_1, Y_4 and Y_5 agonists and antagonists as well as specific binding labelled by [^{125}I]hPP that is insensitive to Y_4 and Y_5 agonists and antagonists [180]. Moreover, Herzog and collaborators have recently demonstrated the existence of specific [^{125}I]PYY sites that were resistant to Y_5 agonists in Y_1, Y_2 and Y_4 triple-knockout mice [181]. Those sites unlikely represent y_6 receptors, since *in situ* hybridization did not revealed positive signal for y_6 receptor mRNA in the hippocampus of the triple knockout mice [181].

NPY receptor localization

Various techniques are presently available to determine the distribution and localization of NPY receptors. These techniques include *in vivo* and *in vitro* bioassays, receptor binding assays, receptor autoradiography, *in situ* hybridization and immunohistochemistry. However, to be fully effective these techniques required highly selective and specific tools. Over the past 15 years, considerable efforts have been done in order to develop selective and specific agonists, antagonists and antibodies to characterize and visualize each NPY receptor subtypes [137].

NPY receptor binding sites have been characterized and their differential distribution investigated using various radioligands including [^{125}I]NPY [182], [^{125}I]PYY [183, 184], [^{125}I][Leu^{31}, Pro^{34}]NPY [185], [^{125}I][Leu^{31}, Pro^{34}]PYY [177, 186], [^{125}I]NPY$_{2-36}$ [187], [^{125}I]PYY$_{3-36}$ [177, 186], [^{125}I]bPP [172], [^{125}I]hPP [171], [^{125}I]GR231118 [140, 141], [^{3}H]BIBP3226 [136], [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY [188] and [^{125}I][cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP [189]. Additionally, the distribution of functional NPY receptors have been addressed by evaluating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) using stable GTP analogues ([^{35}S]GTPγS) following agonist stimulation [190, 191]. However, most agonists used in those studies are known to recognize more than one receptor subtypes and/or radiolabeled molecules possess signal/noise ratio that are too high for detailed receptor binding studies. The development of iodinated non-peptide Y_1, Y_2, Y_4 and Y_5 receptor antagonists will prove most useful to characterize and establish the detailed distribution of each NPY receptor in the CNS and peripheral tissues, since NPY receptor antagonists display better selectivity than agonists for a given NPY receptor subtype.

In parallel, the development of selective and specific NPY receptor antibodies should be most useful to investigate in detail the discrete localization of NPY receptors at the light and electron microscopic level in a variety of tissues. In that regard, it was recently demonstrated that Y_1 receptor antibody directed against its C-terminal region was highly selective [192, 193] as no signal was observed in Y_1 knockout (KO) mice [192]. This was not the case for other Y_1 antibodies directed against the N-terminal or extracellular loop of the Y_1 receptor [192]. Additionally, Y_5 receptor antibodies have recently been developed and used to study the anatomical distribution of this receptor class in the brain [193–195]. To our knowledge, Y_2 and Y_4 receptor antibodies have yet to be reported and/or fully characterized yet.

Among the various NPY receptors, the Y_1 and Y_2 subtypes are the most abundant proteins expressed in all species studied thus far [59, 179, 191]. In peripheral tissues, the Y_1 and Y_2 subtypes are expressed in various tissues including blood vessels, heart, airways, adrenal and pancreatic glands, kidney, urogenital and gastrointestinal tracts [13]. In blood vessels, Y_1 receptors mediate vasoconstriction [196, 197]. The Y_2 receptor subtype is predominantly found at the pre-synaptic level, and is involved in the inhibition of neurotransmitter release, especially noradrenaline [198, 199]. However, post-synaptic Y_2 receptors have also been reported to exist in blood vessels [200].

In the rat CNS, Y_1 receptors are predominantly expressed in the olfactory nuclei, cerebral cortex, claustrum, thalamus, medial geniculate nucleus, brainstem nuclei and lamina 1 and 2 of the spinal cord [42]. Overall, the distribution pattern of Y_1 receptors obtained using different techniques such as receptor autoradiography [140, 177], [^{35}S]GTPγS-associated Y_1 receptor activation [191], *in situ* hybridization [169] and immunohistochemistry [192] is highly similar. Additionally, there is evidence that the Y_1 receptor subtype may act as an autoreceptor in the CNS [201, 202] in addition to its better established post-

synaptic localization. The recent development of highly specific and selective Y_1 receptor antibodies [192] will be very useful to evaluate the distribution of Y_1 receptors at the electron microscopic level, and to determine the cellular phenotype(s) that express this subtype. Already, there is some evidences that Y_1 receptors are not limited to neuronal cells but can also be expressed in glia [203].

The distribution seen for Y_2 receptor is markedly different from that observed for Y_1 receptors in the CNS [42]. High levels of Y_2 receptors are expressed in the lateral septum, hippocampus, septofimbrial nucleus, stria terminalis, brainstem nuclei and lamina 1 and 2 of the spinal cord, while moderate levels are detected in various hypothalamic nuclei and substantia nigra, pars compacta. Moreover, as also seen for the Y_1 receptor subtype, the overall distribution of Y_2 receptors established using radiolabeled probes such as [^{125}I]PYY(3–36) [148, 177], [^{35}S]GTPγS in the presence of C2-NPY to activate Y_2 receptors [191] or *in situ* hybridization [169, 191] is rather similar. However, species differences exist in the level of expression of Y_1 and Y_2 receptor proteins [42, 179, 204].

The expression of the Y_4 and Y_5 receptor mRNAs and proteins has also been reported in both central and peripheral tissues. However, levels are usually lower than that of the Y_1 and Y_2 subtypes. Northern blot analysis revealed the presence of Y_4 receptor mRNA in the brain, lung, gastrointestinal tract and testis [81], while *in situ* hybridization demonstrated only low levels of expression of the Y_4 receptor mRNA in the rat brain [169].

The distribution of Y_4-like receptor binding protein has been investigated using [^{125}I]PP [171, 172, 174]. A very discrete labeling was obtained with these probes with moderate to very high amounts of binding found in the medial preoptic area, paraventricular nucleus of the hypothalamus, interpeduncular nucleus, nucleus tractus solitarius and area postrema. Interestingly, the medial preoptic area, paraventricular nucleus of the hypothalamus and interpeduncular nucleus contained much lower amounts of specific [^{125}I][Leu^{31}, Pro^{34}]PYY [172, 177] and [^{125}I]GR231118 [140, 141] binding sites even if these two radioligands possess high affinities for the Y_4 receptor subtype [142, 175]. Moreover, it was recently been shown that agonist-stimulated [^{35}S]GTPγS failed to detect Y_4 receptor activation with rPP, GR231118 and [Leu^{31}, Pro^{34}]NPY [191]. This could be taken as evidence for the existence in these regions of yet another receptor that is preferentially recognized by PP-related molecules (see above).

In situ hybridization signals of the Y_5 receptor mRNA were observed in the external plexiform layer of the olfactory bulb, anterior olfactory nuclei, olfactory tubercle, piriform cortex, hippocampus, suprachiasmatic and arcuate nuclei of the rat brain [169, 205, 206]. In human, strong *in situ* hybridization signals of Y_5 receptor mRNA have been found at the level of the arcuate nucleus, while low levels were observed in most other human brain structures [207].

A few years ago, we reported that [^{125}I][Leu^{31}, Pro^{34}]PYY specifically bound to at least two different populations of sites (Y_1 and Y_5) in the rat brain

using either BIBP3226 [178] or BIBO3304 [176], two Y_1 receptor antagonists [126, 208] as blocking agents. Competition binding assays of sites labelled by [^{125}I][Leu^{31}, Pro^{34}]PYY in the presence of Y_1 blockers revealed a ligand selectivity profile similar to that reported for Y_5 receptors and those sites were found to be located in the external plexiform layer of the olfactory bulb, lateral septum, dentate gyrus, nucleus tractus solitarius and area postrema [178]. However, under these conditions, the possible labelling of the Y_4 receptor could not be fully excluded as [^{125}I][Leu^{31}, Pro^{34}]PYY also possesses a rather high affinity for this subtype [175]. Most recently, we have developed [^{125}I][hPP(1–17), Ala^{31}, Aib^{32}]NPY as the first Y_5 radioligand [188]. However, this probe displayed rather high non-specific binding rendering it less than optimal for autoradiographic studies. We have thus radiolabeled [cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP and characterized its binding properties in rat brain homogenates and transfected cell lines. Our results demonstrated that [^{125}I][cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP binds with high affinity to the Y_5 receptor, but not to the Y_1, Y_2 and Y_4 subtypes. Furthermore, receptor autoradiography studies have shown that significant amounts of specific [^{125}I][cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP binding sites are found in the lateral septum and area postrema of the rat brain, while very low levels of specific sites were observed in other brain structures [189]. This receptor distribution is somewhat distinct from that previously reported using [^{125}I][Leu^{31}, Pro^{34}]PYY under blocking condition. Additionally, while significant amounts of specific [^{125}I][Leu^{31}, Pro^{34}]PYY/Y_1-insensitive sites (Y_5-like) were found in the nucleus tractus solitarius [178], we were unable to detect significant amounts of specific [^{125}I][cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP binding sites in this nucleus [189]. Moreover, Shaw and collaborators were not able to observe any increase in [^{35}S]GTPγS binding using [cPP(1–7), NPY(19–23), Ala^{31}, Aib^{32}, Gln^{34}]hPP in the rat brain [191]. It thus appears that the distribution of sites previously reported for the "Y_5-like subtype" may in fact represents a heterogeneous population of sites.

Messenger RNA for the y_6 receptor is expressed in the hypothalamus and in the kidney of the mouse [95]. However, there is no conclusive evidence that the y_6 receptor protein in indeed expressed in mouse, rabbit or dog. The fact that mice may express functional y_6 receptor protein is one of the major concerns for the interpretation of results obtained in this species, especially in regards to receptor knockout studies.

General Conclusion

The recent development of selective agonists and antagonists for the various NPY receptor subtype family has greatly facilitated the characterization of the subtype(s) involved in a given effect of NPY, PYY and the PPs. On the other hand, some of these molecules still lack full selectivity and specificity. For

example, BIBP3226 and BIBO33034, two Y_1 receptor antagonists without significant affinity for Y_2, Y_4 and Y_5 receptors were shown to display some affinity (100 nM) for NPFF receptors [209, 210]. Additionally, CGP71683A, a Y_5 antagonist devoid of significant affinity for Y_1, Y_2 and Y_4 receptors [176] was reported to display high affinity for muscarinic and adrenergic receptors, and serotonergic uptake carrier proteins [211]. Accordingly, extra care must be taken in the interpretation of results obtained using these molecules, especially *in vivo* assays. Furthermore, data obtained in mice should take into account the possible involvement of the y_6 receptor usually expressed in this species [93]. It is expected that over the next few years, use of selective NPY receptor subtype agonists and antagonists, receptor antibodies, knockout and transgenic animals (rat and mice) will help to delineate the precise role of this family of peptide in the organism.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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Molecular ligand-receptor interaction of the NPY/PP peptide family

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Introduction

The three peptides neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) sharing a similar structure belong to the NPY hormone family and activate G-protein coupled NPY receptors including several subtypes, namely Y_1, Y_2, Y_3, Y_4, Y_5 and y_6. Structure-affinity and structure-activity relationship studies of peptide analogues gave an insight in the individual requirements concerning the bioactive conformation of the ligand at the various receptors and supplied important information for the development of subtype selective compounds with function as agonists but also as antagonists. Based on these results initial receptor mutagenesis investigations were performed leading to the identification of some key residues of the ligand-receptor interaction. This will help to evolve reliable 3D models of NPY receptors and can be further used for NPY docking studies. To understand the complex mechanism of G-protein-coupled receptor (GPCR) activation the role of receptor dimerization in this process has to be clarified. Recently, also for NPY receptors homo- such as heterodimerization has been shown. In this article, we summarize characteristic features of the subtype specific ligand-receptor interaction elaborated so far.

NPY, PP and PYY a peptide ligand family

Neuropeptide Y (NPY) represents together with peptide YY (PYY) and the pancreatic polypeptide (PP) a ligand family of neuroendocrine hormones (NPY family) which has been of increasing interest in the last 20 years. These peptides are formed by proteolytic processing of preprohormones [1, 2]. The almost exclusively in endocrine pancreas and intestinal cells expressed PP was the first member of this peptide family that was identified and sequenced after finding as a contamination in extracts of chicken insulin [3, 4]. Evolution studies concerning the NPY peptide family revealed PP as the most rapidly evolving neuroendocrine peptide among tetrapods with only 50% identity between

mammals, birds and amphibians [5]. Only seven residues (Pro^{5}, Pro^{8}, Gly^{9}, Ala^{12}, Tyr^{27}, Arg^{33} and Arg^{35}) are invariant among species studied so far [6] (Fig. 1). PP mainly acts as hormone which is released in response to meals and thereby regulates pancreatic and gastric secretion [7, 8]. Beside this metabolic function the existence of PP binding sites in several rat and human brain regions also suggests an effect on brain. An important role with respect to controlling the secretory function of the cortex in a paracrine manner was proven [9]. PYY was first isolated from porcine intestine and is similar to PP, a hormone that is released in the gastrointestinal tract for the regulation of meal digestion [10, 11]. Additionally, PYY could be found in neurons, which also indicates a neuronal function for PYY [12]. Compared to PP, PYY is less variable between different species, particularly in mammals that have nine differences to the gnathostome ancestor [5]. NPY was first characterized from porcine brain [13, 14] and has remained extremely well conserved during evolution with no gnathostome species differs from the ancestral gnathostome sequence at more than five positions [5, 15]. It is widely distributed within the central and peripheral nervous system. NPY represents one of the most abundant neuropeptides in the brain [16] and plays an important role in the regulation of many physiological mechanisms, such as food intake [17–19], thermogenesis [20], memory [18, 21] and reproduction [22]. The latter results from the stimulatory effect of NPY on luteinizing hormone (LH) secretion. In the periphery, NPY could be proven in sympathetic nerves, co-stored and co-released with norepinephrine [23], and in nonsympathetic neurons of several organs. For example NPY, like PYY, has an influence on blood pressure, which is exerted through vasoconstriction in skeletal muscles [24], heart [25], kidney [26] and brain [27]. As common structural features NPY, PYY and PP consist of 36 amino acids (except chicken PYY, which is 37), are C-terminally amidated, show several tyrosine residues and have a considerable amino acid homology [28]. Seven residues are constant between all species of NPY, PYY and PP; these are Pro^{5}, Pro^{8}, Gly^{9}, Ala^{12}, Tyr^{27}, Arg^{33} and Arg^{35}.

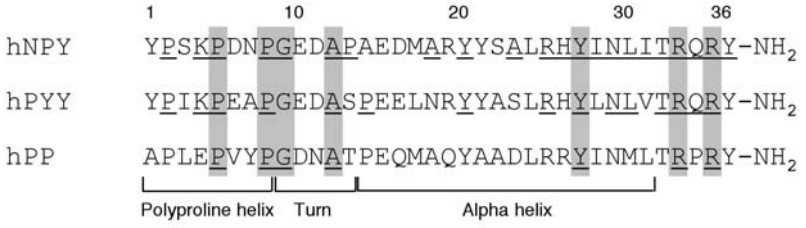

Figure 1. Alignment of the human neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) amino acid sequence. Constant positions in all species for the three peptides are underlined. The seven constant residues within the NPY-family are indicated (boxed). The schematic structure shown at the bottom corresponds to the x-ray structure of avian PP [30].

Structure of the ligand

Corresponding to the high degree of amino acid homology between the peptides of the NPY family it was assumed that they are able to adopt similar secondary structures [29]. The first structural idea is based on X-ray crystallography using crystals of symmetric dimers of avian (turkey) PP (aPP). It comprises an extended type II polyproline helix (residue 1–8) that is followed by a turn (residue 9–13) and an ambiphatic α-helix (residue 14–31) [30]. The well defined tertiary structure is characterized by a typical hairpin-like fold, also named PP-fold, which is stabilized by hydrophobic interactions between residues of the N-terminal part and the α-helix. The five most carboxy-terminal residues are less structurally defined and oriented in opposite direction with respect to the α-helix. Nuclear Magnetic Resonance (NMR) studies support a similar model for human NPY, a polyproline stretch (residue 1–10) followed by a tight hairpin structure (residue 11–14) and two short α-helices (residues 15–26 and 28–35). Hydrophobic interactions promote the packing of the two helices in a typical β-hairpin fold. Other NMR studies suggest a dimerization of NPY that is characterized by an antiparallel, hydrophobic packing of two helical units with the N-terminal segment poorly defined [31–34]. Latest results indicate that NPY exists as dimer only at high, millimolar concentrations. Dimer stabilization results from intermolecular hydrophobic interactions whereas the α-helical segments are in parallel and antiparallel orientation. As NPY is active *in vivo* at nanomolar concentration a shift of the dimerization equilibrium occurs towards the monomeric form. Based on fluorescence resonance energy transfer studies a folding back of the N-terminal tail onto the C-terminal α-helix (PP-fold) could not be confirmed for monomeric NPY. The monomeric structure of NPY in the presence of micelles reveals a flexible N-terminal segment (residue 1–13) and an α-helical C-terminus (residue 14–36) that is orientated parallel to the micelle surface. Accordingly, the C-terminal residues 32–36 become α-helical in the membrane bound state in contrast to the flexibility in solution [35–37].

The Y Receptors

NPY, PYY and PP bind to a network of receptor subtypes that belong to the rhodopsin-like superfamily of G-protein coupled receptors. Currently five different receptor types have been cloned in mammals (Y_1, Y_2, Y_4, Y_5 and y_6) that can be distinguished by their affinity for NPY, PYY and PP. They have been described pharmacologically indicating an overlapping binding pattern for the three peptide ligands. Since the Y_1, Y_2 and Y_5 receptor differ more from each other than any other G-protein coupled receptors with only 27–31% overall identity and 40–43% in the transmembrane (TM) helices this partly common binding profile is surprising [38]. An additional Y_3 receptor has been postulated from pharmacological studies using various tissue preparations. The y_6

receptor is expressed functionally in rabbit and mice whereas in primates only mRNA for the truncated form of y_6 is present. In rat, this receptor subtype is absent as well [39–41].

Activated NPY receptors stimulate inhibition of intracellular cAMP accumulation via pertussis toxin-sensitive G-proteins (G_i/G_o) [42–44]. Investigations in smooth muscle identified a co-expression of Y_1, Y_2 and Y_4 receptors that exhibited distinctive patterns of coupling to G-proteins [45]. All three subtypes were negatively coupled to adenylyl cyclase via one or more isoform of G_i (mainly G_{i2}) and it was shown for the first time that Y_2 and Y_4 are able to couple to G_q and stimulate IP_3 formation and intracellular Ca^{2+} mobilization, additionally. Furthermore also for the Y_1 receptor a coupling to phosphotidylinositol hydrolysis could be shown depending on the cell type in which the receptor is expressed [42, 46, 47]. Other signaling pathways such as activation of mitogen-activated protein kinase in gut epithelial cells [48], inhibition of K^+ and Ca^{2+} channels have been observed in the vasculature [49] and in neurons [50], respectively [51].

The Y_1 receptor

The Y_1 receptor was the first NPY receptor that has been cloned [52] and it displays 90–96% overall identity across mammals [38]. The human Y_1 consists of 384 amino acids [43] and three splice variants were characterized that yield multiple promoters with tissue specific expression patterns [53]. In contrast to the other NPY receptors, the coding region of Y_1 harbors an intron of about 100 base pairs in all species investigated so far. Interestingly, this intron has been shown to enhance the expression of the Y_1 and Y_5 receptor *in vitro* [54]. The Y_1 receptor is expressed in vascular smooth muscle cells [55–57], cerebral cortex [58, 59], colon [60] and human adipocytes [61]. The cellular receptor distribution provides hints about its function. Most of the vascular [62–64] and antinociceptive effects [65, 66] of NPY contribute to the Y_1 receptor. In addition, this subtype is involved in the feeding response [67–69], together with the Y_5 receptor. Several physiological functions, such as decreased anxiety [70] and depression [71] underlay the regulation by Y_1 receptors. Recent studies emphasize the importance of NPY in the consumption of ethanol and confirmed a Y_1 receptor participation in this process [72, 73]. Studies using radioligand binding [74], confocal microscopy with fluorescent ligands [75] or green fluorescent protein tagged receptor [76] showed rapid Y_1 receptor internalization upon agonist stimulation. In agreement with the high internalization rate it was furthermore proven that agonists induce a rapid Y_1 receptor association with β-arrestin 2 that is known to mediate internalization of GPCRs [77]. The human cell lines SK-N-MC [78, 79] and erythroleukemia (HEL) cells [80, 81] stably express Y_1 receptors such as the rat cell line PC12 [82].

The Y_2 receptor

The Y_2 receptor gene was first cloned from human SMS-KAN cells [83] and is also available in a human astrocytoma cell line, LN319 [84], as well as in CHP234 [85, 86]. The human Y_2 receptor consists of 381 amino acids. Like Y_1, the Y_2 receptor shows a high conservation in mammals with more than 90% identity [87–90] and about 80% identity when comparing mammalian and chicken Y_2 [91]. The Y_2 receptor is located in sympathetic and parasympathetic nerve fibres [92, 93], hippocampus [94, 95], intestine [96] and certain blood vessels. The typical responses associated with this receptor are enhanced memory retention [97] and presynaptic inhibition of neurotransmitter release, e.g., noradrenaline [57, 98]. The latter may give an explanation for some opposing relationships between generally postsynaptic Y_1 and presynaptic Y_2 receptor. For example Y_1 specific agonists are anxiolytic [99] whereas Y_2 agonists seem to be anxiogenic [99, 100]. Additionally, vascular effects are mediated via Y_2 receptors and this receptor subtype is also involved in angiogenesis [101] as well as in effects related to the circadian rhythm [102, 103]. In contrast to Y_1, the Y_2 receptor does not appear to internalize after prolonged agonist stimulation or does so only very slowly [74, 76]. This is in agreement with a extreme slow and slight association with β-arrestin 2 [77].

The Y_3 receptor

Despite a variety of attempts, the Y_3 receptor has not been cloned until now. This receptor subtype has only been suggested from pharmacological studies of many tissues characterized by its at least 10-fold lower affinity for PYY than for NPY [104, 105]. Since no specific agonists or antagonists have been identified controversial discussions about the real existence of such a "Y_3 receptor site" were induced. The Y_3 receptor is supposed to be expressed in the nucleus tractus solitarius (NTS) of the rat brain stem in which it should be responsible for hypotension and bradycardia [105]. Furthermore, a localization of the Y_3 receptor was suggested in the human adrenal medulla [106], in rat cardiac membranes [107] and in bovine chromaffin cells [108–110]. Additional effects associated with this receptor are inhibition of catecholamine release [110] and modulation of the arterial blood pressure [111].

The Y_4 receptor

The Y_4 receptor was the third NPY receptor that was cloned (after Y_1 and Y_2). As it is characterized by a high affinity for PP it was originally designated as "PP1" receptor [112, 113]. The human Y_4 receptor consists of 375 amino acids. Based on the lower degree of overall sequence identity between different species (74–86%) the Y_4 receptor represents one of the most evolving

GPCRs known so far [38]. In addition, seven silent polymorphisms have been found in the Y_4 receptor. Summarizing, there is a system comprising three of the most conserved receptors (Y_1, Y_2, Y_5) binding a very conserved peptide (NPY) and one of the least conserved receptors (Y_4) interacting with one very rapidly evolving peptide (PP). Y_4 receptor existence could be proven in a variety of tissues, e.g., brain, hypothalamus, skeletal muscle, thyroid gland, heart, prostate, stomach, small intestine, colon and pancreas [112, 113]. The effects that are transmitted via this receptor subtype might be these described for PP, such as inhibition of pancreatic secretion, gall bladder contraction [114, 115] and stimulation of LH secretion [116]. Concerning the ability of Y_4 receptor internalization upon agonist stimulation controversy results are available [74, 117]. Investigations of Y_4 receptor association with β-arrestin 2 showed an intermediate behavior compared to Y_1 and Y_2 [77].

The Y_5 receptor

The Y_5 receptor was first cloned from a hypothalamic rat cDNA library [118]. There its receptor gene encodes for a 456 amino acid protein whereas in another report a 445 amino acid protein was identified [118, 119]. Concerning the human Y_5 receptor also different numbers of amino acids are described (see Tab. 1). However, the Y_5 receptor is much larger than the other NPY receptors. The chromosomal localization of the human Y_5 receptor is overlapping and in the opposite orientation to the Y_1 receptor, suggesting a co-regulation of these two subtypes [120, 121]. An interesting structural feature of the Y_5 receptor represents its large third cytoplasmic loop with about 100 residues more than the other NPY receptors. However, the C-terminus is much shorter than in Y_1, Y_2 and Y_4 receptor subtype. The Y_5 receptor shows a lower overall identity with 82–95% between different mammals compared to Y_1 and Y_2 [38, 121]. This is mostly due to the great variability in the third intracellular loop that may induce differences between species in the preference or regulation of G-protein coupling [38, 121, 122]. The Y_5 receptor is expressed in the hypothalamus where it is involved in the stimulation of appetite, together with Y_1 [118]. Borowsky could show that the Y_5 receptor is widely distributed in the human brain, especially in the cortex, putamen and caudate nuleus [120]. In the periphery this subtype can be found in the intestine, ovary, testis, spleen, pancreas, skeletal muscle and liver. Some effects that are transmitted via the Y_5 receptor are reproduction through inhibition of LH secretion [123], regulation of brain seizures [124] and a possible involvement in the regulation of circadian rhythm [102, 125]. A human endometrial cancer cell line stably expressed this receptor after transfection, HEC-1B-hY_5 [126]. Also for Y_5 receptor internalization after agonist stimulation could be shown but it was much slower compared to the internalization of the Y_1 receptor [127]. Concerning association with β-arrestin 2 the Y_5 receptor showed a rapid response [77].

Table 1. Characterization of neuropeptide Y receptor subtypes

Receptor	Y_1	Y_2	Y_4	Y_5
Amino acids in human	384	381	375	445 or 455
Tissue expression	Vascular smooth muscle cells, cerebral cortex, colon, human adipocytes	Nerve fibres, hippocampus, intestine, blood vessels	Hypothalamus, skeletal muscle, thyroid gland, stomach, small intestine, colon	Hypothalamus, cortex, intestine, ovary, spleen, pancreas, skeletal muscle
Prominent actions	Analgesia; anxiolysis; vasoconstriction; food response	Enhanced memory; decreased neurotransmitter secretion; angiogenesis; regulation circadian rhythm	Pancreatic secretion; gall bladder contraction; LH secretion	Reproduction; brain seizure; regulation circadian rhythm
Endogenous agonists order of potency	NPY; [Pro^{34}]NPY; PYY >> C-terminal fragments of NPY or PYY; PP	NPY; PYY ≥ NPY(2–36); NPY(3–36) >> [Pro^{34}]NPY; PP	PP >> PYY; NPY >> PYY or NPY fragments	NPY; PYY; [Pro^{34}]NPY; NPY(2–36); NPY(3–36) > PP
Selective agonists	[Phe^{7},Pro^{34}]NPY; [Leu^{31},Pro^{34}]NPY/PYY; [Arg^{6},Pro^{34}]NPY	NPY(3–36); NPY(13–36); [$Ahx^{5–24}$]NPY	PP; 1229U91	[Ala^{31},Aib^{32}]NPY; [Leu^{31},Pro^{34}]NPY; [D-Trp^{34}]NPY
Selective antagonists	BIBP3226; SR120819; 122991	BIIE0246	-	L-152,804
Signal transduction	cAMP, IP_3, Ca^{2+}	cAMP, IP_3, Ca^{2+}	cAMP, IP_3, Ca^{2+}	cAMP
Prototypical cell line	SK-N-MC, HEL, PC12	SMS-KAN, LN319, CHP234	-	HEC-1B-hY_5

Structural properties of Y receptors

Several modeling studies of the Y_1 receptor and one model of the Y_2 receptor revealed a counter-clockwise arrangement of the seven transmembrane helices when viewed from extracellular side [128–131]. Furthermore TM1 and TM4 were most frequently exposed to the lipid bilayer. The helical packing is guaranteed by interactions between residues of different helices. Some of these residues that were identified by mutagenesis could be confirmed by modeling, e.g., a direct interaction between Asp^{86} (TM2) and Asn^{316} (TM7). Further contacts include a hydrophobic interaction between Tyr^{47} (TM1) and Leu^{303} (TM7) such as a hydrophilic interaction between Thr^{212} (TM5) and Asn^{282} (TM6) [128, 132]. The structure of bovine rhodopsin is characterized by an additional α-helix immediately after TM7 that is located on the inside of the membrane [133]. Sequence comparison revealed that this helical element is also present in the Y receptors. Like many rhodopsin-like receptors the NPY receptors display two conserved extracellular cysteines in the first two extracellular loops (ELCs). It is supposed that they are involved in the formation of a disulfide bond as it is known from the X-ray crystallography structure of bovine rhodopsin [133, 134]. In the hY_1 receptor models the disulfide bond was made between Cys^{113} that is close to the extracellular end of TM3 and Cys^{198} in the second extracellular loop [128, 129, 132]. This disulfide bridge may be important for receptor stabilizing in the correct conformation for ligand binding and receptor activation. Interestingly, the Y_1, Y_4 and y_6 receptor might have an additional cysteine bridge, formed by one cysteine residue in the N-terminus and the other in EC3 [135]. One cysteine in the C-terminal part anchors the tail to the inside of the membrane by palmitoylation in a similar fashion as bovine rhodopsin.

Molecular characterization of ligand-receptor interactions

Structure-affinity/activity-relationship studies (SAR)

Structure-affinity relationship studies supply helpful information on the components involved in a specific ligand-receptor interaction and the conformational requirements of the ligand that are necessary for a successful binding.

Structure-affinity/activity-relationship studies for peptide ligands to Y_1 receptors

The Y_1 receptor is characterized by a high affinity for NPY and PYY whereas PP shows only moderate binding at this receptor subtype [43]. To determine the minimal ligand sequence that is required to elicit activity truncated analogues were tested. N-terminal truncations of NPY and PYY resulted in a decrease of

binding affinity at Y_1 receptor. Shorter segments, like NPY(3–36), NPY(13–36) and NPY(18–36) have only micromolar affinities that identifies the N-terminal region to be essential for Y_1 interaction [136]. Furthermore all C-terminally truncated analogues were completely inactive, e.g., NPY(1–12) and NPY(1–24) [137]. This suggests that several ligand parts are involved in binding at the Y_1 receptor. Furthermore, the replacements of large segments in the central region led to a significant loss of affinity. The connection of N- and C-terminal fragments through a spacer for instance 6-amino hexanoic acid (Ahx) indicated the peptide [Ahx^{8-20}]-pNPY as most potent among a series of centrally truncated analogues [138]. Another possibility to link N- and C-terminal segments is given by the introduction of a disulfide bridge. Optimizing such analogues by the exchange of the chirality of the cysteine residues, the spacer and the position of cyclization resulted in the peptide [Cys^2, Cys^{27}]-NPY showing an improved affinity at the Y_1 receptor [139, 140]. Replacement of Gln^{34} of NPY and PYY by proline had no effect on Y_1 affinity [141]. Consequently, disruption of the C-terminal helix favors the bioactive conformation of the ligand. However, the introduction of D-Pro^{34} resulted in a lower affinity at the Y_1 receptor compared to the corresponding alanine mutant indicating that the orientation of the induced turn is crucial [136, 140]. The facts that Pro^{34} was accepted and that modifications at position six and seven showed only moderate influence on affinity at Y_1 were used for the development of Y_1 receptor selective ligands such as [Phe^7, Pro^{34}]-NPY, [Arg^6, Pro^{34}]-NPY and [Leu^{31}, Pro^{34}]-NPY [78, 142]. Since substitution of Arg^{33} and Arg^{35} for alanine provoked the most significant loss of affinity these two basic residues represent the most sensitive amino acids in the C-terminal decapeptide. Replacements of Tyr^{36} by alanine and D-Tyr^{36} resulted in a loss of affinity. Modification of the C-terminus of NPY by the exchange of Tyr^{36} by various amines, alcohols and modified tyrosine residues turned out that the C-terminal tyrosineamide of NPY plays an important role for Y_1 affinity [143]. The most efficient binding revealed pNPY(1–36)-thioamide and pNPY(1–35)-tyrosinol with a 10- to 100-fold better affinity compared to pNPY(1–36)-methylester. This suggests that the amino part is more involved in binding than the carbonyl group. Consequently, the C-terminal region of NPY is playing a major role in receptor recognition. Based on this many low molecular mass Y_1 antagonists were developed that mimic the C-terminal portion of NPY, e.g., BIBP3226 and SR120819A [144, 145].

Structure-affinity/activity-relationship studies for peptide ligands to Y_2 receptors

In accordance with Y_1, the Y_2 receptor subtype displays high affinity for NPY and PYY [98]. However, one can distinguish both through their different affinities for specific NPY analogues. A feature of the Y_2 receptor is its resistance to N-terminal deletions in contrast to Y_1 [29, 98, 136]. Thereby the removal of Tyr^1 and Pro^2 showed no effect on binding at Y_2 and NPY(13–36), NPY(18–36)

as well as NPY(22–36) still interact with an only 10 times less efficiency. This indicates that the N-terminal part of NPY is not involved in the recognition by the Y_2 receptor. The introduction of certain central truncations (NPY 1–4, 31–36) resulted in a loss of affinity and activity, whereas another analogue [Ahx^{5-24}]-NPY was able to bind at the Y_2 receptor [146, 147, 171]. The results of the L-Ala scan showed less effects compared to Y_1. Pro^5 is the only amino acid in the N-terminal part that showed a severely changed affinity after substitution by alanine. Like for the Y_1, the guanidino groups of Arg^{33} and Arg^{35} are important for the ligand-receptor interaction. The C-terminal localization of the most critical residues suggests that the α-helix is a stringent requirement for Y_2 binding [140]. This was confirmed by the loss of Y_2 affinity after the introduction of the turn inducing proline at position 34 [34, 148]. Another feature that is in contrast to Y_1 is the high affinity of the analogue [D-Tyr^{36}]-NPY indicating that the side chain orientation of this residue is not very important [139].

Structure-affinity/activity-relationship studies for peptide ligands to Y_4 receptors

The Y_4 receptor is characterized by a preferential binding of PP compared to NPY and PYY [28, 149]. Structure-affinity and activity relationship studies revealed species specific variations for the Y_4 receptor that are the result of dramatic differences in receptor sequence [112, 135, 150]. Accordingly, the guinea pig Y_4 receptor resembles more the human Y_4 than does the rat and mouse one with respect to mRNA distribution and pharmacological profile [135]. The guinea pig, human and rat Y_4 receptor bind gpPP, hPP, rPP and cow PP in the low picomolar range, whereas pNPY and pYY display one or two orders of magnitude lower affinities (K_i = 30–700 pM). Previous studies showed only very weak binding of NPY and PYY to the rY_4 (Ki > 150 nM) [112]. Explanations for such discrepancies may be differences between used radioligands and variations between the properties of several cell lines. Deletion of the N-terminal tyrosine of pNPY resulted in a decrease of affinity at gpY_4, hY_4 and rY_4, in which gpY_4 seems to be slightly more sensitive [135]. Also when the first four residues of bovine PP were removed binding at the hY_4 was reduced [151]. Bard et al. could see no effect on binding at hY_4 after the truncation of hPP to PP(2–36) [113]. However, further N-terminal deletion of hPP was disruptive for hY_4 affinity. This is in agreement with a 12- to 40-fold reduction in binding of pNPY(13–36) at the Y_4 receptor of guinea pig, human and rat [135, 151]. Deletion of Tyr^{36}amide induced a substantial reduction in affinity at hY_4 [151]. Alanine substitution in position 34 of pNPY was tolerated by rY_4 while affinity for hY_4 and gpY_4 decreased about 50-fold [149]. Replacement of Ile^{31} or Gln^{34} in pNPY and pPYY by leucine and proline, respectively ([Leu^{31}, Pro^{34}]-pNPY; [Leu^{31}, Pro^{34}]-pPYY) lead to an unchanged affinity at hY_4 and gpY_4 compared to pNPY and pPYY. At the rY_4 receptor these analogues turned out to be even better ligands than the natural peptides [112, 135]. Other studies

also described a modestly increase of [Pro^{34}]-NPY and [Pro^{34}]-PYY binding for the hY_4 [113, 151]. All three receptors don't accept alanine in position 33, 35 and 36 while Ala^{33} substitution had the largest effect with 2,000- to 15,000-fold lower affinity than NPY [149]. Consequently, the C-terminal hexapeptide LTRPRY-amide present in almost all mammalian PPs seem to be an important region in the interaction with Y_4 receptors. Together with the reduced affinity of N-terminal truncated peptides it indicates that both the C- and N-terminus are required for full potency. This is similar to the Y_1 receptor suggesting a comparable binding domain between these two subtypes.

Structure-affinity/activity-relationship studies for peptide ligands to Y_5 receptors

Together with Y_1 and Y_2, the Y_5 receptor displays a higher affinity for NPY and PYY than for PP [119]. Comparable to Y_2 the removal of the first amino acid of NPY (NPY(2–36)) and two N-terminal residues of PYY (PYY(3–36)) had no effect on affinity at gpY_5 and rY_5 [118, 152]. However, deletion of the N-terminal residues 1–21 from NPY influenced binding profoundly with an affinity in the low micromolar range. Among a series of truncated analogues this one with the longest N- and C-terminal fragment ([Ahx$^{9-17}$]-pNPY) showed the highest affinity but still displayed 14-fold reduction compared to NPY [153]. This is in agreement with a more than 1,000-fold decrease of Y_5 receptor binding of truncated hPP analogues ([Ahx$^{5-24}$]-hPP, [Ahx$^{5-20}$]-hPP, [Tyr$^{5-20}$]-hPP). The L-Ala scan revealed that the three N-terminal located proline residues (Pro^2, Pro^5, Pro^8) are necessary for hY_5 affinity with an increasing importance from position 2 to 8 [153]. A similar pattern was obtained for substitutions of the tyrosine residues indicating that Tyr^{20} Tyr^{21} and Tyr^{27} are involved in binding whereas the exchange of Tyr^{27} has the most significant effect of a 600-fold reduced affinity. The replacement of Arg^{25} and Arg^{33} and especially Arg^{35} by L-Ala resulted in a very low affinity in the micromolar range [153]. In contrast, [D-Trp^{32}]-NPY, [D-Trp^{34}]-NPY, [Leu^{31}, Pro^{34}]-NPY, [Ala^{31}, Aib^{32}]-NPY represent potent Y_5 agonists [118, 152–155]. As C-terminal fragments containing Ala^{31},Aib^{32} (e.g., [Ala^{31}, Aib^{32}]-NPY(18–36)) showed high affinity at the Y_5 receptor it is suggested that this motif is able to induce and stabilize the required bioactive conformation of the C-terminal ligand part. Biophysical investigations of this analogue revealed a structure composed of an α-helix ending with a 3_{10}-helical turn of the residues 28–31, followed by a not well defined structure of the last five residues.

Receptor mutagenesis

The Y_1 receptor is the only NPY receptor subtype that was intensively investigated by site-directed mutagenesis until now. Thereby a variety of residues of

the human and rat Y_1 receptor were substituted, in most of the cases to alanine, in order to identify the key ligand-receptor binding points. Based on the finding that positively charged residues close to the joint N- and C-terminal ends of NPY are essential for ligand binding an electrostatic ligand-receptor interaction was postulated. Initially, the negative charged residues in the N-terminal region (5 residues) and the EC-loops of the hY_1 receptor (EC1: 2; EC2: 6 and EC3: 1 residue) were screened for possible binding partners of NPY. Mutations in the N-terminus (E10A, E20A, E29A, D31A, D32A) had no effect on NPY affinity suggesting that these residues are not involved in ligand binding through ionic interaction. The substitution of four amino acids located in the extracellular loops resulted in a loss of ligand affinity, thus they were proposed to interact with NPY (hY_1: Asp^{104} (EC1); Asp^{194}, Asp^{200} (EC2) and Asp^{287} (EC3)) [131]. Later studies confirmed the results of the mutation D104A and D287A [156] and also for the homologous residues in the rat Y_1 receptor an involvement in ligand binding was proven [132]. Whereas the investigation of the mutants D104A and D287A expressed in *E. coli* resulted in an identical binding pattern, the substitution of Asp^{194} and Asp^{200} to alanine displayed a wild-type-like character [157]. Re-expression of these mutants in mammalian cells could confirm their functionality. Binding of PYY at the hY_1 mutant D194A is only slightly affected [158] and the corresponding substitution in the rat Y_1 receptor only led to a 2.7-fold NPY decreased affinity, too [132]. Additionally, Sautel and colleagues reported that D200A bound NPY with the same affinity as the human wild-type receptor [156] and the homologous mutation in the rat Y_1 receptor supplied a slightly reduced binding (5.2-fold) [132]. However, Du et al. showed a loss of PYY binding for D200A [128]. Another discrepancy concerns Asp^{205} that after replacement by alanine revealed no PYY binding [128] or only a 8.5-fold reduction in NPY affinity [131, 156]. Consequently, solely Asp^{104} and Asp^{287} could be identified from several groups as essential receptor residues for ligand binding. However, type of interaction and ligand binding partner so far could not be identified. Recent mutagenesis studies of the hY_2, hY_4 and hY_5 receptor revealed the homologous aspartic residue of hY_1-D287 as being essential for ligand interaction of all receptor subtypes and accordingly to play a general role in ligand binding (unpublished data).

Further mutagenesis experiments tried to confirm docking studies of NPY in a hY_1 model that hypothesized a hydrophobic pocket formed by side chains of amino acids in TM1, 2, 6 and 7 for binding of the amidated C-terminal Tyr^{36} of NPY [131]. Thereby Tyr^{100} in TM2, His^{298} at the top of TM7 and Phe^{286} in TM6 were identified as essential binding partners for this NPY residue through the formation of two hydrogen bonds and one aromatic interaction, respectively. Tyr^{100} and His^{298} were replaced by a variety of amino acids but no substitution could regain complete binding [159]. Other studies also using the mammalian system confirmed these results [156–158]. Expression of H298A in *E. coli* resulted in a loss of NPY affinity, too [157]. Although no NPY binding was detected for F286A, Du and colleagues showed an unaffected PYY

affinity at this mutant. Additionally, substitution of His^{298} for glycine was combined with wild-type-like PYY binding [128]. Alanine replacement of Trp^{163} (TM4), Phe^{173} (TM4), Gln^{219} (TM5), Asn^{283} (TM6) and Trp^{288} (TM6) displayed a loss of ^{125}I-NPY binding, suggesting that these residues are also involved in the interaction with NPY [156]. In contrast, the introduction of F173A mutation had no effect on PYY binding [128]. Additionally, for the mutations W106A, D86A, C113S and F221A no PYY affinity was detected. It has to be pointed out, however, that the loss of affinity of some mutants can be due to an overall change in receptor conformation. Especially, mutated residues located deep in the transmembrane region like Trp^{163} can induce such structural changes. That is supported by the fact that a peptide ligand is suggested to bind preferentially at the top of the transmembrane segments.

In conclusion, despite the many contradictions in the various mutagenesis studies one can summarize that the binding domains for NPY and PYY can be found in loops such as upper TM regions and also appear to be very similar for both peptide ligands (Fig. 2). The discrepancies between the described mutagenesis studies may be the result of several factors, including transfection of different cell lines (HeLa *versus* COS-7, HEK293), utilization of different tracers (^{125}I-NPY, ^{3}H-NPY *versus* ^{125}I-PYY) and assay conditions. It is also possible that the molecular environment shows some differences in the used cell lines and thereby affects the pharmacological profile of the same receptor in different ways.

Receptor mutants can not only be used to determine the binding of an agonist but also information about the antagonist binding site are helpful for understanding of receptor interaction and activation mechanism. A comprehensive study investigated the affinity of the nonpeptide antagonist BIBP3226 at a variety of hY_1 receptor mutants [156]. Thereby it was recognized that the residues Trp^{163}, Phe^{173}, Gln^{219}, Asn^{283} and Asp^{287} are not only essential for ^{125}I-NPY binding but also for ^{3}H-BIBP3226 affinity that indicates a partial overlapping of agonist and antagonist binding site. The only mutation showing a loss of BIBP3226 binding and wild-type affinity for NPY and PYY was Y211A (TM5) [128, 156]. The mutant F286A had a five-fold decreased affinity for BIBP3226 whereas it did not display any NPY binding. In contrast to the identified binding sites of NPY, Tyr^{100}, Asp^{104}, Trp^{288} and His^{298} are not involved in the interaction of the antagonist BIBP3226. Two recent neuropeptide Y_1 antagonists are J-104870 and 1229U91 [158]. Identically to BIBP3226, affinity of the nonpeptide J-104870 was influenced by W163A, F173A, N283A and D287A mutation. The peptide 1229U91 was designed to mimic the C-terminal regions in NPY and PYY and showed a partially different binding site at the hY_1 receptor with a reduced affinity for the mutants Y47A (TM1), N299A (TM7) and L303A (TM7). Similar to BIBP3226 the antagonist 1229U91 displayed a decreased binding when Trp^{163} and Asp^{287} were mutated to alanine. Since the mutation of hY_1:Trp^{163} has a negative influence on the binding of NPY and on several Y_1 selective antagonists and as this well conserved receptor residue is located in the lower site of TM4 according to Y_1

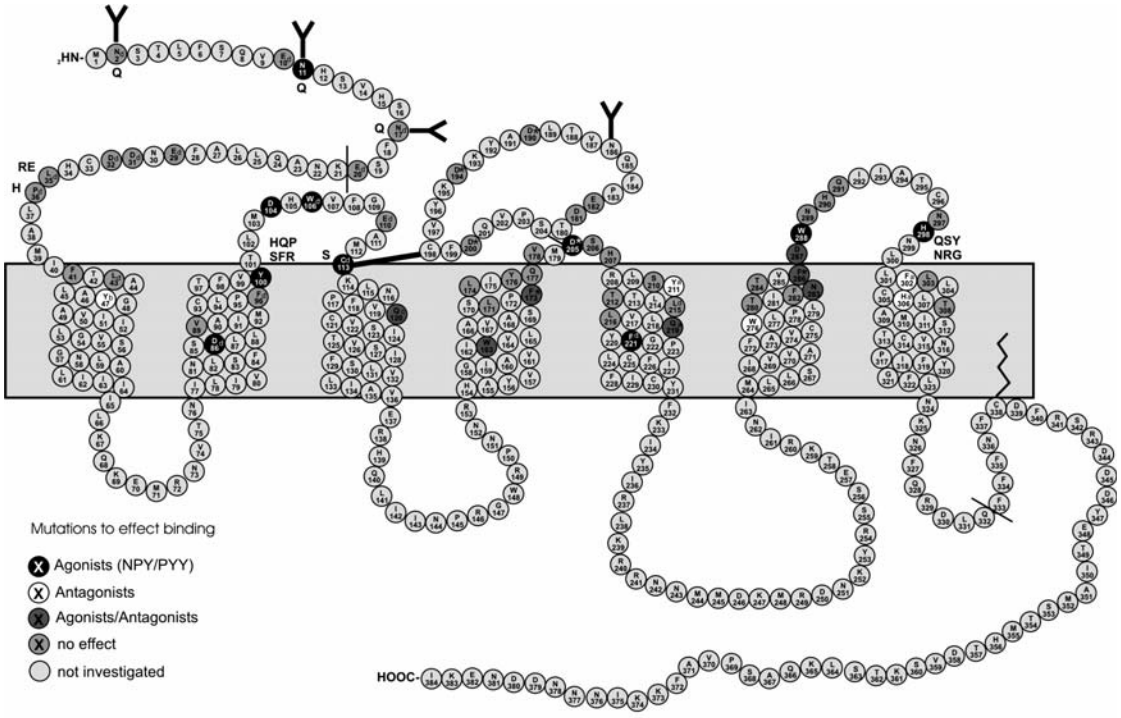

Figure 2. Summary of structural and mutagenesis studies performed at the human and rat Y_1 receptor. Amino acids were mutated to alanine except in some positions. Those are indicated by amino acids next to the original sequence. Special cases: (a) Mutations affect binding of BIBP3226 but not 1229U91 and J-104870, (b) Mutations affect binding of BIBP3226 and 1229U91 but not J-104870, (c) when Gln120 was mutated to tyrosine, no binding of PYY was detected whereas mutation to glutamate abolished BIBP3226 binding, (d) mutation where only the antagonist binding was investigated. Deletion of the C-terminus (hY_1-Δ333-384) did not affect binding whereas deletion of the N-terminus (rY_1-Δ2-20) abolished NPY binding. A star (*) indicates that there are differences between the data from different mutagenesis studies at that particular amino acid.

models, this position might be important for maintaining a precise overall receptor conformation [156, 158].

The first mutagenesis study of the Y_2 receptor also defined the binding site of a selective Y_2 antagonist called BIIE0246 [130]. It was based on the surprising result that the human Y_2 and the chicken Y_2 receptor (chY_2) showed significant differences in their pharmacological profile. Accordingly, the chY_2 receptor was characterized by an affinity as well as activity for the selective Y_1-like receptor agonist [Leu^{31}, Pro^{34}]-NPY and displayed no binding of BIIE0246 in contrast to hY_2 receptor [91]. A combined replacement of three residues of the hY_2 receptor by the homologous amino acids of the chY_2 sequence resulted in a decreased affinity for BIIE0246 as low as chY_2. The reciprocal mutagenesis revealed that in all mammalians conserved residues Gln^{135} in TM3 (His^{139} in chY_2), Leu^{227} in TM5 (Gln^{231} in chY_2) and Leu^{284} in TM6 (Phe^{288} in chY_2) of the hY_2 receptor are responsible for BIIE0246 binding [130].

One additional characteristic feature of G-protein coupled receptors that can be investigated by receptor mutagenesis is the importance of receptor glycosylation for the recruitment to the cell surface and for ligand binding. The deletion of all three putative sites for N-linked glycosylation (Asn^2, Asn^{11}, Asn^{17}) in the N-terminus of the rY_1 receptor ([rY1del(Asn2–Glu20)]) led to a complete loss of binding and a strongly impaired membrane surface localization [132]. Other biochemical studies could confirm NPY receptor glycosylation. Photoaffinity labeling and receptor solubilization resulted in a 70 kDa glycoprotein for Y_1 [160], a 40–60 kDa glycoprotein for the Y_2 receptor [160–163] and a 60–80 kDa protein for the Y_4 receptor [117, 164]. The calculated molecular weight of the Y receptors corresponding to the receptor amino acid sequence deduced from the translated cloned cDNA nucleotide sequence is in most cases significantly lower than the experimentally determined value confirming NPY receptor glycosylation. The large range in the molecular mass of some Y receptors is probably caused by differences in tissues and species that were investigated [165].

Furthermore a direct influence of the N-terminal receptor region in the binding process was concluded after finding that the introduction of the FLAG-epitope (DYKDDDDK) epitope between residue 2 and 3 of the hY_1 receptor led to a complete loss of affinity [131]. The addition of the N-terminal haemagglutinin (HA) sequence following the initial methionine, however, had no influence on rY_1 receptor binding [166]. Whereas the N-terminus seems to contribute to a certain degree to ligand binding, the complete C-terminus could be deleted without any negative influence on NPY affinity to the hY_1 receptor [131]. When the FLAG epitope was positioned at the receptor C-terminus also no effect on binding was detected [130, 132, 158, 159] such as for the addition of six histidine residues [131]. Furthermore it was possible to functionally express Y receptors as C-terminal fusion proteins with the green fluorescent protein and its variants [54, 167]. However for the rhesus Y_4 receptor tagged with GFP lower affinities compared to wild-type receptors and no signal trans-

Table 2. Modifications of the Y1 receptor

Modification (epitope)	Sequence/amino acids	Localization[Ref]	Effect on receptor	Purpose
FLAG	DYKDDDDK 8 aa	between first and second residue [131]	complete loss of affinity	receptor localization
		C-terminus [131, 156, 159]	no influence on binding	
Haemagglutinin (HA)	YPYDVPDYA 9 aa	N-terminus [166]	no influence on binding	
Hexahistidine (His-tag)	HHHHHH	C-terminus [131]	no influence on binding	receptor purification
Renilla luciferase	259 aa	C-terminus [77, 164]	3–10-fold lower binding affinity	receptor dimerization
Green fluorescent protein and its variants (GFP/CFP/DsRed/YFP)	239 or 226 aa	C-terminus [54, 76, 77, 164, 167]	no influence on binding/signaling [167] 3–10-fold lower binding affinity/no signaling [164]	transfection efficiency, receptor localization, receptor dimerization*

* explanation: dimerization is the purpose of green fluorescent protein and its variants

duction was found [164]. This indicates that the attachment of the large GFP molecule consisting of 239 amino acids can have a negative effect on G-protein coupling in some cases.

Modifications in the intracellular receptor part like the mutation of the palmitoylation site (Cys^{337}) in the hY_1 receptor resulted in a mutant with unchanged affinities for Y_1 agonists and antagonists, but without the incorporation of palmitic acid a dramatic reduction in coupling to G-protein was found [166]. Based on this result, it is postulated now that receptor acylation represents a significant mechanism for the regulation of G-protein activation efficiency and receptor desensitization.

Receptor dimerization

Homodimerization

Another interesting feature of GPCRs that has advanced rapidly in the recent past is their ability to form dimers or higher-order oligomers [168–170]. Since most current methods are not able to distinguish between dimers and larger oligomers we refer to the term dimer. Thereby homo- such as heterodimerization is described for GPCRs. Several investigations suggest that dimerization is important for receptor maturation, folding and surface expression. This is based on results that many GPCRs form dimers during biosynthesis as early as in the ER, and are then called constitutive dimers [171–174]. By dimerization specific ER retention signals or hydrophobic patches can be covered and this will guarantee a proper folding and ER export [175, 176]. In contrast, there are also examples for agonist mediated dimerization [177–179]. Thereby agonist binding may have an increasing or decreasing effect on dimerization itself. However, many investigations have come to the conclusion that dimerization is not influenced by ligand binding [167, 180, 181]. Consequently, no general consensus has yet been established. Furthermore an involvement of dimerization on receptor function was proven. Thereby ligand binding properties [182, 183] or signalling specificities [190, 191] can be changed in the oligomeric receptor state compared to the monomer. There still is much work to be done concerning receptor oligomerization, e.g., the identification of a conserved dimerization interface and the role of oligomeric assembly. Currently, a variety of methods are available to detect receptor dimerization including affinity labeling, chemical cross-linking, co-immunoprecipitation and Western blotting.

First speculations about dimerization of Y receptors are based on studies with the Y_1 receptor antagonist and Y_4 receptor agonist GR231118, a homodimeric peptidergic compound, showing 250-fold higher affinity than the monomer [184, 185]. Additionally, truncated NPY analogues ([P^{30}, C^{31}, F^{32}, L^{34}]-NPY(28–36), [P^{30}, C^{31}, W^{32}, L^{34}]-NPY(28–36)) bind to the hY_1 receptor after dimerization with a 10-fold increased affinity. Dimerization of NPY receptors has been investigated by fluorescence and bioluminescence reso-

nance energy transfer (FRET/BRET) [164, 167]. The FRET technique is based on a non-radiative energy transfer between two different chromophores [188]. Thereby one fluorescent probe (donor) emits light at a certain wavelength possible to excite the other fluorescent probe (acceptor). Then emission of the acceptor at its characteristic emission wavelength can be determined and represents FRET. Prerequisites for such an energy transfer are that the two fluorescent molecules are in close proximity (10–100 Å) and are favorably orientated (Fig 3). Additionally, there has to be an overlap between the emission spectra of the donor and the excitation spectra of the acceptor. Since FRET depends on the distance between two fluorescent molecules it is a useful technique to determine protein–protein interactions. Therefore the chromophores only have to be attached to the proteins of interest. BRET is another method used to identify interactions of two partners. It is a process that also occurs in many organisms that emit light (e.g., *Renilla reformis*) [186]. In contrast to

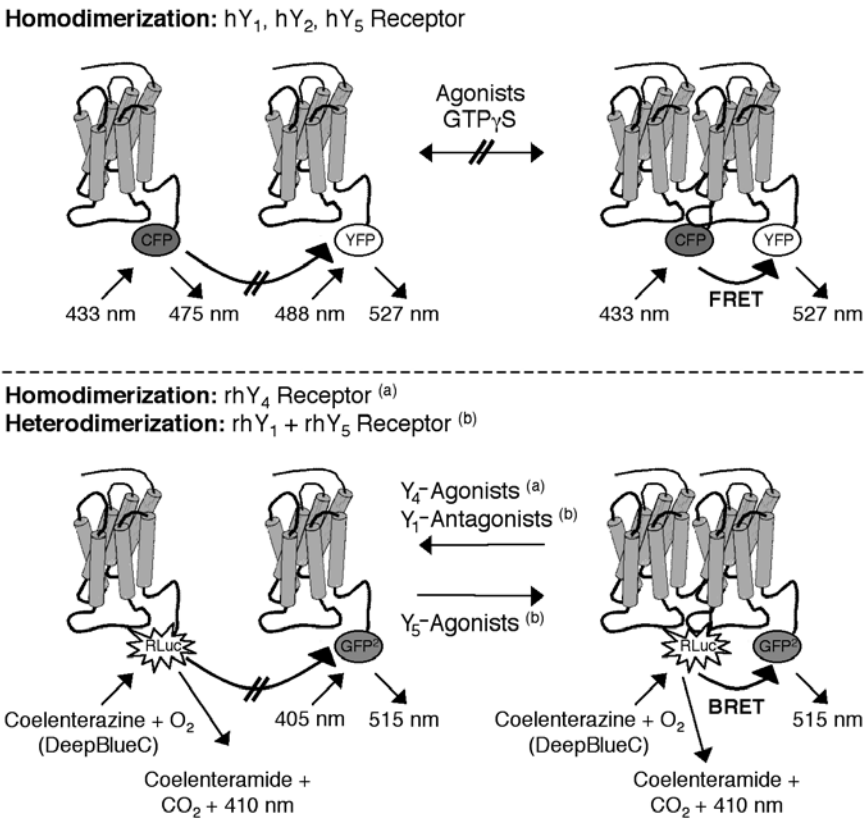

Figure 3. Summary of dimerization studies performed at Y receptors. Two different methods using resonance energy transfer (FRET/BRET) are indicated. The influence of agonists, antagonists and Gα-protein on dimerization is shown (arrows).

FRET, the BRET technique uses the enzyme luciferase (*Firefly* or *Renilla* luciferase) as donor catalyzing a substrate conversion (D-Luciferin or Coelenterazine) that is associated with emission of blue light. This again excites GFP (or YFP) as acceptor resulting in the re-emission of light in the green spectra (or yellow) that can be measured to determine the BRET level. Similar to FRET bioluminescence energy transfer is only possible when luciferase and chromophore are in close proximity (10–100 Å; [190]).

To investigate Y receptor homodimerization by FRET, NPY receptors were C-terminally fused to green fluorescent protein (GFP) or its spectral variants (CFP, YFP, DsRed) [167]. Thereby GFP and DsRed such as CFP and YFP represent so-called FRET pairs. By using fluorescent microscopy and spectroscopy homodimerization of Y receptors was proven and found to depend on the subtype. Quantification based on a hY_2 receptor construct fused to a FRET positive control (YFP-CFP tandem) revealed 26% dimerization for hY_2, but 44% and 41% for hY_1 and hY_5, respectively. Another approach showed homodimerization for the rhesus Y_4 receptor (rhY_4) by BRET. Thereby the Y receptor was C-terminally fused to *Renilla* luciferase (Rluc) or a mutant variant of GFP (GFP2) [164]. Furthermore a modified version of the Rluc substrate (DeepBlueC) was used to increase the spectral resolution. Receptor homodimerization measured by BRET was confirmed by cross-linking combined with Western blotting. Concerning the influence of the natural ligand and Gα-protein binding on dimerization FRET studies of hY_1, hY_2 and hY_5 revealed no effect [167]. Based on this it was speculated that Y receptors already assemble as dimeric units in ER and are then transported to cell membrane as homodimers. However, investigations of rhY_4 showed a concentration dependent decrease of BRET ratio after incubation with the specific ligands hPP and 1229U91 indicating agonist induced homodimer dissociation [164]. Since this phenomenon could not be seen with low affinity Y_4 ligands it might be a receptor mediated response. Another possibility for the ligand decreased BRET response could be conformational changes in predimerized receptors.

Heterodimerization

Unlike this, heterodimerization of rhY_4 with the rhY_1, rhY_2 and rhY_5 receptor could not be detected. However, investigations using rhY_1 together with rhY_5 produced a significant BRET ratio (unpublished data, 7th International NPY Meeting, Coimbra, 2004). Additionally, heterodimerization of rhY_1 and rhY_5 was decreased by Y_1-selective antagonists (BIBP3226, LY366258) and increased by Y_5 agonists (PP(1–17)NPY(18–36)), whereas NPY and PYY showed no effect. Differences with regard to interference of homodimerization by ligands can be the result of variations in experimental set-up, such as the applied detection method and a different cellular system. In particular, the variation of expression in different cells, of numerous scaffold and adaptor proteins can modify GPCR trafficking and signaling specificity [191]. Another

aspect that influences dimerization studies might be the level of receptor expression. It has been pointed out that receptor expression level can alter dimerization level [192]. Furthermore overexpression of a recombinant protein in a cell line can force the receptor contact in a non-natural fashion [193]. Taken together, corresponding controls are necessary to confirm that the experimental conditions reveal reliable results. This can be done by corresponding controls, e.g., for FRET/BRET co-expression of two less related receptors where a dimerization is unlike as negative control [164] and a tandem construct of the acceptor and donor as positive control [164, 167].

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Physiological and pathophysiological perspectives of the NPY family of peptides

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Modulator role of neuropeptide Y in human vascular sympathetic neuroeffector junctions

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Modulator role of NPY in sympathetic co-transmission

Shortly after its discovery, the physiology of NPY was linked to blood pressure regulation and peripheral resistance control. NPY antibodies revealed its co-localization with catecholamines in the central and peripheral nervous system [1–4]. Neuro-anatomical NPY localization preceded functional studies. Immunostaining revealed a dense network of NPY-ergic nerve terminals surrounding mammalian and human blood vessels [5–8], where NPY co-localized with either catecholamines or the noradrenaline (NA) synthesizing enzymes, tyrosine hydroxylase or dopamine β-hydroxylase [6, 9, 10]. As a logical extension of these findings, pharmacologists at the Karolinska Institutet and Lund's Medical School labored ardently with bioassays searching for a role of NPY in vascular tone regulation [8, 11–13]. Soon it was recognized that the long-lasting pressor effect of NPY was lost by the simple cleavage of the amide at the COOH terminus of NPY, i.e., NPY free acid [14], an indication that the peptide likely mediates its pressor effect by the activation of a specific receptor different from the adrenoceptors [15].

Two years after NPY's discovery, it was clear that NPY increased vascular resistance by a direct effect on microvessels, while its modulator role evidenced as an augmented vascular contractility to exogenous NA, an effect that occurred without NPY causing *per se* a rise in vascular tone [8]. Years later, the modulation was extended to adenosine 5' triphosphate (ATP)-evoked vasoconstrictions [16], implying that the modulator role of NPY was exerted on ATP and NA, the two sympathetic co-transmitters. These findings established the notion of NPY as a postjunctional modulator of sympathetic nerve activity. Simultaneously, pharmacological, biochemical and molecular biology grounds concurred to identify the Y_1 receptor in the modulator role of NPY, providing the cell biology framework to study ultimately, its mechanism of action. At about the same time, it was recognized that NPY, together with peptide YY and pancreatic polypeptide, conform a novel family of brain peptides, which act on a set of at least five cloned receptors, Y_1, Y_2, Y_4, Y_5 and Y_6, each

with differential affinities for these peptides [17]. All together, these receptors, as well as their endogenous ligands, were found widely distributed in the brain and in the periphery [18, 19].

The release of NPY from human vascular biopsies supports its integrand role in sympathetic vascular reflexes

Critical to the classification of NPY as a modulator of the vasomotor action of ATP and NA as sympathetic co-transmitters, was the demonstration that NPY is released from human perivascular sympathetic nerve endings. NPY is synthesized from a precursor protein, which is then packed in secretory granules in the Golgi and travels, via axonal transport, to the sympathetic nerve terminals [20, 21]. Likely, the precursor protein is cleaved to mature NPY during axonal transport to the nerve terminal where in addition, vesicular transporters dynamically load the synaptic vesicles with recycled NA and nucleotides [22–24]. Using human saphenous biopsies, Rump and von Kugelgen [25] were among the first to demonstrate the functional role of ATP as a sympathetic co-transmitter. A few years later, Racchi et al. [26] determined, using human saphenous vein biopsies, that NPY facilitated the ATP, the NA, and the vasomotor responses elicited by electrical depolarization of perivascular sympathetic nerve terminals. This was about the first evidence that NPY may participate in the physiology of human sympathetic vascular reflexes enhancing venous return. However, in view of prevailing technical difficulties, few laboratories approached the direct quantification and analytical identification of the peptide released by transmural electrical nerve terminal depolarization, a criterion required to establish the modulator role of NPY in human vascular sympathetic reflex co-transmission.

In a series of laborious studies, our laboratory described and characterized the content and release of hNPY from the perivascular nerve endings surrounding human saphenous vein segments [27] or mammary and radial vessels [28] obtained from patients programmed for myocardial revascularization surgery. These studies detailed the chromatographic identification of hNPY extracted from human vascular biopsies and documented its release to the tissue media, which depends essentially on extracellular calcium, the frequency of nerve terminal depolarization, and the duration of the electrical stimulation period. Using a combined radioimmunoassay plus high performance liquid chromatography (HPLC) procedure, Donoso et al. [27, 28] identified an oxidized byproduct of NPY which has the same biological potency as native NPY, as a modulator of ATP and NA-elicited contractions. This NPY and its metabolite act on the Y_1 receptor, as identified by the tissue expression of its mRNA. The release protocols established that the procedure used to electrically depolarize the tissue nerve endings evoked the release of a small fraction of the tissue-stored NPY, a finding that added to functional results highlighting the role of NPY as a modulator of human vascular sympathetic reflexes.

Relevant to the physiology of human vasomotor reflexes, our findings indicate that both arteries and veins are modulated by NPY to a similar extent, implying that NPY may participate in the regulation of both human blood flow distribution and venous return. In support of this notion, Figure 1 illustrates a HPLC-RIA chromatogram that exemplifies the analytical procedure used to chemically identify and quantify the released material from these biopsies. In addition, Figure 1 summarizes a set of experiments showing that the release of ir-NPY from segments of human mammary artery or saphenous biopsies depends on the frequency of nerve terminal depolarization.

Multiple NPY receptors in human blood vessels

Out of the five known NPY receptor clones, human blood vessels express the Y_1 and the Y_2 receptor subtypes. All NPY receptors belong to the family of G-protein coupled receptors; the Y_1 and Y_2 receptors are coupled to Gi/o proteins, which means that the intracellular transduction mechanism is exerted by regulation of the cyclic adenosine monophosphate (cAMP) content. In vascular sympathetic neuroeffector junctions, endogenous or exogenous NPY modulates sympathetic vasomotor reflexes by acting on vascular smooth muscles through the Y_1 receptor, while the Y_2 receptor subtype is a presynaptic receptor that regulates the release of sympathetic co-transmitters.

Within the past decade, relatively selective agonists/antagonists have become available and have greatly assisted in the Y_1 and Y_2 receptor identification. The first tools that guided NPY receptor classification were synthetic NPY structural analogs, or truncated fragments with preferential affinity for the Y_2 receptor [29, 30]. While [Leu^{31}, Pro^{34}]-NPY is the prototype Y_1 receptor structural analog, the endogenous NPY_{3-36} or PYY_{3-36} fragments, and other truncated peptide fragments were instrumental as selective Y_2 ligands. Non-peptide competitive antagonists with nanomolar affinity and selectivity for the Y_1 receptor, include BIBP 3226 and BIBO 3304 [31–33], and peptide Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH_2, cyclic(2,4')diamide (1229U91), which is commercially available with a similar, though less, affinity than the non-peptide compounds [34, 35]. Likewise, BIIE0246, was described as a competitive, high affinity, and selective Y_2 non-peptide antagonist [36]. These compounds are valuable tools to assess the role of the Y_1 and Y_2 receptors in human blood vessel physiology.

Y_1 receptor, a postjunctional modulator of sympathetic vascular reflexes

In humans, as in experimental animals, two distinct and complementary mechanisms account for the rise in vascular resistance elicited by NPY, which are mediated by Y_1 receptor activation. In resistance vessels, and likely in the microcirculation, NPY ensues a direct vasocontractile action [37]. The intra-

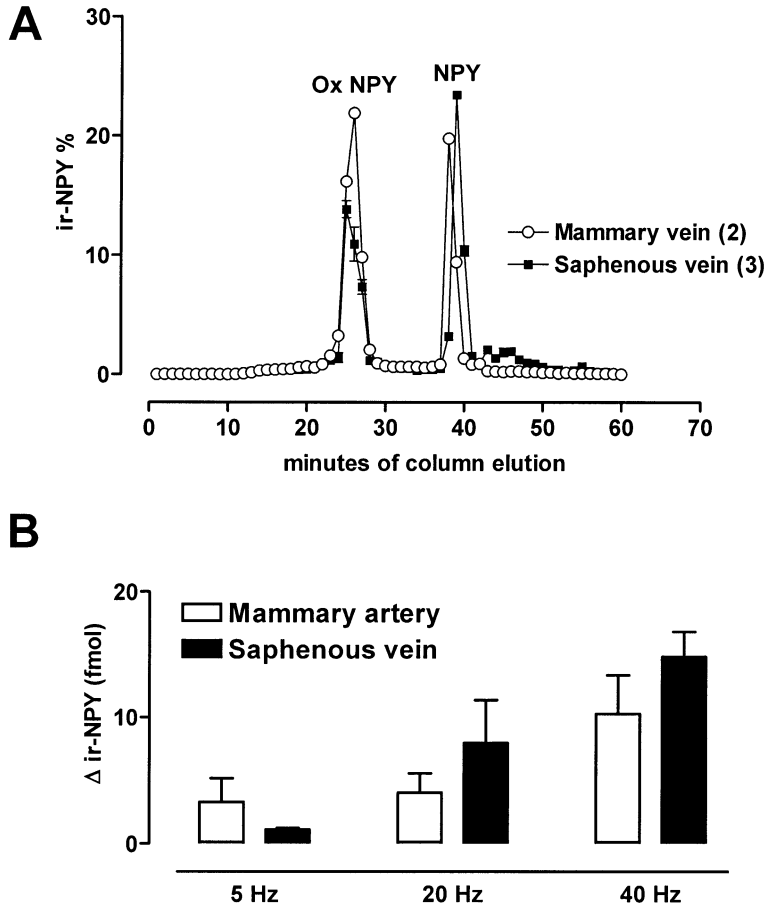

Figure 1. Chemical identification of the electrically evoked ir-NPY released from the sympathetic nerve terminals of human vascular biopsies. Panel A: Combined HPLC-RIA chromatogram of the material released by electrical depolarization of the nerve terminals of mammary vein biopsies (n = 2) and saphenous vein biopsies (n = 3). The retention time of standard peptides identified in the released material native NPY plus a by-product, which corresponds to oxidize-NPY (ox-NPY). Panel B: The extracellular release of ir-NPY depends on the frequency of nerve depolarization as evidenced by the graded peptide release elicited by transmural electrical nerve stimulation (60 V, 1 msec). Columns indicate the mean values; bars SEM (n = 4–6) per protocol. The correlation coefficient between the amount of extracellular ir-NPY released and frequency of nerve stimulation in the mammary artery was $r = 0.58$ ($P < 0.043$) and $r = 0.77$ ($P < 0.003$) in saphenous vein biopsies respectively.

venous administration of NPY significantly increases human vascular resistance, an effect likely interpreted as due to direct contraction of small resistance vessels; however, this effect may be compounded by the facilitation of the vasomotor effect of ATP and NA. In other conductance vessels, vasoconstriction ensues as a consequence of its modulator action on sympathetic co-transmitters, as illustrated in the tracings shown in Figure 2, and the studies by

Racchi et al. [26] and Donoso et al. [27, 28]. In the latter human biopsy studies, NPY acted indirectly, modulating the vasomotor action evoked by sympathetic co-transmitters. Several arguments support the view that the modulator action of NPY is due to the activation of the Y_1 receptor. Consonant with the pharmacology of the Y_1 receptor, BIBP 3226 reduced the modulator action of exogenous NPY (see tracing B in Figure 2), as was also observed by Racchi et al. [26] and Mezzano et al. [38]. Moreover, RT-PCR identified the mRNA coding for the Y_1 receptor (Fig. 3) in these prototype blood vessels, as was also detected in other human blood vessels [27, 28, 39, 40].

In vivo studies with BIBP 3226 are scarce, since BIBP 3226 administration to experimental animals in several models of hypertension did not yield clinically relevant results. However, Doods et al. [41] observed that Y_1 receptor antagonism had no major influence on the basal blood pressure but resulted in an attenuation of the stress-induced hypertension, a finding that strongly supports the view that NPY is mainly released during intense sympathetic nervous system activation. Although Erlinge et al. [42] demonstrated a significant rise in plasma ir-NPY in patients with severe hypertension, the administration of a Y_1 receptor antagonist did not result in a lowering of systemic blood pressure, a finding that complicated the determining role of NPY in primary hypertension [43].

The presynaptic Y_2 receptor in vascular neuroeffector junctions

The Y_2 receptor has received less experimental attention in contrast to the Y_1 receptor, which has been investigated in functional control studies, in animal models of vascular diseases, as well as in human clinical research. On the basis of pharmacological studies with peptide fragments truncated at the amino terminal, which lacked a direct vasomotor effect, Grundemar and Hakanson [29] concluded on the existence of a second, separate NPY receptor, named Y_2. This receptor was postulated to be of presynaptic origin and to regulate the release of sympathetic transmitters at the nerve endings, explaining its efficacy to block neurotransmission in several bioassays, including the vas deferens neurotransmission [44]. Specific antibodies soon evidenced its brain distribution [45]. Assessing directly or indirectly transmitter release, it became possible to evaluate the functionality of the Y_2 receptors. We assessed the influence of human Y_2 receptors by recording isometric muscular contractions elicited by electrical nerve terminal depolarization of rings of human saphenous vein or radial arteries. A prototype of these experiments is shown in Figure 3, where the frequency-dependent contractions elicited by electrical depolarization of the tissue nerve terminals is reduced by NPY, a finding that may be interpreted as an indication that the activation of a presynaptic Y_2-operated mechanism, reduces co-transmitter release. This interpretation is consonant with the view that the BIBP 3226-induced blockade of Y_1 receptors also reduces the magnitude of the electrically evoked contractions (Fig. 3), but by an entirely distinct mechanism; further validating the notion that the modulator role of NPY in

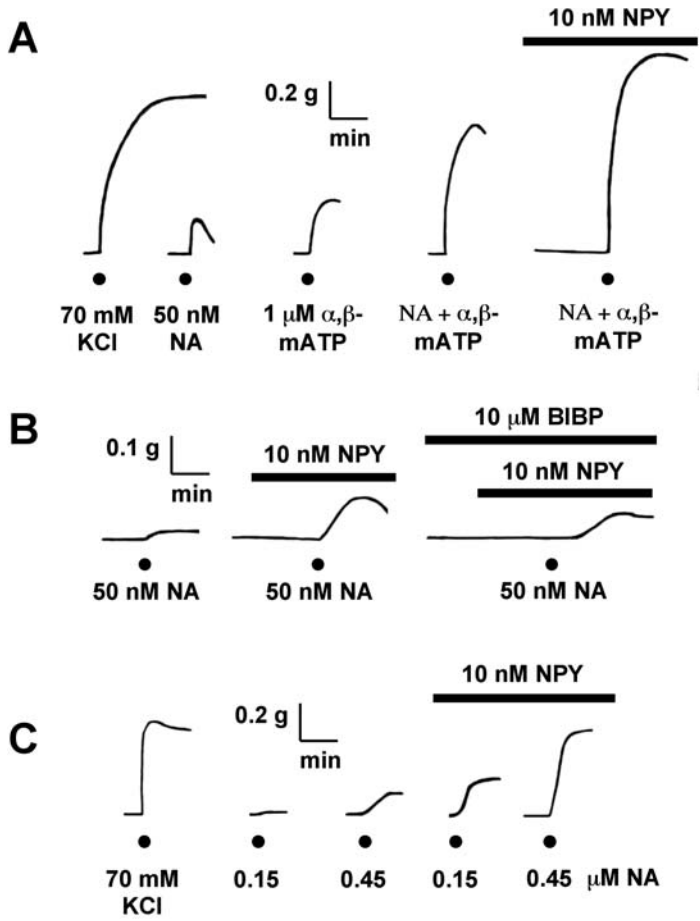

Figure 2. Postjunctional modulator role of NPY evidenced by the facilitation of the vasomotor effect of ATP and NA; activation of Y_1 receptors. Representative tracings show isometric contractions of the circular layer from human saphenous vein biopsy rings (panels A, B) or a human radial artery segment (panel C). 10 nM NPY potentiated the NA, or the NA plus α,β-methylene ATP (α,β-mATP); BIBP 3226 reduced the facilitator action of NPY on NA-evoked contractions.

humans is also mediated by pre- and post-junctional receptor-operated mechanisms. The identification of the mRNA coding for the Y_2 receptor in human thoracic and saphenous veins (Fig. 3) further supports the role of this receptor in human vascular sympathetic reflexes.

Vascular role of NPY in health and disease

The rather subtle role of NPY in the physiology of vascular tone maintenance may be exacerbated under intense sympathetic discharges [46] or pathophysi-

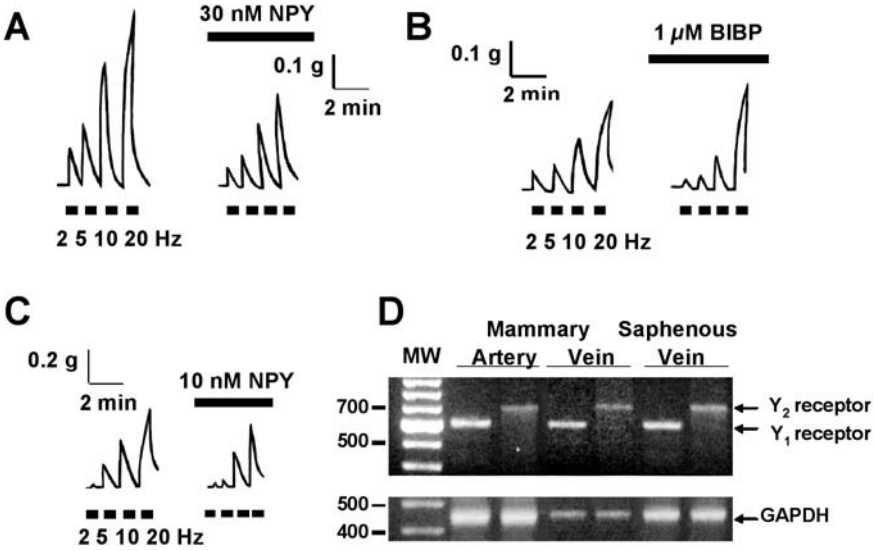

Figure 3. Presynaptic modulator role of NPY, activation of Y_2 receptors. Representative tracings of isometric contractions from the circular layer of human saphenous vein (panel A, B) or radial artery (panel C) biopsies subjected to transmural electrical nerve depolarization with frequencies of pulses varying from 2–20 Hz. Each train was delivered for 30 s. Panel D shows RT-PCR that allowed the identification of the mRNA coding for the Y_1 and the Y_2 receptors in human mammary vessels and a segment of the human saphenous vein. Total RNA was extracted from the biopsies upon the human biopsies reached our laboratory, and the material was processed as described by Donoso et al. [27, 28].

ological conditions that imply prolonged sympathetic discharges, which may be extended chronically, e.g., in stress or pain [47]. This concept may be a determinant in severe cardiac and/or vascular disease, as suggested by reports that patients with cardiac infarction [48], eclampsia [49], pheochromocytoma [50, 51], or pathologies characterized by a sudden surge of circulating ir-NPY plasma levels.

Consistent with the significance of NPY to vascular sympathetic reflexes, several studies measured ir-NPY in human plasma under a variety of physiological and pathophysiological conditions, such as eclampsia and pre-eclampsia [49, 53], or strenuous exercise [54, 55], including stressful conditions [52]. Childbirth is among the most potent stimuli to increase circulating human plasma ir-NPY [56]. Likewise, hypoxia, a stimulus that ensues strong and persistent sympathetic reflex activation, is characterized by an important rise in plasma levels of ir-NPY [55].

A bias common to all these studies is that the rise in the plasma peptide is derived from nerve terminals, and is therefore an indication of repetitive and intense sympathetic tone, or secreted from the adrenals, since the gland is a rich source of NPY [57]. The paroxysmal episodes of high blood pressure characteristic to pheochromocytoma might be compounded to the NPY facili-

tation of sympathetic co-transmitter vasoconstriction. Interestingly, Tabarin et al. [58] detected NPY fragments in the plasma of humans suffering from pheochromocytoma; likewise, Hegbrant et al. [59] also found NPY fragments in the plasma of hemodialyzed patients, indicating that NPY is metabolized *in vivo* and that the truncated peptide forms may have pathophysiological relevance. Grundemar and Hakansson [29] first reported that synthetic truncated NPY fragments are biologically active and possess a defined Y_2 receptor pharmacology.

An integrated overview of sympathetic intracellular events

The prevailing view that sympathetic transmission relies on the sole basis of NA as the sympathetic transmitter is no longer accepted. Recent editions of classical physiology/pharmacology textbooks [60] put forward the notion that sympathetic co-transmission involves the concerted postjunctional action of ATP plus NA, and its modulation by NPY [47]. Consonant with this hypothesis, vascular smooth muscles are equipped with a collection of ionic channels gated by extracellular ATP [61] and belong to the P2X family of purinoceptors, which lead to cell depolarization. Moreover, several adrenoceptors and their subtypes are expressed in vascular smooth muscle, including isoforms of the α_1 and α_2-adrenoceptors, which activate essentially G proteins, linked to the intracellular mobilization of calcium stores via the activation of endoplasmic reticulum inositol triphosphate (IP_3) receptors. The vascular neuroeffector junctions combine fast excitatory P2X receptors with the metabotropic α-adrenoceptors; the combination of which mobilizes ions and metabolic reservoirs, causing a fast and modulated vasomotor response. Therefore, while ATP results in the depolarization of the smooth muscle within milliseconds, NA mobilizes intracellular calcium stores plus the activation of protein kinase C, elements necessary to boost contractile events. These dual and complementary mechanisms, orchestrated within a defined temporary sequence and distinctive time frame, establish the foundations for co-transmission. Consonantly, the co-application of ATP plus NA, mimicking sympathetic co-transmission, results in a synergic muscular response, highlighting the physiological implications of co-transmission; recordings in Figure 2 illustrate this principle. In this synaptic scenario, the co-release of NPY facilitates further, by as yet unknown mechanisms, the contractility of the vascular smooth muscle ensued by either ATP or NA alone, or the orchestrated action of these co-transmitters.

Concluding remarks

The modulator role of NPY as a functional integrand of sympathetic reflexes is firmly established. There is consensus that NPY acts on pre- and post-junc-

tional mechanisms and that it regulates the strength of sympathetic signalling pathways. The clinical relevance of NPY in sympathetic co-transmission, and its eventual role in the regulation of human vascular sympathetic reflexes, particularly following strong and maintained sympathetic discharges, awaits a full understanding of the molecular basis underlying its signalling transduction, thus defining eventual therapeutic strategies of clinical relevance.

Summary

Reverse transcription polymerase chain reaction (RT-PCR) studies identified the mRNA coding for the Y_1 and Y_2 receptors in human mammary artery/vein and saphenous vein biopsies. Y_1 receptors are expressed in vascular smooth muscles and potentiate the contractile action of sympathetic co-transmitters, adenosine triphosphate (ATP) and noradrenaline (NA); BIBP 3226, a competitive Y_1 receptor antagonist, blocked the neuropeptide Y (NPY)-induced modulation. The Y_2 receptor is expressed in sympathetic nerves terminals and modulates the pool of sympathetic co-transmitters released at the neuroeffector junction. NPY plays a dual role as a modulator of sympathetic co-transmission; it facilitates vascular smooth muscle reactivity and modulates the presynaptic release of ATP and NA. Sympathetic reflexes regulate human vascular resistance, where NPY plays a modulator role of paramount importance following increased sympathetic discharges, such as stress and vascular disease.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
from Genes to Therapeutics
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NPY and NPY receptors: presence, distribution and roles in the regulation of the endocardial endothelium and cardiac function

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Endocardial endothelium

The endocardial endothelial cells (EECs) constitute a uniform and continuous layer of flat polygonal cells that line the cavity surface of the heart (Fig. 1) [1]. The luminal surface of the majority of EECs has a variety of microappendages or microvilli which project into the heart cavities [1, 2]. The very large contact surface area of the endocardial endothelium (EE) offers a very high ratio of cav-

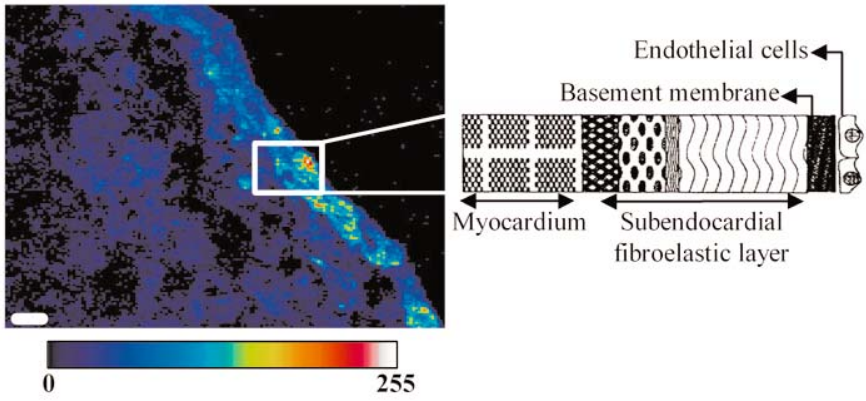

Figure 1. (Left panel) Slice of a 20 week-old human fetal heart at the level of the ventricle labeled with hNPY coupled to fluorescein. Please note that the density of the NPY receptors is higher at the level of the endocardial endothelium when compared to the adjacent cardiomyocytes. (Right panel) Schematic representation of the endocardial endothelial cell layer, extracellular matrix and adjacent cardiomyocytes. The endocardial endothelium forms a continuous monolayer of cells that line the cardiac cavities. These cells are separated from the adjacent cardiomyocytes by an extracellular matrix that contains a basement membrane consisting of a thin basal lamina and reticular lamina with fine collagen fibers. Adjacent to the basement membrane is a dense subendocardial fibroelastic layer that contains collagen, elastic fibers, fibroblasts, blood vessels as well as a nerve plexus. The white scale bar in the left panel represents 5 μm.

ity surface area to ventricular volume, particularly in the right ventricle, suggesting an important sensor role for the EE [2–4]. The presence of structured contractile proteins within EECs indicates the possibility of shape changes as well as some degree of motility [4–5]. Gap junctions are present between EECs [6] where they play a role in rapid intercellular electrochemical coupling. EECs also have tight junctions (zonula occludens), which are simple with one or two junctional contact points that serve as a selective barrier to the diffusion of molecules and limit the paracellular transport through the intercellular spaces or clefts [3, 7]. EECs' Golgi apparatus and endoplasmic reticulum are well developed with a high number of mitochondria that surround the nucleus [6, 8] suggesting that these cells are highly active metabolically. Endothelial markers such as von Willebrand's factor and a secreted form of vimentin identified by the PAL-E antibody [9] are also abundant in EECs [1, 6].

Cardiac endothelial cells of the myocardial capillary (MCEs) and the EECs share many common features, such as regulating normal cardiac growth [10–13], contractility performance and rhythmicity which must be clearly distinguished from the functional role of coronary vascular endothelial cells in the heart [13]. However, even if similarities exist between MCEs and EECs, differences between these two endothelial cell types also do exist such as eNOS expression and gap junctions are very important in EECs but very limited in MCE cells [3, 13]. Furthermore, EECs differ from all other cardiac and vascular endothelial cells (VECs) by their higher sensitivity to circulating factors such as angiotensin II (Ang II) [14], endothelin-1 (ET-1) [15] and neuropeptide Y (NPY) [8], as well as by their key localization at the entrance and exit of the pulmonary circulation [13]. The subendocardial space or extracellular matrix located between the EECs and the cardiomyocytes provides additional interactions for the EECs with the subendocardial nerve plexus, which comprises sympathetic nerve fascicles and fibers that are able to release various peptides including NPY [8, 16] (Fig. 1). For more information on the endocardial endothelium and its role in the regulation of heart function, please refer to a recent review by Brutsaert [13].

EECs and heart function

EECs as well as coronary blood vessels, can exercise substantial control over the contractility of cardiomyocytes by releasing various factors such as ET-1 [13], nitric oxide (NO) [13, 17, 18] and NPY [8]. Thus, EECs may act as an intermediate for neurotransmitters and humoral agents regarding their contribution to the control of heart muscle contraction [13, 16]. There has been *in vitro* evidence that EECs participate in the inotropic response to several circulating and locally released factors such as atrial natriuretic peptide (ANP) [19], phenylephrine [13] or reactive oxygen radicals [20]. Thus, an indirect dialogue between EECs and cardiomyocytes in the heart is more widespread than was originally thought. In addition, only in the presence of EECs (but not VECs or

fibroblasts), can cardiomyocytes maintain their adult phenotype [13, 21]. This highly suggests a difference between EECs and VECs in directly regulating, not only the cardiac function, but also the remodeling of heart cells. We thus cannot extrapolate from our large knowledge concerning VECs to EECs. These later cell types seem to be unique since they are able to release a highly important regulator of excitable cells such as NPY.

Ca^{2+} transport in EECs

Similar to VECs [22–24], EECs do not possess T or L-type Ca^{2+} channels. The resting influx of Ca^{2+} seems to be carried in through the voltage dependent steady-state nifedipine insensitive, but PN200-110 sensitive, R-type Ca^{2+} channel [22–24]. The biphasic increase in $[Ca]_i$ due to humoral stimulation is the most crucial step of the signal transduction pathway in many cell types including EECs. In EEC monolayers of rabbit cardiac valves, a biphasic increase in $[Ca]_i$ was observed with acetylcholine, bradykinin, histamine and adenosine triphosphate (ATP) but not with thrombin [25]. Also, in our laboratory, in EECs isolated from 20 week-old fetal human heart, NPY, ET-1 and Ang II seem to induce an increase in $[Ca]_i$ via the stimulation of resting R-type Ca^{2+} channels. In addition, the secretory process of EECs seems to be Ca^{2+}-dependent [26]. Even more recently, it was shown that in smooth muscle cells isolated from the rabbit stomach, the Y_2 and Y_4 receptor types are coupled to Gq suggesting that in this specific tissue, the NPY-induced change in intracellular Ca^{2+} is IP_3-dependent [27].

Neuropeptide Y and its receptors

NPY and structurally related peptide YY (PYY) and pancreatic polypeptides (PPs) are abundant and ubiquitous [28, 29]. Indeed, many investigators have suggested that NPY is one of the most abundant mammalian neuropeptides identified to date in the heart and brain [28, 30]. The structure and parts of its precursors are well conserved throughout evolution, suggesting an important role(s) in cell function. NPY has been known as a sympathetic co-transmitter, vasoconstrictor stress mediator, neuroendocrine modulator [31] and stimulator of obesity [32]. It is also found in platelets of some species and immune cells [31]. NPY activates multiple heptahelical Gi/o-coupled receptors, termed Y_1–y_6. Rat, murine and human Y_1 [33, 34], Y_2 [35–37], Y_4 [38], Y_5 [39] and y_6 [40, 41] receptors have been cloned, while the Y_3 receptor has not. All the NPY receptors, except for the y_6 receptor, which is only functional in the mouse, are linked to the inhibition of adenylyl cyclase [35]. Few studies have also shown that NPY activates mitogen-activated protein kinase [42] and protein kinase C [43]. The main functions of these receptors are reviewed in details elsewhere (please refer to Dumont et al. [29, 30]). The receptor types responsible for the

major effect of the peptide in the cardiovascular system possesses potent vascular mitogenic and angiogenic activities and are the Y_1, Y_2 and Y_5 [31, 44–47] receptors.

NPY and cardiac muscle

The hearts of several species, including humans, have been shown to contain high amounts of NPY immunoreactivity; this peptide being more abundant in the atria than in the ventricles [48]. In rodents, NPY immunoreactivity has been reported to be present in sympathetic nerve fibers that innervate coronary arteries and cardiomyocytes [48–51]. High concentrations of NPY were also found in nerve fibers near the sinus and atrioventricular node conductive tissues and in the endocardial layer [48–50]. It is important to mention that NPY is not only present in sympathetic nerve fibers, but also in intrinsic cardiac nerves [52].

Binding studies demonstrated that both the Y_1 and the Y_2 receptors are present in rat ventricular membranes [53]. Opposing effects on contraction of rat cardiomyocytes were observed when these cells were subjected to [Leu^{31}, Pro^{34}]NPY and $NPY_{13–36}$, suggesting the presence of both the Y_1 and the Y_2 receptors in this preparation [54]. Y_1 and Y_2 receptor immunoreactivities were observed on both the atrial and ventricular cardiomyocytes [55]. However, the Y_1 receptors had a higher density in subendocardial, as compared to subepicardial vessels of the left ventricular wall [55].

NPY can alter cardiac function indirectly, via central effects by causing changes in afterload due to its own vasoconstrictory activities or by potentiating those of other vasoconstrictors [56]. NPY can also modify cardiac function directly by inducing coronary constriction as well as potentiation of noradrenaline-induced constriction [57].

The reported effects of NPY on cardiac contraction vary depending on the species and the tissue used. For example, in whole isolated heart from guinea pig, rabbit and rat, the infusion of NPY decreased contractile force and blood flow [58–61]. However, NPY had no effect on inotropy in papillary muscles from cat, guinea pig and rat [49, 62]. On the other hand, in isolated atria or strips of atrial tissue, NPY had negative inotropic effects in the dog and rat [61, 63], whereas positive inotropic effects were obtained in guinea pig [60, 64]. Furthermore, inotropy was not affected by NPY in human right atrial strips [63]. NPY was found to induce negative inotropic effects in rat cardiomyocytes [65, 66], whereas both inhibitory and stimulatory effects were observed in guinea pig cardiomyocytes [65, 67]. In spontaneously contracting single ventricular cardiomyocytes isolated from 10 day-old embryonic chicks, NPY (10^{-10} M) increased the frequency of calcium transients during spontaneous contraction which was mainly mediated by the Y_1 receptor [68]. However, in resting non-contracting cardiac cells, NPY induced a dose-dependent increase of steady-state resting cytosolic and nuclear calcium that

was partially blocked by BIBP3226, a selective Y_1 receptor antagonist, implying the presence of other receptor types such as the Y_2 and/or Y_5 receptors in these cells [68].

NPY is also able to stimulate hypertrophy of adult ventricular cardiomyocytes [69]. NPY was also proposed to be a marker for determining the condition of patients suffering from heart diseases. Indeed, high levels of NPY in the plasma were reported in patients with hypertension, cardiac hypertrophy and congestive heart failure suggesting that overstimulation of myocardial NPY receptors can be one cause of pathological myocardial growth [69–71]. Recently, a common single nucleotide polymorphism in the signal peptide of the NPY gene was found in humans, that makes the peptide more releasable and associates with elevated total and LDL cholesterol as well as accelerated atherosclerosis [72]. In addition, recent work by Michalkiewicz's group [73] showed that transgenic rats overexpressing NPY (2 times higher than normal) in many tissues, including the heart, exhibited higher total vascular resistance and blood pressure compared to their non-transgenic siblings [73].

Localization of Neuropeptide Y and the Y_1 receptor in EECs as well as their role in the regulation of intracellular calcium

Our recent work showed that right ventricular EECs isolated from 20 week-old fetal human and adult rat hearts also express NPY and the Y_1 receptor [8]. This peptide is present not only at the cytosolic level, but also at the level of the nucleus of human and rat right ventricular EECs (Fig. 2A). Similar to NPY, Y_1 receptor immunostaining is also present in EECs and exhibits the same distribution; higher fluorescence labeling being observed at the level of the nucleus, and more particularly at the perinucleoplasm and nuclear membranes levels, while lower levels were detected in the cytoplasm and the plasma membrane (Fig. 2B). Thus, NPY was found to be present near its receptor suggesting that this peptide can be made available to stimulate its receptors at the plasma membrane and nuclear membranes levels. Similar to what was reported for HUVECs [74] and rabbit vascular endothelial cells [75, 76], it was recently demonstrated that not only NPY is present in the cytosol of EECs, but also the Y_1 receptor [8]. In accordance with the nuclear localization of NPY and the Y_1 receptor, there is plenty of evidence in the literature reporting the presence of enzymes, channels, exchangers, pumps, hormones such as ET-1 and Ang II as well as G-protein coupled receptors (for detailed review please refer to Bkaily et al. [77]). Furthermore, several studies demonstrate that nuclear G-protein coupled receptors such as the prostanglandin E_2 EP_3 [78] and ET-1 receptors [79, 80] are functional. Based on these facts, it seems that NPY can act, not only on its receptors at the plasma membrane level, but also on its receptors present at the nuclear membranes' level. Thus, some of the effects of NPY in EECs could also be mediated via its receptors at the level of the nuclear envelope membranes, something which offers the possibility that NPY could mod-

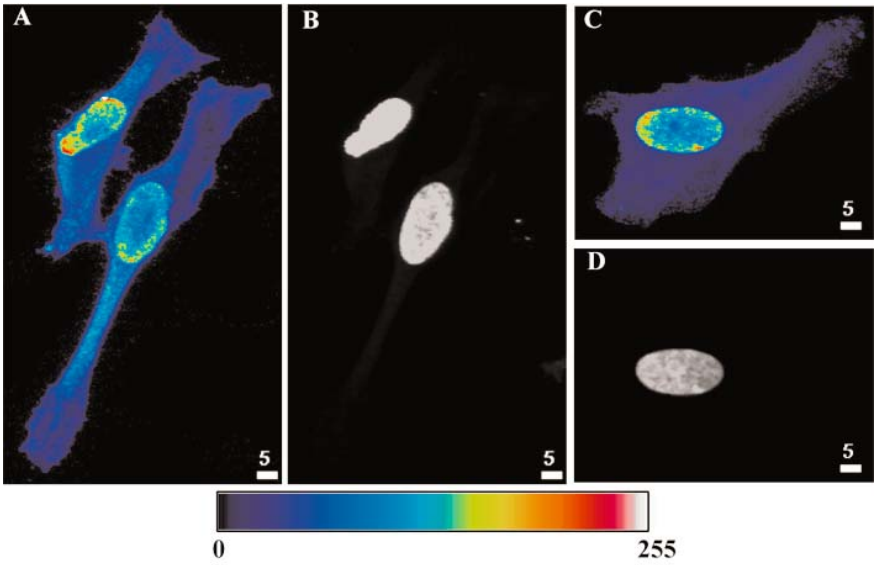

Figure 2. Confocal microscopic top view 3D images of anti-NPY (A) and anti-Y_1 receptor (C) immunoreactivities in 20 week-old human fetal ventricular endocardial endothelial cells showing higher labeling of NPY and the Y_1 receptor in the nucleus compared to the cytosol. For both NPY and the Y_1 receptor, note the labeling at the level of the nuclear envelope membranes. (B and D) Nuclear staining of the cells using Sytox green (100 nM). In A and C, the color scale represents the level of fluorescence intensity of the NPY and the Y_1 receptor antisera from 0 to 255, respectively. In B and D, the colors have no meaning. The white scale bar represents 5 μm.

ulate the expression of certain genes, as was reported for the prostaglandin E_2 EP_3 receptors [78]. In addition, like AT_1 [81, 82] and ET-1 receptors [79, 80, 82], nuclear envelope membranes' Y_1 receptors may also have a higher sensitivity to their ligand and may exert specific functions that are different from Y_1 receptors present on the plasma membrane. In addition, it was shown that the NPY-induced increase in $[Ca]_c$ and $[Ca]_n$ was partially blocked by the selective Y_1 receptor antagonist, BIBP3226, suggesting that the Y_1 receptor is indeed implicated in this effect without excluding the possibility of the participation of other NPY receptors [8]. It is clear from these results that NPY plays an important role in the regulation of intracellular free Ca^{2+} in EECs. This may suggest that upon an increase of nucleoplasmic free Ca^{2+} levels, nucleoplasmic NPY can be released inside the nuclear envelope space (perinucleoplasm) by Ca^{2+}-dependent exocytosis to activate NPY receptors present at the inner nuclear envelope membrane level. Along the same line, following an increase in nuclear envelope free Ca^{2+}, NPY present in the nuclear envelope space (perinucleoplasm) can also be released into the cytosol to activate NPY receptors present on the outer nuclear envelope membrane. The activation of NPY receptors, both at the outer and inner nuclear envelope membranes, can contribute to the regulation of perinucleoplasmic and nucleoplasmic free Ca^{2+} levels [23].

In addition, this high increase of cytosolic and nuclear Ca^{2+} could contribute to angiogenesis [74].

Our recent results demonstrate that the excitation-secretion coupling state of these cells can be modulated by an increase in intracellular Ca^{2+} and confirm that EECs do not only secrete ET-1, NO and prostanoids, but also NPY. Thus, this latter peptide may also be considered an EEC-derived factor. In accordance with these results, it was demonstrated that HUVECs can also release NPY during cell growth by yet unidentified factors [75] confirming that under certain conditions, NPY can be released from endothelial cells. Thus, EECs may contribute to local as well as systemic release of factors via a Ca^{2+}-dependent mechanism that can alter the excitation-secretion state of these cells as well as excitation-contraction coupling of cardiomyocytes [18, 68].

Discussion and conclusion

There is not doubt today that NPY does play a role in regulating cytosolic and nuclear Ca^{2+} in heart cells including ventricular cardiomyocytes and EECs. The recent work in the literature including ours, agree that the major effect of NPY in both ventricular cardiomyocytes and EECs is mediated primarily by the activation of the Y_1 and Y_2 receptors. However, other NPY receptors may also contribute to the peptide's effect on cardiomyocytes and EECs such as the Y_5 receptors. The mechanisms by which these effects take place could be via indirect activation of the voltage dependent resting R-type Ca^{2+} channels. One important aspect that should be mentioned is that EECs could be an important source of NPY which may highly contribute to both circulating NPY as well as locally released NPY. Furthermore, the presence of different types of NPY receptors in the nuclear membranes may suggest that these receptors may also contribute to the modulation of nuclear function and more particularly to the regulation of nucleoplasmic Ca^{2+} metabolism which is known to modulate nuclear function and transport. It is very likely that overactivation of different NPY receptors in both cardiomyocytes and EECs and more particularly the Y_1 and Y_5 receptors could be implicated in hypertrophy and cardiac heart failure. We are quite sure that future work in the field of NPY and cardiac function will help us to better understand the implication of this peptide in cardiac physiopathology.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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Neuropeptide Y and sympathetic control of vascular tone in hypertension

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Introduction

It is now well accepted that sympathetic nerves express as many as three neurotransmitters: norepinephrine (NE), neuropeptide Y (NPY) and adenosine-5'-triphosphate (ATP) [1–4]. Evidence for the existence of these three co-transmitters has come from studies showing that they are located in sympathetic nerves, that they can be released under appropriate conditions, that the application of each mimics a phase of sympathetic nerve stimulation, and that each phase can be blocked with an appropriate antagonist. NPY is known to be co-localized with NE and ATP in perivascular nerves innervating a variety of blood vessels. NPY exerts prejunctional modulatory effects on transmitter synthesis and release [5]. The peptide is also involved in cardiovascular regulatory mechanisms. Studies with selective NPY-Y1 antagonists provide evidence that the principal postjunctional receptor that produces direct contractile effects or potentiation of the contractile effects of other vasoactive substances is of the Y_1 subtype. Similarly, studies with selective Y_2 antagonists suggest that the principle prejunctional receptor is of the Y_2 subtype, both in the periphery and central nervous system (CNS) [5]. Other NPY receptor subtypes may also be involved in the prejunctional and postjunctional actions of NPY although information is incomplete.

It appears well accepted that essential hypertension is a multifactorial disease involving many alterations in the nervous system and endocrine system as well as alterations in vascular smooth muscle function. Despite the complex nature of the pathophysiological mechanisms contributing to hypertension, there is considerable evidence for an involvement of increased sympathetic nerve activity in various experimental hypertensive models as well as human hypertension [6–11].

For instance, a study by Esler has demonstrated that NE release from renal nerves is elevated in young borderline hypertensive patients and they also have altered spillover of central monoamines from subcortical regions of the brain [12]. Neonatal sympathectomy of the Spontaneously Hypertensive Rat (SHR)

produces long-term reductions in blood pressure [13]. Moreover, interruption of brain neurotransmitters or lesions of specific areas involved in sympathetic regulation prevents hypertension in the SHR as well as DOCA-salt hypertension [14–16]. The purpose of the present paper is to discuss and summarize data suggesting an involvement of NPY and sympathetic control of vascular tone in hypertension. The reader is also referred to several recent reviews which address various aspects of the topic [17–22].

Evidence for a role of NPY in the development and/or maintenance of essential hypertension and experimental hypertensive models

NPY tissue levels and innervation

There have been numerous reports of a denser innervation of NPY containing nerves in both prehypertensive as well as adult SHR compared to normotensive controls. This has been demonstrated in cerebral blood vessels [23, 24] mesenteric arteries [25–28] the caudal artery [28] and intralobar and interlobar arteries in rat pancreatic islets [29]. In several cases the increased innervation increased with age in the SHR although the hyperinnervation preceded hypertension or associated medial hypertrophy. It was observed that there is a co-segregation of NPY hyperinnervation of the vasculature with the hypertensive phenotype, evident as early as one month of age in the hypertensive strains [28].

There have been similar reports of higher NPY levels in several tissues such as the median preoptic and arcuate nucleus [30]; ventromedial hypothalamic nucleus and locus coeruleus [31]; mesenteric and femoral artery, jugular vein and vena cava [22]; urinary bladder, urethra and prostate [32] of SHR compared to Wistar-Kyoto (WKY). On the other hand, there have also been reports of lower NPY levels compared to normotensive controls, especially in the CNS and spinal cord. For instance, NPY-ir in the cortex, cervical and thoracic spinal cord of SHR was less than WKY [33]. Similar lower concentrations of NPY were seen in the pons/medulla oblongata [34]. Lower levels of NPY have also been seen in the atria, kidney and adrenals of SHR compared to WKY [35]. Moreover aortic co-arctation induced hypertension resulted in a decrease in NPY-ir fibers in the kidney and renal artery as well as in mesenteric arteries [36]. Experimental hypertension using the 2 kidney 1 clip model decreased NPY mRNA in both kidneys while there was no change in DOCA-salt hypertension [37].

Plasma NPY levels

Although there are reports of no significant differences in plasma concentrations in hypertensive patients [38, 39] a large number of studies have reported

that plasma NPY levels are elevated in patients with essential hypertension [40–46] and in pheochromocytoma [47–49]. Moreover, plasma NPY is elevated in hypertensive patients undergoing various stressors or exercise [43, 50, 51]. Although these results are consistent with a role of NPY in contributing to the maintenance/development of hypertension one caveat is that circulating levels of NPY have been reported to be still elevated more than controls despite a marked reduction in blood pressure [40, 46]. In contrast, treatment of SHR with nitrendipine, captopril or both drugs, in doses which lowers blood pressure, also decreased serum NPY levels [52].

Platelet NPY is also higher in hypertensive patients [53] and in SHR [54–56]. Higher plasma levels of NPY have also been reported in stroke prone SHR [57] as well as SHR [52] and both NE and NPY plasma levels increased significantly in parallel with blood pressure during DOCA-salt hypertension [58]. Moreover tissue NPY content was decreased in the mesenteric artery and heart ventricle after 1–3 weeks of DOCA-salt treatment, but the content in the adrenal gland was not significantly different. It was concluded that in the DOCA-salt hypertensive rat, increased plasma NPY-li levels originate primarily from sympathetic nerves since those levels were correlated exclusively with circulating NE levels and were associated with a reduction in NPY-ir in heart and mesenteric artery. Basal plasma concentration of NPY-ir in arterial blood was also higher in renal hypertensive rabbits compared to normotensive controls [59].

Functional NPY responsiveness

Exaggerated contractile responses to NPY in various experimental hypertensive models have been well documented. For instance, pressor responsiveness to NPY increased in parallel with the development of hypertension in the SHR [60, 61] and infusion of NPY led to greater increases in blood pressure in this model relative to several control strains. The ability of NPY to potentiate nerve-induced or agonist induced increases in perfusion pressure of the perfused mesenteric arterial bed is enhanced in beds obtained from 8–10 week old SHR compared to normotensive controls [62–65]. Moreover the pressor response to microinjections of NPY into the posterior hypothalamic nucleus was potentiated in the SHR [64, 66].

Similar enhanced pressor responses to central injections of NPY have been seen in the SHR. It has been reported that the intracereboventricular injection of NPY had an increased potency associated with a longer duration of the pressor response in SHR compared to WKY. This was accompanied by an increased density of Y_2 receptors (vasopressor effects) in the nucleus tractus solitarius (NTS). This suggests a dominance of the Y_2 over Y_1 receptor in the SHR [67]. A similar increase in Y_2 receptor mRNA was observed in the NTS 2 h after aortic co-arctation hypertension and correlated with the rapid increase in blood pressure in this model [68].

In other systems where NPY has been shown to produce depressor responses there is an attenuation of the effect of NPY in SHR. The intrathecal (Int) administration of NPY into conscious or anesthetized rats at the level of T_4 or T_{10} produced a decrease in blood pressure accompanied by a decrease in total peripheral resistance, a decrease in renal sympathetic nerve activity without a change in baroreflex sensitivity [62, 69–71]. The depressor effect of Int NPY was significantly reduced when examined in 10-week-old SHR in contrast to WKY, Sprague Dawley or DOCA-salt hypertensive rats [69]. Injections of NPY into the NTS also induced a depressor effect that was also attenuated in SHR compared to WKY [72].

The use of selective NPY Y_1 antagonists has provided evidence for an important role of NPY in the activity of the sympathetic nerve activity. For instance, the long-lasting vasoconstriction induced by high frequency stimulation of sympathetic nerves of the guinea pig vena cava *in vitro* was significantly attenuated in the presence of SR 120107 [73] or BIBP3226 (both selective NPY-Y_1 antagonists) [74, 75]. It was also observed that sympathetic nerve stimulation (NS) produced concomitant vasoconstriction and NPY-ir release in the isolated perfused mesenteric arterial bed. The NS, as well as NE and ATP, induced vasoconstriction was potentiated by a NPY-Y_1 agonist and attenuated by BIBP3226 [76]. Similar results have been seen *in vivo*, where BIBP3226 inhibited the vasoconstrictor response to high frequency stimulation of sympathetic nerves in nasal mucosa, hindlimb and skin [74]. These results clearly suggest that endogenous NPY, acting on a Y_1 receptor, plays a role in producing long-lasting vasoconstriction in these organs or tissues.

As already mentioned, NPY is well known to potentiate the contractile response to electrical stimulation or vasoactive agents in isolated blood vessels. Administration of NPY antiserum to tissues not previously exposed to NPY significantly reduced the response to field stimulation. The antiserum depressed the response to field stimulation in caudal arteries from SHR but not those from WKY. This suggests that the enhancement of the response to field stimulation is enhanced in hypertensive animals [77].

With this in mind, it has been disappointing that several groups have reported that acute or short-term administration of the NPY selective Y_1 antagonist BIBP 3226 does not lower blood pressure in the Goldblatt Hypertensive rats [78] or SHR [78, 79] although it clearly antagonizes the contractile or pressor effects of NPY. In contrast the infusion of the more potent and selective Y_1 antagonist BIBO3304 for 4 weeks blunted development of hypertension in the 2 kidney, 1 clip renovascular hypertensive model [80] as well as attenuating the acute pressor response to NPY. There are no reported studies in which Y_1 antagonists have been continuously administered over a similar or longer time period in the SHR model. Therefore the fact that acute or short-term treatment of Y_1 antagonists does not decrease basal blood pressure in the SHR must be viewed with caution and shouldn't be interpreted as a lack of a role for NPY in the development or maintenance of hypertension in this model. An additional explanation for the failure of the acute administration of BIBP3226 to

lower basal blood pressure in the SHR is there might be an atypical (non Y_1) NPY receptor subtype that mediates the vascular effects of endogenously released NPY [79, 81]. It is also possible that the NPY receptor could be located intrajunctionally whereas the classical NPY-Y_1 receptor could be located extrajunctionally and respond to exogenous administration of NPY and BIBP3226 as is thought to exist for the α_1 adrenoceptor.

Modulation by NPY or NE release in hypertensive models

Neuropeptide Y negatively modulates NE release from sympathetic neurons as well as central monoaminergic neurons [5]. In contrast to the potentiation of nerve stimulation induced vasoconstrictor responses mentioned previously, the ability of NPY to decrease the evoked release of NE from nerve stimulation induced release of NE from the perfused mesenteric arterial bed was significantly attenuated in preparations obtained from the SHR compared to WKY or Sprague Dawley (SP) age matched normotensive rats [62–65]. A similar attenuation of the ability of NPY to inhibit NE release has also been observed in the CNS. The electrical stimulation of NE from slices of hypothalamus and medulla oblongata obtained from SHR was greater than in WKY [82–84]. NPY produced an inhibition of the evoked release of NE from slices obtained from both brain regions and this inhibition was attenuated in slices obtained from SHR compared to normotensive controls [82–84]. Using microdialysis or push–pull cannulae, it has been observed that there was a greater spontaneous or stimulation induced release of NE from the paraventricular nucleus of SHR or aortic banded rats compared to WKY or SD controls [85, 86]. The ability of NPY to decrease the release of NE from the PVN of SHR was attenuated in the SHR and aortic banded rats compared to normotensive controls [86].

Genetic studies

The NPY gene locus has been shown to be co-segregated with elevated blood pressure in the SHR, specifically in chromosome 4 [87]. This suggests that the NPY locus may be a candidate for a hypertensive effect in the SHR and that using a genomic screening approach may be valuable is clarifying the difference in blood pressure between SHR and WKY. More recently, studies on the NPY gene in humans have been carried out and it has been observed that a polymorphism exists in the coding region for the NPY gene [89]. It is known that the human NPY gene is located on chromosome 7p15.1 [88] and there is a polymorphism that includes a thymidine (1128) to cytosine (1128) polymorphism (T1128C) which results in a substitution of leucine (Leu 7) to proline (7) in the signal part of prepro NPY [89]. A recent study investigated the significance of the NPY T1128C polymorphism for cardiovascular and cere-

brovascular outcome in a cohort of hypertensive patients, that is, is it associated with development of myocardial infarction and stroke in a prospective cohort of hypertensive patients [90, 92, 93]. The frequency of the NPY T1128C polymorphism was found to be 8.4% among patients with a myocardial infarction (MI) or stroke as compared to 5.1% in the control group ($p = 0.040$). The difference remained significant after adjustment for the cardiovascular risk factors: age, sex, smoking status, body mass index, systolic and diastolic blood pressure, presence of diabetes, total cholesterol, HDL, LDL and triglycerides. The authors concluded that this study indicates that the NPY T1128C polymorphism is an independent predictor for MI and stroke in a Swedish hypertensive population.

The Leu(7)-to-Pro(7) polymorphism has been linked to both type 1 [106] and type 2 diabetes [107, 108] atherosclerosis [109] and coronary heart disease [110]. These studies suggest that the NPY T1128C polymorphism may be a strong independent risk factor for various cardiovascular diseases. The overall significance of this finding is unclear however since the frequency of the T1128C polymorphism shows a geographic distribution with most of the positive associations found largely in Nordic countries such as Finland, Sweden and the Netherlands [91]. Moreover in a recent retrospective study Yamada et al. [111] were unable to find an association between NPY T1128C polymorphism and MI in Japanese subjects although the frequency of T1128C polymorphism is low in that country.

A recent editorial in the *Journal of Hypertension* states: "Identification of an association between the T1128C polymorphism and cardiovascular risk factors may only be the initial step towards understanding the physiological and pathological roles of NPY in humans" [91].

More translational and basic studies are required to focus on the genetic impact of this polymorphism on normal cardiovascular and blood pressure control and the development of cardiov ascular disease, as well as the cellular and molecular mechanisms involved.

Role of NPY in stress induced hypertension

There is clear evidence that various forms of stress can induce transient elevations of blood pressure. Folkow [95] has long maintained that repetitive (stress-induced) sympathetic stimulation which is able to induce transient increases in blood pressure, heart rate and cardiac output will lead to structural adaptation of the blood vessels and the heart (for review see [94]). The question of whether stress can lead to persistently elevations of blood pressure is still unclear, however.

We and others have obtained results clearly implicating NPY in stress-induced hypertension. Zukowaska-Grojec has demonstrated that NPY plays a role in the pressor response to cold-water pressor test [96]. These investigators observed that there was an increase in plasma NPY-ir in rats exposed to cold-

water stress (COLD). Administration of BIBP3226 tended to decrease the stress-induced pressor response and significantly attenuated the post COLD elevation of blood pressure. The COLD induced fall in the superior mesenteric artery blood flow and the increase in the mesenteric vascular resistance was either reduced or eliminated by BIBP3226.

Chronic cold stress (4 °C, 1–3 weeks) has been shown to produce hypertension in rats [97, 98]. We observed that chronic cold stress (4 °C) produced a sustained increase in mean arterial pressure in both normotensive and borderline hypertensive rats (BHR) [99]. The high blood pressure in BHRs was also significantly reversed by the Y_1 selective antagonist BIBP3226. Chronic cold stress potentiated the pressor response to rats to subsequent acute stress (the cold water stress).

Chronic cold stress (4 °C, 1–3 weeks) also induced a marked increase in gene expression (adrenal medulla; superior cervical ganglia), tissue content (mesenteric arterial bed) and nerve stimulation induced overflow of NPYir from the perfused mesenteric arterial bed [99, 100]. These results suggest that chronic cold stress-induced hypertension is mediated by elevated NPY release and vascular tone as a result of increased NPY gene expression and storage.

Since insulin-induced hypoglycemia has been shown to produce increased sympathetic output and elevated blood pressure, the role of NPY in the hypertensive response to insulin was investigated [101]. Subcutaneous injection of insulin to rats produced a sequential increase in plasma NPYir, NPY mRNA abundance in the adrenal and superior cervical ganglia and adrenal NPYir, strongly suggesting that NPY release, biosynthesis and storage are elevated following hypoglycemic stress. These results are consistent with an important role for NPY in insulin-induced hypertension. NPY has also been implicated in other types of stress. For instance, the effect of BIBP3226 and its inactive enantiomer, BIBP3435, on a mental stress paradigm was investigated in SHR. The stress (air jet) induced maximum increase in heart rate was significantly reduced by BIBP 3226. These results suggest a role for NPY in the response to mental stress [102]. Odar-Cederlöf and colleagues investigated the role of NPY in fluid overload induced hypertension in hemodialysis patients [103]. They observed that the plasma concentrations of NPY correlated well with the degree of fluid overload and the mean arterial pressure. In stepwise multiple regression analysis, NPY alone explained blood pressure elevation when analyzed with fluid overload and angiotensin II, renin, NE and epinephrine levels. These investigations hypothesize that fluid overload in dialysis patients is a stress-inducing state that activates the sympathetic nervous system and releases NPY. They further conclude that over hydration is a stress situation that creates a vicious cycle through the release of NPY. NPY exacerbates hypertension and increases cardiac load and thus the risk of heart failure. This in turn increases the stress and further sympathetic nerve stimulation.

Studies examining the role of central NPY neurons following stress have also been carried out [104–105]. Activation of brainstem and arcuate nucleus (ARC) NPY neurons and levels of NPY mRNA in the ARC were measured in

response to restraint stress in adult SHR and two normotensive controls [104]. FOS immunohistochemistry and NPY *in situ* hybridization to identify activated NPY neurons were examined in the NTS, ventrolateral medulla (VLM) and ARC. In the NTS and VLM of restrained rats approximately 33% and 75%, respectively of NPY neurons were activated, but no differences among strains was found. In the ARC about 36% of neurons activated by restrain contained NPY mRNA. NPY mRNA levels were significantly elevated in SHR's compared to controls. Restraint led to significant decreases in mRNA levels. These results suggest that NPY likely participates as a neurotransmitter in the autonomic pathways utilized during stress and originating in the NTS, VLM and ARC. The decrease in NPY gene expression in the ARC which was measured following restraint stress argues against a role for NPY from the ARC in activating sympathetic activity or the hypothalamo-pituitary adrenal (HPA) axis. Others have observed that exposure to restraint results in significant changes in prepro-NPY mRNA expression in specific nuclei of both WKY and SHR that are components of not only the central circuitry regulating the stress response, but also the neural network modulating autonomic function [105].

Evidence consistent with or suggestive of a role for NPY in the development and/or maintenance of hypertension or stress induced hypertension in humans or experimental hypertensive models

Taken together there is considerable evidence implicating peripheral and central NPY in the development and/or maintenance of hypertension in humans and experimental animal models of hypertension. The evidence supporting this is summarized below:

- The NPY gene locus is co-segregated with elevated blood pressure in the SHR
- Evidence exists that the NPY T1128C polymorphism is associated with development of myocardial infarction and stroke in a prospective cohort of Swedish hypertensive patients
- There is a co-segregation of NPY hyperinnervation of the vasculature which precedes the development of hypertension in many cases
- There is elevated NPY levels in certain central nuclei associated with pressor responses and lower NPY levels in areas associated with depressor responses in hypertensive models
- There are elevated plasma as well as platelet levels in patients with essential hypertension and in animal models of hypertensions including SHR, stroke prone SHR, DOCA-salt hypertensive rats and renal hypertensive rabbits
- Exaggerated vascular responsiveness to NPY exists in various experimental hypertensive models; moreover vascular and pressor responsiveness to NPY increase in parallel with the development of hypertension in certain models such as SHR

- Enhanced pressor responses to NPY are also seen following central administration in experimental hypertension; in contrast in areas mediating depressor responses to NPY, there is an attenuation of the depressor response
- The selective Y_1 antagonist BIBO3304 blunted the development of hypertension in 2 kidney, 1 clip hypertensive models; NPY antiserum depressed the neurogenic response to field stimulation in caudal arteries obtained from SHR but not WKY
- The prejunctional inhibitory effect of NPY on sympathetic neurotransmission in blood vessels is attenuated in SHR compared to normotensive rats; a similar attenuation of NPY's inhibitory effect on NE release was seen in brain slices (medulla oblongata; hypothalamus) or central pressor regions (paraventricular hypothalamic nucleus) in the SHR
- NPY is clearly implicated in contributing to the development of stress-induced hypertension in both animals and humans
- In the SHR, NPY has been shown not only to potentiate adrenergic responses but attenuates the development of compensatory adrenergic desensitization, thus perhaps promoting the maintenance of elevated blood pressure

Evidence inconsistent with a role for NPY in the development/maintenance of hypertension and future directions

Despite a great deal of evidence in favor of a role for NPY in contributing to the development or maintenance of hypertension in humans and animal models, definitive proof is still lacking. It is not clear if the elevation of plasma NPY and tissue levels is the cause or result of elevated blood pressure, although in some cases the increased levels precede elevations of blood pressure. It is not clear why specific NPY Y_1 antagonists do not reduce blood pressure in the SHR and why plasma NPY levels remain elevated despite blood pressure reaching normotensive levels. Further studies are needed where NPY antagonists are administered for longer periods of time and in additional hypertensive models other than the SHR. Clearly more translational and basic studies are required to focus on the genetic impact of the NPY T1128C polymorphism on normal cardiovascular and blood pressure control and the development of cardiovascular disease as well as the cellular and molecular mechanisms involved.

The following schema is an attempt to integrate much of the information discussed above. The figure depicts the vascular sympathetic neuroeffector junction (sympathetic terminals containing NE, NPY and vascular smooth muscle) with connections from the CNS. ATP has been left out for simplicity but is also an important player. Large lines depict increased activity, small lines normal activity and dotted lines decreased activity. In animals or patients who become hypertensive, it is hypothesized that there is an enhancement of sympathetic outflow mediated centrally. In central pressor areas such as the rostral

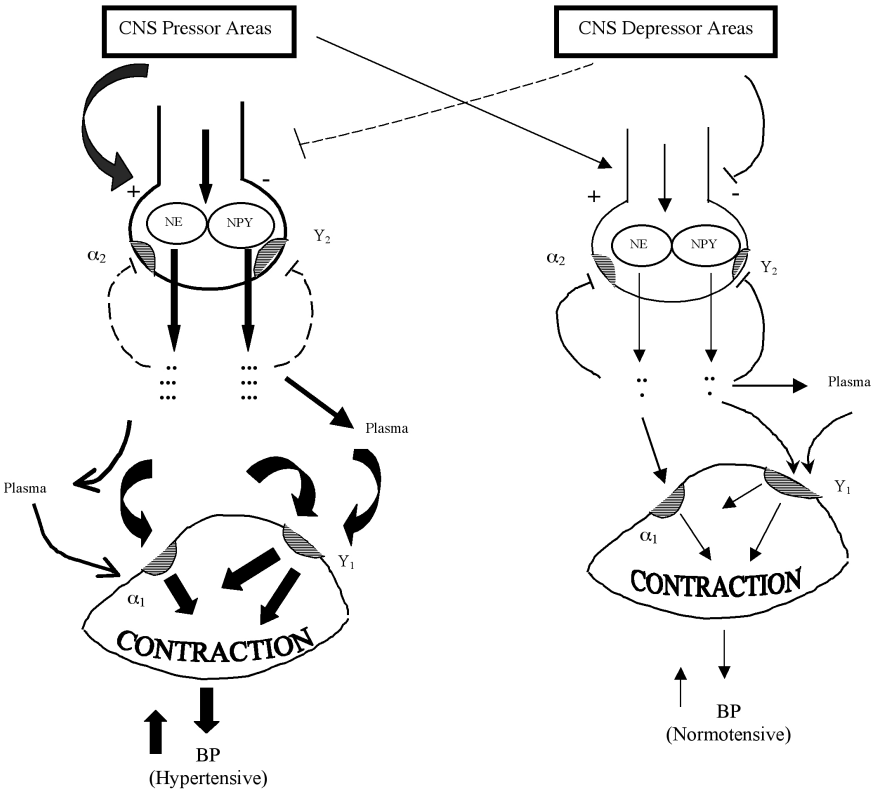

Figure 1.

ventrolateral medulla and posterior hypothalamus there is a greater response to NPY due to enhanced NPY levels or increased NPY activation as well as the action of other mediators. In contrast there is an attenuation of responses from depressor areas such as the nucleus tractus solitarius, caudal ventral lateral medulla and spinal cord with a decrease in sympathetic outflow. An increase from pressor areas coupled with a decrease from depressor areas would result in an increase in nerve impulses arriving at the sympathetic nerve terminals. The increase in nerve impulses coupled with an attenuation of negative feedback modulation would result in enhanced release of both NE and NPY (and perhaps ATP). This increased release would contribute to the elevated plasma levels of NPY and NE commonly reported. Increased levels of NE and NPY at the vascular neuroeffector junction plus enhancement of NPY's ability to potentiate contraction would result in an enhancement of the contraction of vascular smooth muscle and contribute to the hypertensive state.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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Neuropeptide Y and the cerebral circulation

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Introduction

Neuropeptide Y (NPY) is one of the most abundant neuropeptides in both the peripheral and the central nervous systems. In addition, NPY is found in blood and cerebrospinal fluid (approximately 100 pmoles/L). Immunohistochemical studies show NPY to be widely distributed in sympathetic nerves supplying the cardiovascular system including the cerebral arteries. In general [1, 2] perivascular NPY-containing fibers are more abundant around arteries than veins. Larger cortex pial arteries tend to be more densely innervated by NPY-positive sympathetic fibers than smaller arteries; and rostral arteries receive more NPY fibers than caudal vessels. NPY-positive nerve fibers also are closely associated with penetrating arterioles and intraparenchymal microvessels of the brain.

In sympathetic nerve terminals, NPY is packaged with norepinephrine in large, dense core synaptic vesicles, whereas adenosine 5' triphosphate (ATP) is co-stored with norepinephrine in both small and large dense core vesicles. Consistent with this general scheme, NPY immunogold labeling is associated with large granular vesicles in the varicosities of nerves innervating human brain vessels [3].

NPY receptor subtypes

NPY acts on a family of G-protein coupled receptors (Y_1–Y_6) [4]. So far, five distinct NPY receptors have been cloned, and a sixth receptor (Y_3) has been characterized pharmacologically. All of the subtypes appear to act through similar signaling pathways mediated by pertussis toxin-sensitive G proteins (G_i and G_o). The Y_1, Y_2, and Y_5 subtypes preferentially bind NPY and the related endogenous peptide YY (PYY). Y_2 receptors, which were first described as presynaptic receptors, are uniquely activated by C-terminal fragments of NPY, e.g., $NPY_{3–36}$, $NPY_{13–36}$ and $PYY_{13–36}$, which do not affect Y_1 receptors. Specific agonists that are more potent at Y_1 than Y_2 receptors have been synthesized, e.g., [Leu^{31}, Pro^{34}]-NPY and [Pro^{34}]-PYY, which contain a proline

residue instead of glutamine at position 34 of the molecule [4]. The Y_3 and Y_4 receptor subtypes preferentially bind NPY and another related peptide, pancreatic polypeptide (PP), while the pharmacological profile of the Y_6 subtype is still controversial. The cerebrovascular actions of NPY appear to involve primarily Y_1 and Y_2 receptors; there is no evidence at this time that Y_3, Y_4, Y_5 and Y_6 subtypes are present in cerebral blood vessels.

The Y_1 receptor, which is the predominant vascular receptor mediating vasoconstriction [5] is the best studied and understood of the NPY receptor subtypes. The presence of Y_1 receptor protein and mRNA has been demonstrated in human and rat cerebral arteries [6, 7]. Using reverse transcriptase-polymerase chain reaction (RT-PCR), mRNA for the Y_1 receptor is found in isolated human pial vessels and intracortical microvessels as well as cerebrovascular smooth muscle cells in culture. *In situ* hybridization localized Y_1 mRNA to the smooth muscle layer of pial vessels. Neither RT-PCR nor *in situ* hybridization techniques were able to detect mRNA for Y_2, Y_4 or Y_5 receptors, suggesting that these receptor subtypes are absent or expressed in very low concentrations in cerebral vessels. As discussed below, functional studies, however, indicate a role for both the Y_1 and Y_2 subtypes.

Using immunohistochemistry, Y_1 receptor protein can be visualized in smooth muscle cells of rat pial arteries and arterioles [8]. The intensity of immunostaining is highest in arterioles, especially those with a diameter of 15–30 μm, with fewer receptors observed in the arteries on the basal surface of the brain. Y_1 receptor immunostaining is found on all sides of the vascular smooth muscle cells with the highest concentration on the abluminal surface. It is somewhat surprising that, in the rat, NPY-positive fibers are mainly found around the larger arteries exhibiting low levels of Y_1 receptors, while few NPY-positive fibers are observed around Y_1 receptor-rich arterioles [8]. In contrast, other species such as cat and man exhibit dense NPY innervation around pial vessels of all sizes.

NPY-induced constriction

Exogeneous NPY produces a long-lasting response [1, 2, 7, 9], and it potently constricts cerebral arteries in all species that have been examined, including human. For example, in isolated rat cerebral arterioles, NPY causes a profound constriction, to 81% of control diameter; and the concentration of NPY eliciting a half-maximum response is 6×10^{-10} M [10]. In isolated cat middle cerebral artery and pial arteries, constriction to NPY is about half as strong as that produced by K^+ [11]. *In situ*, cat pial arteries constrict markedly following perivascular application of NPY at concentrations of 7 nM or more [12]. The maximum NPY-induced constriction of cat pial arteries *in situ* is about 35%, which is equivalent to or exceeds that of other cerebrovascular constrictor agents such as norepinephrine or angiotensin II. In contrast, the isolated basilar artery of the rabbit responds to NPY with weak contraction. In

general, the ability of NPY to induce arterial vasoconstriction increases as vascular diameter decreases with the greatest effects on small resistance vessels. Pial veins of the cat also respond to perivascular NPY, but much larger concentrations of the peptide are required to produce a significant reduction in diameter [12].

Vasoconstrictor mechanisms

In cerebral arteries, constrictor responses to NPY appear to be mediated by changes in smooth muscle membrane potential [11] and influx of extracellular calcium. In guinea pig basilar artery, NPY evokes a slow, long lasting depolarization (up to 8 mV) of the smooth muscle that is directly correlated with constriction [13]. NPY is known to inhibit ATP-sensitive K^+ channels, causing depolarization of arterial smooth muscle, but this has yet to be demonstrated in cerebral arteries. Membrane depolarization produced by NPY likely activates voltage-operated calcium channels (VOCC). NPY-induced vasoconstriction of cerebral arteries is dependent on the influx of calcium since it is blocked by either removal of extracellular calcium or addition of VOCC blockers, e.g., nifedipine, nimodipine, verapamil and diltiazem [1, 11, 14].

NPY inhibits cyclic adenosine monophosphate (AMP) formation in cerebral arteries, as it does in other tissues [4]. This effect is expected for G_i/G_o-coupled receptors; and it likely contributes to the vasoconstrictor effects of NPY since cyclic AMP is considered a vasodilator. In cat and guinea pig cerebral vessels, NPY decreases basal cyclic AMP content as well as forskolin-induced cyclic AMP production [15].

Inhibition of vasodilatation is another way that NPY increases vascular tone in various vascular beds. Interestingly, NPY inhibits vasodilator responses to acetylcholine, adenosine, norepinephrine (in the presence of phentolamine), substance P, and VIP without affecting calcitonin gene-related peptide (CGRP)-mediated dilation. In pre-contracted basilar arteries from guinea pig, acetylcholine or substance P induces relaxation, and this response is significantly inhibited by NPY. The inhibitory effect of NPY is reversed by the inhibitor α-trinositol. NPY-induced inhibition of adenylate cyclase, decrease in Na^+-K^+ pump activity, or membrane depolarization have been postulated as mechanisms underlying inhibition of relaxation by NPY [16, 17].

NPY-induced dilatation

Although NPY is best known for its ability to increase vascular tone, this peptide can also cause direct vasodilatation *in vivo* and *in vitro* [17]. Injection of NPY into the carotid artery of the cat produces a transient, but significant, increase in cerebral blood volume and flow. Administration of N^G-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase (NOS) inhibitor,

prevents the volume increase, thus implying that NPY elicits transient vasodilatation via production of NO [18]. In isolated guinea pig cerebral arteries, NPY also causes a transient vasodilatation that is correlated with increased cyclic GMP levels [19]. When applied selectively to the lumen of rat middle cerebral artery segments, NPY, [Leu^{31}, Pro^{34}]-NPY, and NPY$_{13–36}$ all produce a concentration-dependent vasodilatation [17]. Pretreatment of the artery with a NOS inhibitor or removal of the endothelium prevents the dilatory response to NPY. In fact when the endothelium is removed, intraluminal NPY agonists produce constriction instead, no doubt by direct action on the smooth muscle [17]. Together, these studies indicate that NPY acts on the endothelium to release NO that in turn stimulates smooth muscle guanylate cyclase leading to relaxation.

There is some evidence for the presence of NPY receptors on endothelial cells. In cerebral arteries, the functional NPY antagonist α-trinositol attenuates NPY-stimulated increases in cyclic GMP formation without affecting basal cyclic GMP levels [19]. BIBP 3226, however, does not affect vasodilatation in response to intraluminal NPY or [Leu^{31}, Pro^{34}]-NPY [17] indicating that Y_1 receptors are not involved. It is hypothesized that endothelial Y_2 receptors are responsible for NPY-induced dilatation; however, other NPY receptor subtypes cannot be ruled out until more discriminating agents, e.g., a selective Y_2 receptor antagonist, are tested. Within the brain, NPY is often co-localized in NOS-containing nerves that appose intracerebral blood vessels [20]. While the functional consequences have never been demonstrated, it is possible that NPY and NO act synergistically to produce local vasodilatation.

Neuropeptide Y and the cerebral circulation

The influence of *in vivo* NPY administration on the cerebral circulation was first examined in the rat. Allen and colleagues [21] reported that a bolus injection of NPY into the carotid artery produced profound reductions in cortical blood flow. Decreases in flow were large in magnitude (up to 98%) and long in duration (at least 2 h). Although an identical protocol was used in another study, such a dramatic or prolonged reduction in cerebral blood flow was not seen [22]. The administration of 1 nM and 5 nM of NPY as a bolus into the internal carotid artery of the rat resulted in a dose-dependent decrease in ipsilateral striatal local blood flow as measured with the hydrogen clearance method. The decrease developed slowly and persisted for at least 2 h without affecting blood pressure. A bolus injection of NPY into the vertebral artery of anesthetized dogs resulted in a concentration-related reduction in vertebral blood flow as measured by electromagnetic flowmetry [23]. The decrease in vertebral blood flow produced by NPY reached its maximum at 3 min and remained depressed for up to 30 min.

Effect of neuropeptide Y on regional blood flow and metabolism

The importance of NPY in the regulation of regional cerebral blood flow (CBF) has been investigated in the rat striatum using quantitative autoradiographic techniques [24, 25]. Since NPY may influence local CBF either by a direct vasomotor action or by directly altering cerebral metabolism with secondary changes in blood flow, the effect of NPY on both striatal blood flow and striatal glucose use was examined [25]. Intrastriatal administration of NPY produced significant reductions of CBF within a limited number of regions of the CNS. The majority (30 of 40) of the regions investigated, however, did not exhibit changes in CBF or in glucose use.

In the caudate nucleus into which NPY had been administered, tissue perfusion was markedly reduced [24, 25]. Reduction in striatal blood flow extended from the most rostral to the most caudal portion of the caudate nucleus. In contrast, the overall rate of glucose utilization in the striatum was only minimally altered by the administration of NPY. Thus, the increase in striatal cerebrovascular resistance occurred independently of local changes in metabolism, indicating that NPY directly alters striatal blood flow. This is a relatively unusual observation, since changes in CBF are generally correlated with alterations in cerebral oxidative metabolism. Arteries that supply blood to the striatum (the middle cerebral and lenticulostriate arteries) also are innervated by NPY-like immunoreactive fibers and respond to neuropeptide Y with a dose-dependent contraction [24]. Together these observations further support a role for NPY in regional cerebrovascular regulation.

It is surprising that NPY caused profound reductions in CBF with minimal changes in glucose utilization in several brain regions far removed from the striatal injection site, e.g., the entorhinal cortex, amygdala, and perirhinal cortex [23–25]. The cause of the marked alterations in blood flow in these extrastriatal regions is uncertain. Since their distance from the injection site (greater than 2 mm) makes simple diffusion of NPY into these regions unlikely, it is improbable that blood flow changes in these regions resulted from a direct vasoconstrictor effect of NPY. It may be that constriction originating in the striatal arteries and arterioles is propagated to regions remote from the injection site. Another intriguing possibility is that the injection of peptide produced alterations in neuronal activity within intracerebral pathways involved in cerebral circulatory control. Thus, the dissociation between local CBF and local glucose may be evidence of a functional role played by cerebrovascular fibers originating within the CNS itself.

Modulation of autoregulation

Direct proof for involvement of NPY in cerebrovascular physiology comes from two sets of results. In the cat, Goadsby and Edvinsson [26] examined cortical microcirculation in conjunction with activation of the sympathetic nerves.

The NPY blocker α-trinositol was found to shift the autoregulation curve to the left, thus showing that part of the protective effect of the sympathetic system was mediated by NPY.

The second study was carried out by Vraamark et al. [27] who studied whole cerebral blood flow in rat using the Kety-Schmidt method. A marked influence on the upper limit of the autoregulation was also observed with the NPY blocker α-trinositol. Evidence now exists that both NPY and norepinephrine participate in protecting the brain against breakthrough of the upper limit of autoregulation in conditions of high blood pressure.

Neuropeptide Y in stroke

The possibility that NPY contributes to the development of cerebral vasospasm has been examined in experimental subarachnoid hemorrhage (SAH) [28, 29]. In the first study Abel et al. [28] observed that NPY in cerebrospinal fluid was markedly increased after injection of autologous blood. In these studies NPY was observed to be a strong vasoconstrictor. Depending on experimental conditions the responses to NPY were modified following SAH. NPY-like immunoreactivity (NPY-LI) also has been measured in CSF from patients with aneurysmal SAH [30, 31]. Both studies found that NPY was not significantly higher in SAH than in controls. However, Juul et al. [30] observed that in some patients there was a correlation between the degree of severity of spasm as studied with transcranial Doppler ultrasound and the content of NPY-LI in the external jugular vein. The possibility that NPY plays a role in a particular subset of SAH patients needs to be explored further.

Further support for a deleterious role of NPY in stroke has appeared [32]. There was increased immunoreactivity for neuropeptide Y (NPY) within the perilesional cortex following experimental middle cerebral artery occlusion (MCAO) or focal excitotoxic damage. NPY administration increased the relative infarct volume and reduced rCBF as observed during reperfusion. These results indicate that peripheral or central administration of NPY impairs reperfusion following experimental MCAO and worsens the outcome of focal cerebral ischemia [32].

Concluding remarks

The presence of NPY receptors on both endothelial and vascular smooth muscle cells indicates that blood-borne NPY as well as NPY stemming from surrounding nerves can affect contractile responses of cerebral vessels. In addition, endothelial cells may themselves be a source of NPY as well as a site of NPY metabolism. Understanding the roles of NPY in cerebrovascular regulation is complicated by multiple receptor subtypes, signalling pathways and cellular responses. Vasodilatory effects and vascular remodeling actions under-

score the fact that NPY cannot just be classified as a potent vasoconstrictor. NPY is an important sympathetic transmitter, but it clearly acts via intracerebral nerves as well to regulate local CBF.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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Neuropeptide Y and the heart: implication for myocardial infarction and heart failure

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Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide first isolated from porcine brain in 1982 [1]. Shortly thereafter another peptide was isolated from porcine intestine that showed high sequence homology with NPY and hence named peptide YY (PYY) [2]. NPY and PYY possess structural elements similar to those of the pancreatic polypeptides (PPs) [3] and thus been included in the same peptide family, called the Y family or NPY family [4] (for further review see Dumont and Quirion, in this book). All these peptides have 36 amino acids, contain several tyrosine residues, have a polyproline type II helix, a beta-turn (amino acid residues 10–13), an amphiphilic alpha-helix (amino acid residues 14–30) and a C-terminal area that appears to adopt a flexible structure [5, 6]. These structural elements are likely to confer a hairpin like-structure, in which N- and C-terminal amino acid residues are in closed proximity as previously shown for avian PP [7, 8].

Localization studies of NPY by immunohistochemical techniques using highly potent and selective antibodies established the abundance of NPY-like immunoreactive (ir) in the central nervous system of mammals, including human [9–11] as well as the peripheral nervous system [12–14]. Other members of the family such as PYY and PP are primarily found in endocrine cells of the intestine [15]. However, PYY-like ir was also shown to be present in the brainstem and various hypothalamic nuclei [16]. For in-depth review of NPY family of peptides, their receptors and fundamental molecular signaling, the reader is advised to refer to the NPY biochemical chapters in this book.

Heart Failure and NPY

Epidemiology

Heart failure is a common clinical syndrome that has enormous impact on the prognosis and lifestyle of the patients. In the USA, more than 4 million people

have heart failure and more than 400,000 new cases are diagnosed each year. The worldwide heart failure patient population is estimated to be over 10 million in developed countries with the forecast for aggressive increase in disease incidence in developing countries. The diagnosis of heart failure is associated with high mortality, about 50% in 5 years and the morbidity of the syndrome has major effects on the quality of life and individual productivity. The overall management and costs of heart failure is one of the most significant factors in healthcare fiscal and management burden with steady growth of prevalence and hospitalization volume. Heart failure has become a primary diagnosis of adult patients discharged from hospitals (for review see [17, 18]).

Pathophysiology

The clinical syndrome of heart failure likely represents summation of multiple anatomic, functional and biologic alterations that interact in a complex manner and in different environments and genetics backgrounds over chronic and variable periods. Heart failure occurs when "abnormalities of cardiac function causes the heart to fail to pump blood at a rate required by the metabolizing tissues". The main cause associated with heart failure is ischemia due to coronary artery diseases, myocardial infarction, hypotension or toxic conditions. Cardiac myopathies, either following inflammatory/infectious (viral, bacterial) conditions or immune reactions or idiopathic (of unknown) can also contribute significantly to chronic heart failure (for review see [18, 19]). Cardiac rhythm disturbances such as chronic atrial flutter and atrial fibrillation also drive cardiac remodeling and exacerbate heart failure. Regardless of the conditions that trigger cardiac damage, progression of heart failure invariably leads to progressive morbidity and ultimately, death.

The neurohormonal model of heart failure

The process of cardiac remodeling following cardiac injury, considered as a main mechanism that leads to heart failure and death, is believed to be driven by multiple factors derived from peripheral and central neuronal elements, immune and inflammatory cells and the endocrine system. These factors include catecholamines (noradrenaline, adrenaline), angiotensin II, vasopressin, prostaglandins, natriuretic peptides, cytokines (TNF alpha, IL-1beta), vasodilator peptides (CGRP, substance P), aldosterone and others. The activation of numerous neurohormonal factors believed to contribute to progression of heart failure have lead to the "neurohormonal model" [19] of heart failure progression that underwrites therapeutic strategies to combat the relentless progression of heart failure. Several important and medically beneficial drugs have been discovered and developed based on current understanding of the "neurohormonal model" such as angiotensin II receptor antagonists and

angiotensin II converting enzyme inhibitors [20], adrenergic beta receptors blockers [21], atrial natriuretic peptide (ANP) agonists [22], and aldosterone antagonists [23].

It is however well recognized nowadays that contemporary drugs that block neurohormonal factors have limited benefits and in fact result in diminished efficacy over time possibly due to: 1) inability to achieve adequate levels of neurohormonal antagonism, 2) ceiling of neurohormonal antagonism, 3) neurohormonal independent mechanism.

In recent years NPY emerged as an additional possible neurohormonal factor that could play a role in cardiac function in health and disease. In this brief review, the case for a role of NPY and related peptides in cardiac function and dysfunction will be reviewed and analyzed. The potential for NPY modulators to join the heart failure therapeutics arsenal is attractive in view of the rapid discovery and development of highly selective NPY receptor agonists and antagonists.

NPY and NPY receptors localization and function in the heart

NPY is found in sympathetic nerve endings of the human heart as well as in many other species [24, 25]. NPY containing sympathetic nerve ending are localized in both the coronary vasculature and within cardiac myocytes. The heart contains also NPY receptors and especially the NPY Y1 and NPY Y2. The cardiac NPY Y1 receptors have been identified by immunocytochemistry in the cardiac vasculature but their presence in normal cardiac human myocytes is less clear [26]. However, pharmacological studies with various NPY agonists suggest that both NPY Y1 and NPY Y2 receptors are likely to be present and functional in cardiac myocytes, at least in rodents [27].

The NPY Y2 receptor is believed to be primarily located on pre-junctional sympathetic nerves where it serves as a negative feedback regulation mechanism for norepinephrine release [28]. More recent studies using specific antibodies rose against extracellular parts of the NPY 1 and 2 receptors have been studied in human *post mortem* cardiac specimens from individuals of no history of cardiac diseases [29]. This study provided strong indication on the presence of both NPY Y1 and especially NPY Y2 receptors in both the atrial and ventricle myocytes and surprisingly, also present on conducting fibers. This study also identified differential densities of Y1 *versus* Y2 receptor distribution on cardiac microvessels with greater fractional Y1 receptor densities in sub-endocardial than the sub-epicardial vessels while the Y2 distribution seemed to be more evenly distributed. These authors also confirmed the presence of Y2 receptors on cardiac nerves in accord with the animal data where NPY is believed to mediate a negative feedback loop on both sympathetic and parasympathetic neurotransmitter release [30]. In rats and guinea pigs, vagal bradycardia was attenuated by Y2 receptor partial agonist [31] while a selective non-peptide Y2 receptor antagonist attenuated Y2 receptor mediated inhi-

bition of cholinergic transmission in these species heart [32, 33]. These data suggest the potential of the Y2 receptors to regulate autonomic input to the heart especially upon sympathetic and baroreceptor activation.

The pathophysiological role of NPY and Y1/2 receptors in the heart is not entirely clear. Y1 receptor stimulation is expected to induce coronary vessel constriction with the potential of inducing ischemia. This possibility is based on studies in patients with angina pectoris where intra-coronary injections of NPY induce myocardial ischemia with typical chest pain and electrocardiograph (ECG) changes [34]. Also, positive correlation between plasma NPY levels and the degree of ST- segment depression after exercise in patients with coronary artery disease has been demonstrated [35].

Taken together, animal and human data regarding the localization and function of the NPY system in the heart suggest a regulatory role on autonomic tone (sympathetic and parasympathetic), spatial regulation of cardiac contractility, microcirculation, angiogenesis and remodeling (for further review see [36, 37]).

NPY and heart failure

As pointed out (vide supra, neurohormonal model of heart failure) numerous neurohormonal mediators have been implicated in the pathogenesis of heart failure. It is therefore of no surprise that NPY has also been a subject of investigation in this condition especially since NPY co-localization and release with the adrenergic neurotransmitters suggested excessive co-release with norepinephrine from the activated peripheral sympathetic system. Indeed, several reports have demonstrated elevated plasma levels of NPY of patients with heart failure, regardless of the etiology of the disease [37–39]. Furthermore, it appears that plasma levels of NPY correlate with the severity of the progression of heart failure and therefore, NPY might serve as an independent prognostic factor for heart failure severity and outcome [40]. Regarding the levels of NPY in the heart of patients with heart failure, little information is available at this time. It appears however, that NPY levels are not elevated and in fact might be rather lower than normal as is also the case with norepinephrine, suggesting that NPY depletion might follow the state of the sympathetic nervous system in general [41]. However, periodic regional ischemic events might contribute to heart failure via release of norepinephrine and NPY as shown in experimental models (pig) where coronary ligation elicited local release of these mediators monitored by microdialysis [42]. The functional significance of elevated local or systemic levels of NPY in the circulation of patients with heart failure are not entirely clear and can only be speculated upon at this time. The complex physiological actions of NPY may suggest potential regulatory actions that may alleviate or exacerbate the heart failure condition depending on the receptor types that NPY activates.

Recent studies by groups of Haramati and Zukowska have brought new insight into the role of NPY receptors in chronic heart failure (CHF). Using an arterio-venous fistula model of heart failure in rats, the investigators have found that cardiac Y1 receptor gene expression decreases in proportion to severity of cardiac hypertrophy and decompensation [43]. Interestingly, at the same time, the Y2 receptor expression increases immensely in failing hearts

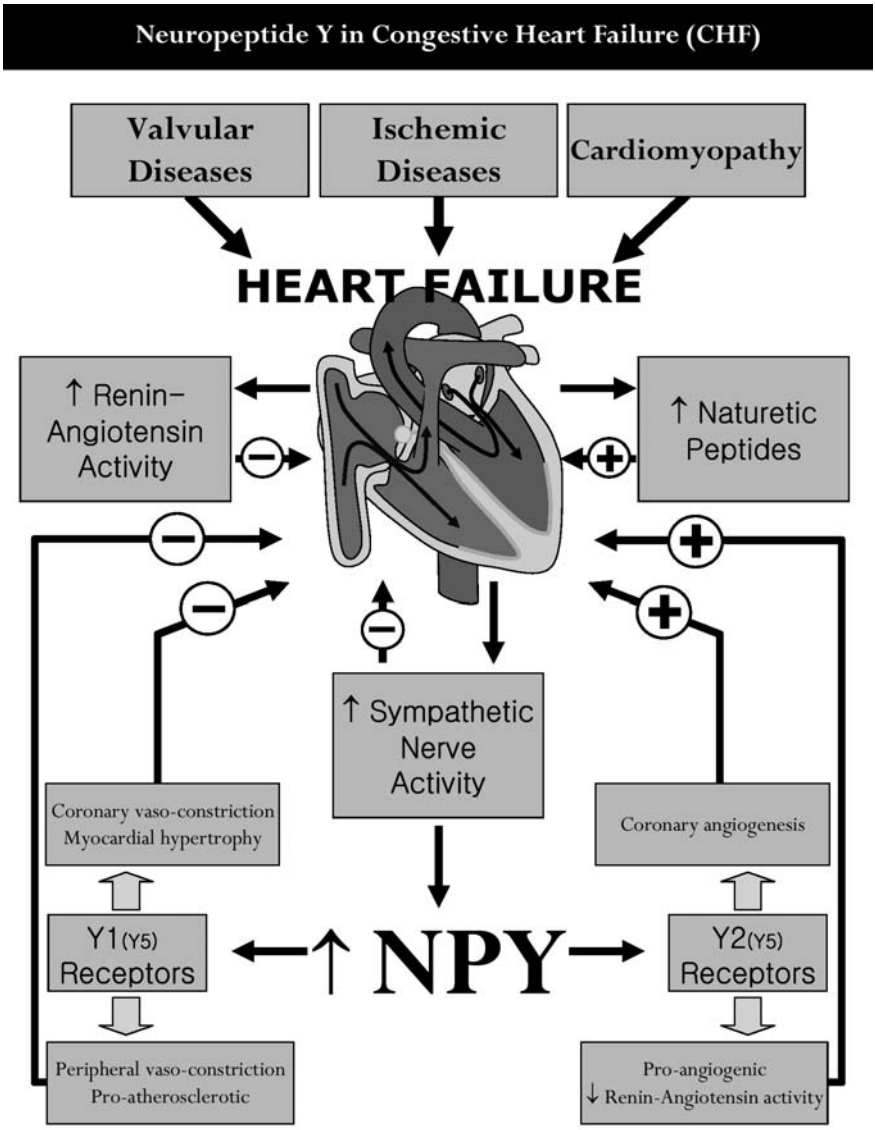

Figure 1.

[43]. Similar patterns of receptor changes were observed in kidneys, and were also proportional to the degree of renal failure [43]. Since Y1 receptor appear to mediate known NPY's growth-promoting activities in blood vessels [44, 45] and myocytes [46] – this receptor may play a pathogenic role in development of cardiac hypertrophy in failing hearts. In contrast, Y2 receptor is strongly implicated in NPY's angiogenic activity [47] suggesting that upregulation of this receptor may play an important compensatory role aimed at improving angiogenesis of the ischemic heart.

NPY may play also a role in mitigating excessive renin-angiotensin system (RAS) challenges by decreasing plasma renin and angiotensin II [48–50]. Likewise, NPY may antagonize excessive adrenergic impact on the failing heart by reducing the release of norepinephrine from pre synaptic nerves terminals [51]. Furthermore, NPY was shown in experimental models to exert diuretic and natriuretic properties [52] and to increase the release of ANP [53] thereby facilitating water and electrolyte clearance and reducing congestion.

Since the RAS and the sympathetic nervous system play an important role in progression of heart failure, as evidenced by the substantial medical benefits of angiotensin II receptor type 1 antagonists, angiotensin I converting enzyme inhibitors and beta-adrenergic receptor blockers, the higher circulating levels of NPY could be considered as a counteracting mechanism and hence of potential benefit in slowing the progression of heart failure. However, NPY, via NPY Y1 receptors, is also a potent vascular constrictor mediator which could contribute to increase in vascular resistance, including coronary vessels constriction which could compromise cardiac blood flow and other essential to essential organs. Furthermore, NPY was shown to exert hypertrophic actions on cardiac myocytes [46, 54] which are likely to be mediated via the Y1 or Y5 receptors. However, NPY was argued to directly mitigate cardiac myocytes hypertrophy induced by beta adrenergic receptors stimulation by norepinephrine [55]; thus, the role of NPY in cardiac remodeling *in vivo* needs to be elucidated. Finally the role of NPY in cardiac function and remodeling in heart failure is further complicated by experimental data suggesting that NPY vascular effects mediated via the Y1 receptor are significantly reduced in heart failure condition [56]. Taken together, evidence in support for a potential role of NPY in heart failure progression and remodeling via actions on vascular and cardiomyocyte function exerted via multiple receptors (Y1, Y2 and Y5) calls for further investigation using more selective and potent research tools.

Perspectives on NPY and cardiac diseases

The presence of NPY and several of its receptors in the normal heart and changes in expression of NPY and NPY receptors in pathophysiological states such as heart failure suggest that this neuropeptide has a local role in normal and pathological states of the heart. Further support for the possibility that the

local cardiac NPY system might influence cardiac function can be derived from demonstration such as increased release of NPY from cardiac tissue stressed by ischemia and the ability of higher than normal levels of NPY to induced cardiac stress condition such as coronary syndrome. In addition, the positive correlation between plasma levels of NPY and the extent of progression of heart failure along with the capacity of this peptide to promote cardiac cell hypertrophy, implicate NPY in cardiac remodeling associated with chronic heart failure and other post-cardiac injury conditions. Also, as a co-transmitter of the sympathetic noradrenergic nerves, 'cross talk' between norepinephrine and NPY in governing cardiac function and dysfunction is an attractive possibility. Finally, NPY is potently angiogenic and its ability to revascularize ischemic tissue via Y2 receptors has been shown in rodent hindlimbs [47, 57]. These receptors are also markedly upregulated by tissue ischemia, including rat heart failure model [43]. Thus, Y2 receptor agonists may become useful as revascularization therapy of ischemic myocardium.

NPY has also discrete and complex biological functions which make interpretation of its precise role in cardiac function quite challenging. Amplification loops as well as modulatory actions shown in isolated or highly regulated systems are difficult to extrapolate into the highly complex *in vivo* conditions. Moreover, condition such as heart failure are associated with numerous, complex and varying systemic and local biochemical changes which render any postulation on the specific role of NPY in such conditions highly speculative. Progress in understanding the diverse role of NPY in cardiac function and dysfunction and translation of this knowledge into therapeutics will require clear advances in the following fronts: 1) better *in vivo* models where NPY and its receptors are genetically modulated locally (in a cell specific manner) so that single elements are manipulated in *in vivo* context and especially during stress and disease conditions; 2) potent and selective NPY receptors agonists/antagonists are critical to assess the potential of any of the NPY receptors to become subject of manipulation in view of realizing therapeutic benefits. It is hoped that such tool reagents will be generated in the near future so that critical testing in experimental and clinical trials can be conducted.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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Role of neuropeptide Y in the regulation of kidney function

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Introduction

Neuropeptide Y (NPY), a 36-residue peptide is a sympathetic co-transmitter stored and released together with noradrenaline by adrenergic nerve terminals of the sympathetic nervous system. Structurally, NPY shares high homology with two other members of the pancreatic polypeptide family, Peptide YY (PYY) and pancreatic polypeptide (PP). These two closely related peptides are produced and released by the intestinal endocrine cells and pancreatic islet cells respectively, and acts as hormones [1, 2]. Although NPY was originally isolated from the brain and is highly expressed in the central nervous system (CNS), it has been clearly demonstrated that NPY exhibits a wide spectrum of biological activities in peripheral organs such as the cardiovascular system, the gastrointestinal tract and the kidney [3–9]. The following chapter will focus primarily on the kidney and summarize shortly our knowledge on the role of NPY in the regulation of renal function. Two excellent reviews on the subject have been published in the past by Presson et al. [8] and Bischoff and Michel [9].

NPY and the kidney

NPY localization in the kidney

Shortly after its original identification in the early eighties by Tatemoto et al. [10], NPY was reported to exist in the dense plexus of nerve fibers around the renal juxtaglomerular apparatus, by immunohistochemical staining [11]. Since then, numerous studies have confirmed the presence of the peptide in the kidney of man, rat, monkey, mouse, hamster and guinea pig [11–14]. The peptide has been localized to adrenergic nerve endings in all segments of the renal arterial system [15, 16], including the juxtamedullary regions, and afferent and efferent arterioles at the vascular pole of the glomeruli [14]. Moreover, using

specific antibodies against the C-flanking region of NPY, positive staining was found in the renal tubules but not in glomeruli of the human kidney [17]. This suggests that the peptide may also be generated in the nephron itself and not only in sympathetic terminals innervating the kidney.

NPY receptors and signaling

The biological actions of NPY are mediated through G-protein coupled receptors that are also activated by the two other peptides family members, PYY and PP [9, 18]. Six receptor subtypes (denoted Y_{1-5} and y^6) have been identified in mammalian tissues [18, 19]. There is convincing evidence for the presence of the Y_1 receptor subtype in the kidney, based on mRNA expression studies by Northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR), and *in situ* hybridization [20, 21]. In addition to the expression in renal blood vessels, media and intima, mRNA of Y_1 was also localized to the renal collecting ducts, loop of Henle, and juxtaglomerular apparatus [21]. Radioligand binding studies as well as pharmacological characterization with selective NPY agonists/antagonists provided further evidence for the existence of the Y_1 and probably Y_2 in the kidney of various species [9]. However, it appears that considerable heterogeneity exists in this regard. Thus, some studies have demonstrated abundant NPY binding in the rabbit kidney and significantly less or negligible binding in the human and rat kidney [9]. In the renal papilla, high-affinity binding sites to the related peptide PYY were identified, and are thought to be of the Y_2 subtype in the rabbit kidney, and of the Y_1 subtype in the rat kidney [22]. Finally, the natriuresis and diuresis caused by NPY in rats have been suggested to be mediated in part by Y_5 receptor, based on pharmacological characterization [23, 24]. Yet, several studies failed to detect mRNA expression of Y_5 outside of the CNS [24, 25]. This might suggest the existence of a novel, currently unidentified, Y_5-like receptor in the kidney, or alternatively, that these effects of NPY could be mediated indirectly by the actions of the peptide in extra-renal tissue [24].

As pointed out earlier, all NPY receptors belong to the family of seven transmembrane domains of the G-protein coupled receptors. Renal NPY receptors preferentially act through the pertussis toxin-sensitive G_i/G_0 family, and are predominantly linked to inhibition of adenylate cyclase [9]. In addition, there is evidence that NPY receptors may be positively linked to intracellular calcium $[Ca^{2+}]_i$ through stimulation of Ca^{2+} channels, stimulation of phospholipase C, and mobilization of Ca^{2+} from intra-cellular stores [18]. Finally, in the isolated kidney preparation, NPY has been reported to stimulate prostaglandin E_2 and I_2 production in a Ca^{2+} calmodulin-dependent manner, suggesting a possible coupling between the NPY receptor and phospholipase A_2 [26].

Effects of NPY on renal function

Renal blood flow and renal vascular resistance

Renal vasoconstriction associated with an increase in mean arterial pressure (MAP) is perhaps the best documented and most consistent finding following exogenous administration of NPY [8, 9]. Numerous studies utilizing both *in vivo* and *in vitro* techniques have demonstrated the capacity of the peptide to reduce renal blood flow (RBF) and increase renal vascular resistance (RVR) in various species including rat, rabbit, pig and man [23, 26–34]. Compared with other blood vessels, such as the mesenteric and hindlimb vascular beds, the kidney appears to be uniquely sensitive to the vasoconstrictor effect of the peptide [31]. Intrarenal infusion of NPY reduced RBF to a greater extent than systemic infusion of the peptide [29]. In the split hydronephrotic rat kidney, systemic infusion of low non-pressor doses of NPY produced a non-uniform pattern of vascular reactivity, causing a significant constriction of the proximal and distal parts of the arcuate artery with all doses [35]. No constriction was seen at the interlobular artery or the larger part of the afferent arteriole. The very distal part of the afferent arteriole adjacent to the glomerulus and the proximal efferent arteriole responded in a similar way to the arcuate arteries [35]. This pattern suggests a differential sensitivity of various segments of the renal vasculature to the vasoconstrictor effect of the peptide. The NPY-induced increase in RVR appears to be mediated by the Y_1 receptor subtype, since it could be mimicked by the Y1-agonist [Leu(31), Pro(34)] NPY, and blocked by the selective Y1-receptor antagonist BIBP 3226 in various species [36, 37]. Moreover, studies in the isolated perfused kidney of rat demonstrated that NPY-induced vasoconstriction could be inhibited by pertussis toxin treatment [38], by the Ca^{2+} channel blockers, diltiazem and nifedipine and also by removal of Ca^{2+} from the perfusates [26]. Additional studies by Bischoff et al. [9] also suggested that release of Ca^{2+} from intracellular stores may be responsible for the sustained phase of vasoconstriction during continuous infusion of the peptide. Taken together, these findings suggest that both inhibition of adenylate cyclase and alterations in $[Ca^{2+}]_i$ may be involved in mediating the renal vasoconstrictor effect of NPY. The $[Ca^{2+}]_i$ dependence of NPY mediated vasoconstriction may also explain the finding that in various vascular beds including the kidney, NPY can potentiate the effects of other vasoconstrictor agents [4]. In particular, studies in the isolated perfused rat kidney demonstrated that the renal vasoconstriction elicited by norepinephrine, arginine vasopressin, and by angiotensin II was enhanced by NPY [26]. Similarly, NPY potentiated the renal vasoconstricting effect of the α_1-agonist methoxamine, and this effect could be blocked by the Y_1 antagonist BIBP 3226 [39]. This phenomenon may be of importance in pathophysiological situations with high sympathetic outflow and increased demand for vasoconstriction.

Glomerular filtration rate

Despite the potent vasoconstrictor effect of NPY on renal vasculature, it appears that this effect is not associated with a similar reduction in glomerular filtration rate (GFR). Indeed, most of the studies in which this parameter was evaluated show only minor or no alterations in GFR in response to NPY administration [23, 29, 30, 40]. In the study of Evequoz et al. [41] it was shown that NPY infusion in the rat did not alter GFR when given alone. However, when GFR was increased by prior administration of the β-receptor agonist isoproterenol, NPY caused a significant reduction in GFR [41]. The finding that GFR was minimally affected by the same doses of NPY that caused a substantial reduction in RBF might suggest that the peptide has a greater vasoconstricting effect on the efferent than on the afferent arteriole. Indeed, this notion is compatible with the finding of Dietrich et al. [35] in the split hydronephrotic kidney that NPY constricted only the very distal part of the afferent arteriole but not its larger more proximal part. Recently, it has been shown that the sympathetic innervation of the glomerulus consists of two distinct populations of axons, type I and II [42]. Type I axons almost exclusively innervate the afferent arteriole, whereas type II axons are equally distributed on the afferent and efferent arterioles. Interestingly, NPY was located only in type II but not type I axons [42]. The functional significance of this finding and whether it may account for the preservation of GFR in the face of reduced RBF remain to be elucidated.

Effects of NPY on renal electrolytes excretion

Considering the potent renal vasoconstrictor action of NPY, a decrease in electrolyte and water excretion could be expected following the administration of the peptide. However, the available data at present suggest that NPY may exert either a natriuretic [23, 28–30, 43] or an antinatriuretic [40, 44] action, depending on the experimental conditions and the species utilized. While in early studies, conducted in dogs and primates, NPY tended to decrease sodium and water excretion, studies in the rat kidney demonstrated clearly an increase in the excretion of sodium, water, calcium and perhaps potassium [9, 29, 45]. The natriuretic/diuretic effect was observed in rats during systemic infusion of the peptide, central intracerebroventricular administration, as well as in the isolated perfused kidney [28, 29, 46]. Studies in humans with the related PYY peptide also revealed a significant increase in urinary sodium excretion following intravenous administration of the peptide [34]. While an antinatriuretic response can be easily explained on the basis of the potent vasoconstrictor properties of the peptide and its effect on renal hemodynamics, the natriuresis/diuresis appears to be mediated by a tubular action that deserves additional explanation. Several mechanisms were offered to explain this finding [9]. A potential mechanism that could account for the

NPY-induced natriuresis is the phenomenon of pressure natriuresis, secondary to the NPY-related increase in MAP [47]. However, controlling renal perfusion pressure by a supra-renal aortic clamp and renal decapsulation did not block the NPY-induced diuresis [29]. This suggests that pressure natriuresis is not the predominant factor in mediating the natriuresis/diuresis caused by systemic NPY administration [9, 29]. An interesting observation in the original study of Bischoff et al. [29] was that systemic infusion of NPY produced a greater natriuretic response compared with intrarenal infusion of the peptide at equal doses. In an additional study, the same group demonstrated that the natriuretic effect of NPY could be mimicked by the Y_5 agonist PYY_{3-36}, and not affected by the classical Y_1 antagonist BIBP 3226 [23]. Since the Y_5 receptor is not expressed in the kidney, the authors hypothesized that Y_5-like receptors in extra-renal tissues may be involved in the mediation of NPY-induced natriuresis and diuresis [24]. In an attempt to identify the mediator pathways linking the extrarenal NPY receptors to the increase in renal sodium, water and calcium excretions, additional experiments were performed. Thus, acute renal denervation did not alter the tubular actions of NPY, suggesting that the putative mediator acts as a hormone rather than a neurotransmitter [48]. Moreover, the NPY-induced diuresis and natriuresis were enhanced by the angiotensin II-converting enzyme inhibitor ramiprilat, not modified by the angiotensin II receptor antagonist losartan, and strongly inhibited by the bradykinin B_2 receptor antagonist icatibant [48]. Based on these findings the authors concluded that bradykinin could be the mediator of the tubular effects of NPY. However, more recent experiments by the same group failed to support this conclusion [49]. Thus, infusion of NPY that caused a four-fold increase in sodium excretion did not increase urinary bradykinin excretion. Furthermore, intrarenal infusion of bradykinin did not alter the urine flow rate or sodium excretion [49]. Other potential mediators of the tubular action of are the cyclooxygenase-derived vasodilatory/natriuretic prostaglandins. It has been reported that treatment with indomethacin did not affect NPY-induced alterations in systemic and renovascular hemodynamics, but completely abolished NPY- and PYY_{3-36}-induced diuresis and natriuresis, indicating that cyclooxygenase derivatives may be involved in this action [50]. Finally, although initial studies did not report an increase in potassium excretion following NPY administration, analysis of the data demonstrated a kaliuretic response under several experimental conditions [45]. Both kaliuresis and diuresis were slow in onset (requiring > 45 min to develop fully) and blocked by the cyclooxygenase inhibitor indomethacin [45].

In summary, in addition to its potent renal vasoconstrictor effect, NPY may exert distinct tubular actions. These are species dependent and in the rat are characterized by a slow onset natriuresis, diuresis, calciuresis and kaliuresis. The cellular mechanisms of, as well as the nephron sites at which the tubular actions of NPY are exerted have not been thoroughly elucidated and remain to be determined.

Effects of NPY on renin secretion

The juxtaglomerular apparatus is richly supplied by nerve endings containing immunoreactive NPY [11]. In addition, Y_1 receptor mRNA was detected in the juxtaglomerular apparatus of murine kidney by *in situ* hybridization [21]. However, in a more recent study, Y_1-immunoreactive staining was detected by immunohistochemistry in the juxtaglomerular apparatus of the mouse but not in rats [51]. Indeed, several studies in the past have suggested that NPY may negatively regulate renin secretion via a pressure-independent, pertussis-sensitive mechanism, involving the Y_1-receptor [23, 34, 38, 52–54]. Such an inhibitory effect was reported in the rat, cat, and humans, but not in dogs or primates [40, 44]. Moreover, NPY was able to lower plasma renin in pathophysiological situations characterized by increased activity of renin-angiotensin system, such as renal artery stenosis and postmyocardial infaction [54, 55]. Further support for an inhibitory action of NPY on renin release emerged from recent reports in Y_1 receptor knockout mice. Thus, plasma renin concentrations were significantly increased in Y_1 knockout mice [56]. Furthermore, using the 2 kidney 1 clip (2K1C) model of renovascular hypertension in mice, it was shown that renin secretion was higher in Y_1-deficient mice than in wild type controls [56]. These findings provide further evidence that renin secretion is controlled in part by NPY via the Y_1 receptor subtype, and that this receptor acts preferentially as an inhibitor of renin release. It is possible that such an NPY-related decrease in plasma renin activity may mediate in part the natriuretic/diuretic effect of the peptide in the rat [9].

Miscellaneous renal effects

In 1989 Dillingham and Anderson demonstrated that NPY significantly decreased arginine vasopressin (AVP)-stimulated water transport in perfused rat cortical collecting tubules [57]. Either α-2-adrenergic receptor blockade (yohimbine) or pretreatment of CCT with pertussis toxin abolished the NPY action on AVP-stimulated water transport, suggesting that NPY acts via an α-2-adrenergic receptor coupled to a pertussis toxin-sensitive protein to inhibit AVP-stimulated cAMP formation and water permeability in the collecting duct [57]. It is possible that such an interaction could contribute to the diuretic effect of NPY in the rat.

Studies by Ohtomo and co-workers [58, 59], using isolated permeabilized rat renal proximal convoluted tubule cells, demonstrated that NPY was able to stimulate Na^+, K^+,-ATPase activity. Removal of extracellular Ca^{2+}, addition of nifedipine the L-type Ca^{2+} blocker, or CaMKII-Ala286[281–302] a blocker of Ca^{2+}/calmodulin-dependent protein kinase II, inhibited the NPY-stimulated Na^+, K^+,-ATPase activity, indicating that this effect was Ca^{2+}-dependent [59]. Additional data from this laboratory suggest that NPY may modulate the renal sympathetic tone by shifting the equilibrium between the α- and β-adrenergic

tonus in the regulation of Na^+, K^+,-ATPase activity [60]. The physiological relevance of this phenomenon remains controversial since in the rat NPY has been shown to exert a natriuretic rather than an antinatriuretic effect.

Studies in genetically-manipulated animals

In recent years, studies using genetic approaches in which the gene of NPY or its receptors were deleted or overexpressed have been published. These investigations, in knockout mice and transgenic mice and rats, provided exciting information unraveling novel biological activities of NPY and its receptors [56, 61, 62]. Interestingly, no major impairments or alterations in renal function have been reported in these genetically-modified models [61]. With the exception of the data of Pedrazzini [56] on the elevated plasma renin activity in Y_1 receptor knockout mice, alluded to in the previous section, noticeable alterations in renal hemodynamics or electrolyte excretion have not been reported. It might be argued that renal function has not been thoroughly and specifically studied in these models. However, given the complex and redundant control of renal hemodynamics and sodium excretion, it is also possible that additional regulatory pathways are activated to compensate for the missing renal action of NPY.

Summary

The presence in the mammalian kidney of NPY and at least one of its receptor subtypes has been proven by several independent methodologies. Also, numerous studies using physiological and pharmacological approaches indicated that this peptide has the capacity to alter renal function. In particular, these studies suggest that NPY may exert renal vasoconstrictor and tubular actions that are species dependent, and may also influence renin secretion by the kidney. The question whether NPY plays an important role in the physiological regulation of renal hemodynamics and electrolyte excretion, remains largely unanswered at present. No major impairments in renal function have been reported in genetically models deficient in NPY or its Y_1 receptor. Thus, additional studies are required to elucidate the role of NPY in the physiological and pathophysiological regulation of renal function.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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NPY and neuron–adipocyte interactions in the regulation of metabolism

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Introduction

Adipose tissue and the brain are intricately connected for the regulation of energy metabolism and thermogenesis. The brain consolidates signals from elsewhere in the body to ascertain the overall energy balance of an organism, and then determines whether the body's resources will be utilized for energy conservation or expenditure [20]. Adipose tissue is responsible for the storage of triglycerides during energy excess and the release of free fatty acids to meet the energy needs of the body during times of increased energy demand (for review, see [1]). The stored triglycerides in white adipose tissue (WAT) are hydrolyzed by hormone-sensitive lipase to free fatty acids and glycerol to provide metabolic fuel for other tissues. The lipid in brown adipose tissue (BAT) is used instead predominantly for heat production. The sympathetic nervous system (SNS) innervates both BAT and WAT, and is in part responsible for their thermogenesis or metabolic responsiveness, respectively, by relaying signals from the central nervous system (CNS) [2–4].

One of the key molecules implicated in the regulation of energy metabolism is neuropeptide Y (NPY), a 36-amino acid α-amidated peptide produced by the posttranslational processing of preproNPY, which consists of a signal sequence, NPY, and a carboxyl-terminal peptide [5–7]. NPY is widely distributed in both the central and autonomic nervous systems, and is often found in norepinephrine-secreting neurons [8–10]. In the peripheral nervous system, neurons containing NPY innervate vascular smooth muscle, the heart, gastrointestinal system, spleen, and adrenal glands [7, 11–14]. In human coronary arteries, NPY can trigger potent dose-dependent vasoconstriction [15, 16]. In small cutaneous arteries *in vivo*, NPY acts as a co-transmitter with norepinephrine to trigger vasoconstriction [17]. A similar role for NPY as a co-transmitter with norepinephrine exists in some veins [18]. NPY also functions as a co-transmitter with norepinephrine in mediating the interaction between the sympathetic nervous system and macrophages [19]. NPY is believed to play a role in mediating many of the long-term nonadrenergic actions of sympathetic

neurons [8]. In the CNS, NPY is a potent orexigenic agent, with actions antagonistic to those of leptin [20–22]. Leptin itself is capable of inhibiting the synthesis and release of NPY [23].

NPY's actions are mediated via the NPY receptor family, which currently contains six members isolated by molecular cloning: Y1, Y2, Y3, Y4, Y5, and Y6 receptors [24, 25]. All of the Y receptors are members of the G protein-coupled receptor superfamily, and have been implicated in a wide variety of physiological responses [24]. The Y receptors can be activated by a family of functionally diverse but structurally related peptides, including NPY, pancreatic polypeptide (PP or PPY), and peptide YY (PYY), all of which are synthesized from prepropeptides [26]. The tissue distribution of the Y receptors is broad, with expression both in the central nervous system and in peripheral tissues [24, 26].

Y1 receptors are important in the periphery in the mediation of vasoconstriction [27], and along with Y5 receptors are thought to contribute to the regulation of food intake [24]. In addition, gene knockout experiments have implicated a role for the Y1 receptor in feeding [28] and in antinociception by inhibiting the release of pain neurotransmitters such as substance P [29]. The Y2 receptor is believed to be a presynaptic receptor that suppresses neurotransmitter release [24, 30], and has been implicated as having a role in angiogenesis [31]. The Y4 receptor is the same as the pancreatic polypeptide receptor (PP1), but its specific functions are poorly characterized at the present time [26]. The Y5 receptor has been implicated in mediating feeding behavior [21], although whether it is the sole receptor responsible for NPY's orexigenic actions is still under debate [32, 33]. Whether or not the Y6 receptor has any function *in vivo* has yet to be determined [26, 34]. Pharmacological characterization indicates that there may be more members of this class that have not yet been successfully cloned and characterized [21, 24].

NPY's role in the central nervous system

When injected into the cerebral ventricles or directly into hypothalamic nuclei, NPY results in pronounced hyperphagia [35]. There is also an associated decrease in thermogenesis via inhibition of sympathetic outflow from the central nervous system to brown adipose tissue [36]. With respect to the central nervous system's influence on metabolism and energy balance, the role of NPY is best understood in the hypothalamus.

The majority of neurons containing NPY are located in the arcuate nucleus, and project both within and outside of the hypothalamus, as well as within the arcuate nucleus itself [25, 37, 38]. Hypothalamic NPY expression and release are elevated in states of starvation and other forms of energy deficit, such as diabetes [39–41]. NPY gene expression in the arcuate nucleus is inhibited by leptin and/or insulin [42], the circulating levels of which are both low in starvation states. Administration of insulin and/or leptin either peripherally or in

the central nervous system ordinarily results in the inhibition of feeding and increased energy expenditure [43]. Animal obesity models which lack the leptin molecule (*ob/ob* mouse) or have a mutation in the leptin receptor (*db/db* mouse and *fa/fa* rat) display elevated amounts of NPY activity in the hypothalamus [25, 44]. Administration of leptin to the *ob/ob* mouse results in inhibition of this NPY expression [44].

The predominant hypothalamic receptors by which NPY is thought to mediate hyperphagia are the Y1 and Y5 receptors [25]. Hypothalamic Y2 receptors, affected by its agonist the gut hormone $PYY_{3–36}$, are thought to restrain feeding in a feedback system, since the majority of NPY neurons in the arcuate nucleus co-express the Y2 receptor [45]. Knockouts of the hypothalamic Y2 receptors in mice result in increased food intake [46].

NPY's role in the sympathetic nervous system

The central nervous system communicates to the rest of the body via output tracts which include the autonomic nervous system as well as via motor and sensory neuronal pathways. The central effects of NPY are in particular thought to be mediated by the sympathetic branch of the autonomic nervous system. The NPY-containing neurons of the arcuate nucleus project prominently to the paraventricular nucleus and the dorsomedial hypothalamic nuclei [25], both of which are prominent in the outflow of information to the sympathetic nervous system [47].

Experimental evidence strongly implicates an important role for sympathetic nervous system innervation in the regulation of white adipose tissue (see [48] for review). The exact nature of the innervation is still under investigation. Several possibilities exist for the innervation of white adipose tissue: 1) *en passant* innervation of adipocytes as the sympathetic fibers traverse adipose tissue on their way to other target tissues; 2) direct innervation of adipocytes with clear pre- and post-synaptic units directly to adipose tissue; and 3) innervation of the vasculature of white adipose tissue only, resulting in control of adipocytes via regulation of their perfusing microenvironment [49, 50]. Any of these mechanisms in isolation or in combination could account for the enhancement of white adipose tissue lipolysis that is observed with increased sympathetic nervous system stimulation [4]. In addition, the sympathetic innervation of white adipose tissue may play a key role in the responses of specific fat pads in terms of lipid mobilization and cellularity [50, 51], and may be crucial in understanding why different types of white fat depots have different metabolic responses [49, 52].

By immunocytochemistry, approximately two-thirds of all principal neurons in the adult rat superior cervical ganglion (SCG) contain NPY, most often in conjunction with tyrosine hydroxylase staining [53]. In dissociated cultures of rat neonatal SCG grown under defined conditions, NPY biosynthesis increases 30-fold over a 3 week period, in parallel with increases in norepi-

nephrine production [11, 54]. Levels of both NPY and norepinephrine border on undetectable after 1 week in culture of SCG neurons cultured alone, with increased levels of both developed during the second to third week in culture under serum-free conditions [11]. In SCG cultures incubated with heart cell conditioned medium, the amount of norepinephrine synthesis decreased while NPY biosynthesis remained unchanged [11]. Increasing neuronal density in culture results in decreased levels of NPY expression, as does co-culture with non-neuronal ganglion cells [55]. Addition of nerve growth factor (NGF) to culture conditions induces NPY expression in PC12 cells [56], whereas leukemia inhibitory factor (LIF) decreases NPY expression [57, 58].

To bridge the gap between entirely *in vivo* whole animal models and *ex vivo* studies of adipocytes and neurons in isolation, a co-culture system was developed to provide another model for the study of the innervation of white adipose tissue [59]. In the co-culture system, primary sympathetic neurons are isolated from the SCG of newborn to 2 day old rat pups, then plated onto 3T3-L1A adipocyte monolayers which are 3 days post-induction of adipocyte differentiation. For the first 6 days after initial co-culture plating, cytosine β-D-arabinofuranonucleoside (Ara-C) is included in the medium to eliminate contamination by non-adipocyte, non-neuronal cells.

SCGs in co-culture are capable of secreting the neurotransmitter NPY after 1 and 2 weeks in co-culture [59]. Adipocytes co-cultured with SCG neurons markedly stimulate NPY secretion. When the neurons and adipocytes are co-cultured in the same media, but with a co-culture insert providing a membrane barrier to cell–cell contact, the level of NPY secretion is greater than that seen in SCG cultured alone, although somewhat less than by co-cultures in which cell–cell contact is permitted. Conditioned medium from Day 8 adipocytes, which should be fully differentiated, did not induce the response in SCG neurons cultured alone. These results indicate that the answer is likely to be a complex one, perhaps involving a combination of cell–cell interactions and circulating factors. Elucidation of the nature of the factor would be of interest, since little is known of what specifically induces NPY secretion in sympathetic neurons.

The decreased secretion of NPY in co-cultures in response to insulin treatment [59] is consistent with the reported effect of intracerebroventricular injections of insulin on NPY gene expression in rat hypothalamus [60]. The decreased levels of secretion observed after addition of insulin to the medium are consistent with an effect on the level of NPY mRNA. It has not yet been conclusively established that the insulin effect observed in co-culture is mediated via the adipocytes' signaling to the neurons, although the available evidence suggests that this is the case. While a statistically significant difference is seen between co-culture controls and those which have been treated with insulin, no such difference exists when SCG neurons alone are treated with insulin [59]. The low level of NPY secretion in the SCG neurons cultured alone may make observation of a direct effect on SCG neurons difficult, however. If the insulin signal is communicated to the neurons via the adipocytes,

as opposed to via direct action on the neurons, this is further evidence of reciprocal interactions between the adipocytes and neurons in co-culture.

The SNS and the adipocyte: Evidence of a local feedback loop involving NPY

Classically, the role of the sympathetic nervous system in the regulation of adiposity has been viewed in terms of norepinephrine release and the subsequent stimulation of lipolysis by this agent. The existence of other neurotransmitters within the sympathetic neuron complicates this picture. Considerable investigation has demonstrated a role for NPY in the central nervous system's regulation of energy metabolism and feeding behavior [20, 61, 62], but its importance in metabolism elsewhere in the body is still unknown. Evidence exists that NPY may act to modulate the effects of norepinephrine, with activation-dependent dosing [17]. Centrally, NPY is thought to inhibit the activity of the sympathetic nervous system in general [63]. In the CNS, NPY appears to have a role in preventing starvation, by increasing the drive to eat while decreasing depletion of existing energy stores [25]. This effect appears to be mimicked in the periphery as well. NPY is expressed by sympathetic neurons both *ex vivo* and *in vivo*, and has an antagonistic effect on lipolysis [59]. The specific circumstances under which sympathetic neurons release NPY to adipocytes in the periphery are as yet unknown.

The results from the adipocyte/sympathetic neuron co-culture system indicate that NPY released from sympathetic nerves in the periphery may contribute to the effects observed, thus resulting in a direct effect of sympathetic nerves on the adipocytes. The neurons appear to regulate the level of leptin secretion and the degree of β-adrenergic-stimulated lipolysis in the co-cultured adipocytes. Preliminary evidence indicates that the agent responsible for this effect is most likely NPY, although another factor has not been ruled out.

Likewise, the adipocytes themselves may regulate the actions of the sympathetic neurons via a local feedback loop. In co-culture studies, the secretion of NPY by neurons in co-culture was diminished when insulin was added to the medium [59]. This effect was not demonstrated by neurons cultured in isolation, which suggests that an agent or condition in the co-culture environment mediated the insulin effect. These results are indicative of crosstalk between adipocytes and neurons responsive to the metabolic state of the adipocyte. If the antilipolytic agent insulin is present, the requirement for additional antilipolytic agents such as NPY from the neuron is diminished, resulting in a signal from the adipocyte to the neuron to modulate this factor.

Few studies to date have addressed the issue of whether such a local feedback loop is in place. One group of investigators found that while NPY itself had an antilipolytic effect, leptin stimulated lipolysis in isolated rat primary adipocytes [64]. When adipocytes were exposed to equimolar concentrations of both NPY and leptin, the lipolytic rate was equivalent to the basal rate.

These findings were interpreted as evidence of a local homeostatic mechanism between leptin and NPY [64]. This evidence is consistent with the results of the co-culture system to date.

Evidence derived from the co-culture system supports a role for crosstalk between adipocytes and sympathetic neurons in the periphery (Fig. 1). In the absence of strong signals arising from elsewhere in the body, adipocytes in the biosynthetic/adipogenic state would stimulate NPY production by sympathetic neurons and thereby inhibit lipolysis locally. Likewise, centrally acting NPY would inhibit the sympathetic nervous system's release of norepinephrine. When the body senses a need to switch from a fed to a fasted state, the CNS's activation of the sympathetic system to release large amounts of norepinephrine would override the effects of NPY release, and trigger lipolysis by the adipocyte. Likewise, the release of a bolus of the antilipolytic hormone insulin from the pancreas would indicate that local antilipolytic agents were not immediately necessary, resulting in decreased NPY secretion while insulin levels in the bloodstream were high. At intermediate stages, the adipocyte and the sympathetic neuron would communicate to find the optimal balance between energy conservation in the two cell types and energy release for the needs of the rest of the body.

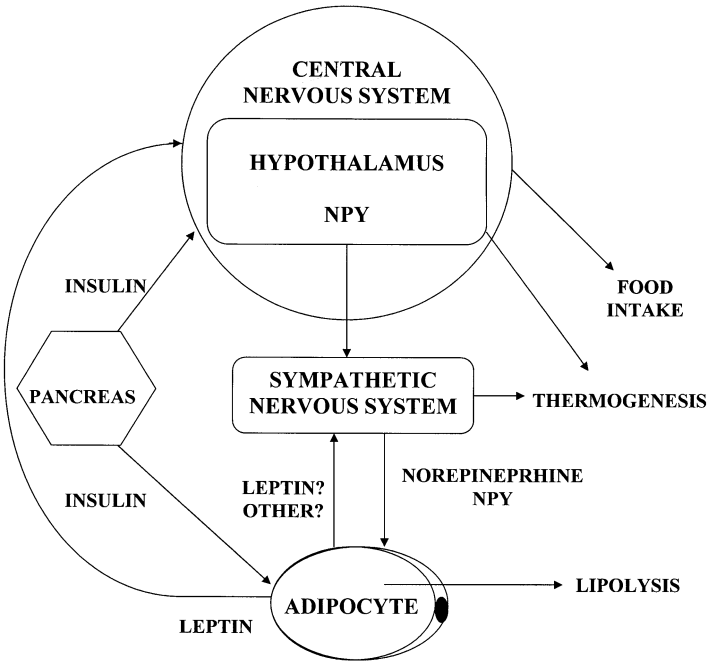

Figure 1 The brain-adipose tissue axis. Interactions among the central nervous system, sympathetic nervous system, the adipocyte, and the pancreas regulate metabolism and feeding behavior. NPY plays a key role in the hypothalamus, and is hypothesized to play an important role in the periphery as part of the sympathetic nervous system's communication with adipose tissue.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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NPY and energy homeostasis: an opportunity for novel anti-obesity therapies

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Introduction

Obesity is one of the most daunting health challenges of the 21st century; left unabated, the increasing rates of obesity in the world will place a severe burden on national healthcare systems. Obesity is a major, chronic health problem which decreases the quality of life and life expectancy and is a strong risk factor for diseases such as type 2 diabetes, heart disease, and stroke, and is associated with certain cancers, osteoarthritis, liver disease, urinary incontinence, sleep apnea, and depression [1]. In the past two decades, the hypothalamus has become a focus to understand the mechanisms underlying energy homeostasis. With the demonstration that neuropeptide Y (NPY) injected into the hypothalamus causes a very potent orexigenic response, and the discovery that leptin acts in the hypothalamus, an assortment of hypothalamic modulators have been described that affect energy homeostasis [2, 3].

Despite the explosion in knowledge about the role of the hypothalamus in regulating energy homeostasis, NPY remains the most potent orexigenic peptide known, with maximal effect when injected into the paraventricular nucleus (PVN) of the hypothalamus [2, 4]. These effects are observed in both satiated and hungry rodents. Moreover, the loss of appetite seen in rats with reduced NPY due to antisense or antibody treatment indicates NPY is an endogenous orexigenic compound [5, 6]. Indeed, chronic intracerebroventricularly (ICV) or PVN injection of NPY leads to hyperphagia, obesity, and hyperinsulinemia [7–10]. However, it should be pointed out that injection of NPY into other brain regions also affects food intake, and NPY may not just affect hunger but the motivation to eat and the rewards of eating [11]. NPY's role in energy homeostasis affects energy output as well as food intake. NPY reduces stimulation of the sympathetic system in adipose tissue, and acts directly as an anti-lipolytic agent, leading to reduced fatty acid oxidation, while stimulating lipid accumulation and *de novo* fatty acid synthesis [12, 13].

NPY, along with pancreatic polypeptide (PP) and PYY, are a family of 36 amino acid ligands that, in both rodents and man, functionally interact with

Table 1. NPY ligands and receptors affect energy homeostasis

Ligand	Function	Receptor and site of action
NPY	Orexigenic peptide	Y1/Y5 in the hypothalamus
	Modulates FAO/lipolysis	Activates SNS decreases energy expenditure
	Antilipolytic actions	Y1 on adipocytes
PYY3-36	Gut peptide/satiety signal	PYY from the gut converted to PYY-3-36 activates arcuate Y2 decreasing NPY release
PP	Pancreatic satiety signal	Activates brain stem Y4, signals hypothalamus

four NPY receptors: Y1, Y2, Y4 and Y5 [14]. All three ligands, and all four receptors, have roles in energy homeostasis acting centrally, peripherally, or both. Table 1 summarizes the role of the NPY pathway in energy homeostasis.

The hypothalamus modulates energy balance, by responding to adipokines (factors released from adipose tissue, such as leptin), gut peptides that modulating satiety and hunger (such as PYY3-36, PP, ghrelin) and acute fuel levels (circulating glucose, free fatty acids). Two prominent hypothalamic neuronal pathways have been elucidated in which signaling is inversely related (for a review see [15]). Leptin inhibited neurons in the arcuate nucleus (ARC) release NPY and AgRP, resulting in an increase in food intake and a decrease in energy output. Counterbalancing this pathway are leptin activated pro-opiomelanocortin (POMC) neurons that inhibit food intake and increase energy expenditure. In addition to endogenous hypothalamic NPY, circulating levels of PYY3-36 and PP also affect energy homeostasis via hypothalamic signaling (see Fig. 1). As will be detailed below, almost every aspect of the NPY pathway, its four ligands NPY, PP, PYY, and PYY3-36 and its four receptors Y1, Y2, Y4, and Y5, provide an opportunity to modulate energy homeostasis. However, the pharmacological potential of modulating the NPY pathway remains unproven for two reasons. First, complex modulation of multiple NPY receptors may be needed to cause significant body weight reduction. Second, both NPY and its receptors are widely distributed in the brain, and NPY has been associated with a variety of behaviors, thus the tolerability of NPY therapeutics remains to be established [11].

NPY and its pathway in the hypothalamus

Within the brain, the concentration of NPY is highest in the hypothalamus where it is found mainly in interneurons located in the ARC that project to the PVN [16]. The ARC responds to neuronal inputs from other brain regions, in particular the brain stem, but also to circulating factors that permeate the medium eminence, a region adjacent to the ARC that lacks an intact BBB. The NPY neurons in the ARC have a variety of receptors that can regulate their action.

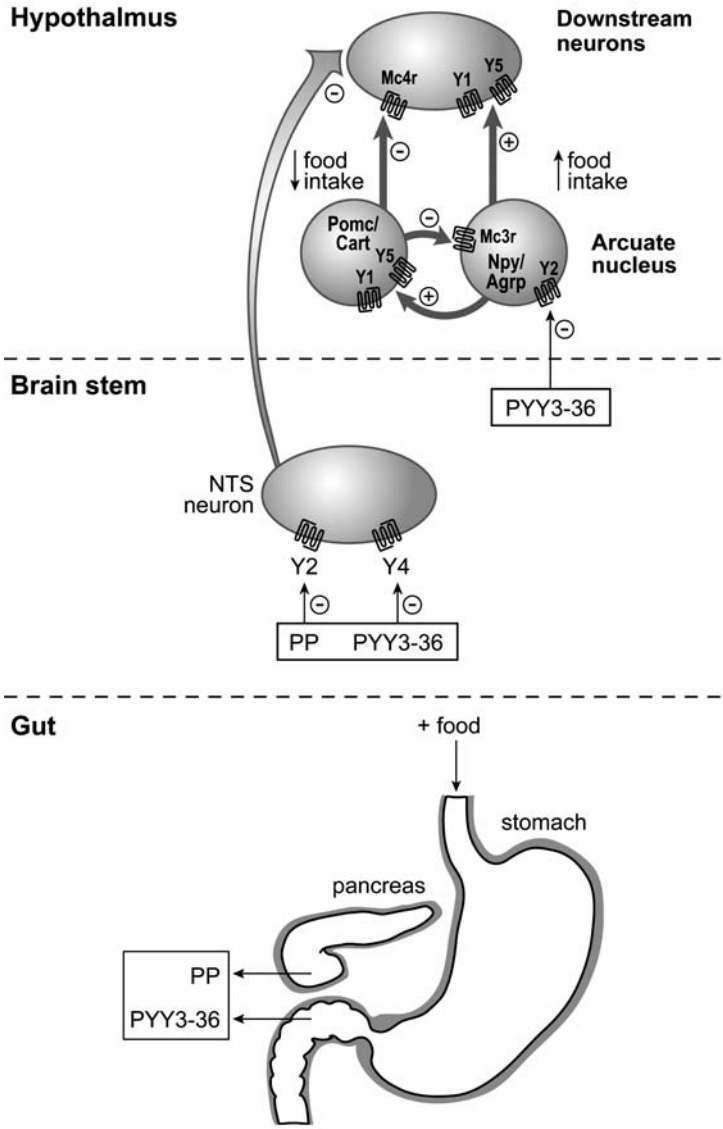

Figure 1. Arrows indicate the positive or negative effects of NPY ligands on energy homeostasis. Ligands include peripheral gut peptides PP and PYY3-36 and hypothalamic neuropeptide NPY, which act on various NPY receptors located in the brain stem or hypothalamus.

Growth hormone secretagogue receptors on NPY neurons are activated by ghrelin released from the stomach, while activation of the leptin, insulin, melanocortin 3, or Y2 receptors inhibits the NPY neurons [17].

The neuronal circuits through which the hypothalamus signals, including the leptin-R containing NPY neurons and the POMC containing neurons, can

under go rapid rewiring when animals are cycled from energy excess (low leptin) to energy restriction (high leptin) [18]. Leptin induces c-Fos activation of ARC neurons containing POMC, but not NPY, consistent with a role of leptin to inhibit NPY neurons [19]. The NPY containing ARC neurons project to the ventromedial (VMH), paraventricular (PVH) and dorsomedial nuclei (DMH) of the hypothalamus [20].

Regulation of NPY pathway by energy state

In addition to the potent orexigenic effect of NPY, changes in the levels of endogenous NPY and its receptors by energy state are strong evidence for involvement of the NPY system in energy homeostasis. Although a long line of evidence has accumulated thus far, these changes might occur directly (activation, inactivation, etc.) or indirectly (compensation, desensitization, etc.).

CNS changes (NPY)

Food deprivation and pregnancy/lactation are spontaneous conditions which stimulate food intake and reduce energy expenditure. Increased immunoreactivity of NPY in the hypothalamic region of rodents after food deprivation [21, 22], and during lactation [23, 24], support a physiological role for endogenous NPY in energy homeostasis. In addition, increased NPY secretion in the PVN during food restriction supports activation of the NPY systems in physiological energy states [25, 26]. Moreover, increased NPY levels return to normal levels after re-feeding and pup-removal [26–28], indicating that hypothalamic NPY is a physiological signal for energy homeostasis.

Additional evidence for a key role of NPY in energy homeostasis comes from analysis of various pathophysiological conditions. The expression levels of NPY in diet-induced obese (DIO) mice positively correlate with the degree of obesity [29]. Moreover, increased levels of NPY were reported in genetically obese models, such as *ob/ob*, A^y, MC4R knockout (KO) and *tubby* mice [29–31]. However, interestingly, expression patterns of NPY in the hypothalamic region varied among these obese mice. In *ob/ob* mice, a significant increase of NPY in the ARC was observed, but no detectable expression in the DMH [31]. In contrast, A^y and MC4R KO mice, which are obese due to disruption of melanocortinergic signaling, showed increased expression levels in the DMH and no changes in the ARC [31]. Furthermore, DIO and *tubby* mice had reduced levels of NPY in ARC, but showed increased levels in DMH [29, 30]. In an anorectic mouse model, *anx/anx* mice which have a poor appetite, immunoreactivity for NPY was increased in cell bodies of ARC and decreased in terminals in the hypothalamus [32, 33].

CNS changes (NPY receptors)

NPY-induced changes in metabolic states are mediated by multiple types of NPY receptors [34–44]. Activation of CNS Y1 and Y5 receptors by injection of NPY related peptides stimulates food intake and reduces energy expenditure [34–38]. In contrast, activation of Y2 and Y4 receptors by peripherally injected agonists reduce food intake and also increase energy expenditure [39–44]. Changes in the level of each NPY receptor may also be informative about the role of NPY in energy homeostasis.

In several animal models, the Y1 receptor expression varied with energy state. A 48 h fast reduced Y1 receptor-imunoreactivity and Y1 mRNA in the ARC of rats [45]. In mice transgenic for a Y1 receptor/LacZ fusion, 72 h fasting reduced expression of the transgene in the PVN, while supplementing the drinking water with glucose increased the transgene expression in the PVN and ARC [46]. Pregnancy, reduced transgene expression in the VMH, but increased it in the PVN and ARC [47]. In contrast, no significant changes of the Y1 receptor were detected in DIO, Zucker fatty, or food restricted lean rats [48, 49].

Changes in Y2 and Y5 expression have also been observed. A significant reduction of Y2/Y5 receptor-like binding sites were detected in DIO and Zucker fatty rats [48, 49], and an increase was observed in food restricted lean rats [49]. Y5 mRNA was down regulated in *ob/ob* mice [50], and expression levels of Y2 and Y5 mRNA were low in DIO-resistant mice when compare to DIO mice [51].

Peripheral role of NPY ligands

Three NPY family ligands are produced by the gut: PP, PYY and a PYY metabolite, PYY3-36. PP and PYY3-36 are both satiety signals released from the gut in response to a meal.

PP is primarily found in the pancreas, but PP cells are also found in other areas of the gastrointestinal (GI) tract. PP is released into circulation by the islets of Langerhans where PP containing cells are located in the periphery of the islet. The amount of PP released is proportional to the caloric intake and studies in rodents have shown this release dependent on a vagal tone, since vagotomy abolishes post-prandial PP release [52]. PP is a selective ligand for the Y4 receptor, though some forms of PP also activate the Y5 receptor [35]. PP is unable to cross the blood:brain barrier (BBB), but cFos studies indicate PP activates neurons in the area postrema, a region deficient in the BBB and a region containing Y4 receptors [52].

Studies in mice have shown a significant role for PP in regulating food intake [42, 53]. Peripherally administered PP to fasted lean mice reduced food intake in a dose dependent manner over 4 h [53]. A more comprehensive study in *ob/ob* mice confirmed the anorectic effects of PP extend to 24 h, and found

that the peptide also increased oxygen consumption and delayed gastric emptying; while chronic administration of PP led to weight loss [42]. The mechanism of PP action in fasted lean mice involved decreases in orexigenic peptides (NPY, orexin, ghrelin), an increase in anorectic peptide urocortin, and activation of a vagovagal reflex arc [42]. Transgenic mice overexpressing PP in pancreatic islets had PP levels elevated 20-fold, resulting in reduced food intake, lower bodyweight and less adiposity than littermate controls [41].

Recently PP was shown to also affect appetite and food intake in normal weight humans [43]. A 90 min infusion of PP caused PP levels to rise 10-fold, resulting in decreased hunger (as measured by a visual analog scale). Actual food intake decreased by 21% at a measured buffet meal, and a 23% decrease in 24 h food intake as measured by analysis of food diaries [43].

The gut peptide PYY is produced in the enteroendocrine cells in the ileum and colon where it is secreted in proportion to the size of the meal and, through the action of dipeptidyl peptidase-IV, PYY is converted to its major circulating form PYY3-36; which is elevated within 15 min of a meal [54]. In rodents, PYY3-36 injected peripherally inhibited food intake and reduced weight gain [40, 54, 55], although this claim is controversial. Unlike PP, PYY3-36 does penetrate the BBB and PYY3-36 appears to act within the ARC, since arcuate cFos levels increases after peripherally administered PYY3-36 [40, 56]. Although PYY3-36 is a potent ligand for multiple NPY receptors, the anorectic actions of PYY3-36 are thought to be mediated via the Y2 receptor since, PYY3-36 does not affect food intake in Y2 KO mice [40]. Indeed, direct intraarcuate injections of PYY3-36 causes a decrease in food intake [40], in contrast to ICV injection of PYY3-36 which cause the opposite effect, possibly through the actions of Y1 and Y5 [35, 57]. Thus, it was suggested that PYY3-36 acts via the Y2 receptors on NPY neurons in the ARC to reduce NPY release [40].

PYY3-36, like PP, is effective in reducing appetite and food intake in humans [40, 58]. Hunger, as measured by a visual analog score, decreased in lean humans after a 90 min infusion of PYY3-36 that raised circulating levels five-fold; and food intake at a buffet meal deceased by 36% [40]. Although obese subjects had 50% lower PYY3-36 at base line, infusion of PYY3-36, which increased plasma levels five-fold, resulted in a decrease in the buffet meal by 26% and a decrease in hunger score by 29% [58].

Phenotype of KO mice deficient in components of the NPY pathway

To further elucidate the role of the NPY system in energy homeostasis, mice deficient in NPY, as well as the Y1, Y2, Y4, and Y5 receptors have been made (for a review see [59]). Surprisingly, the phenotype of the knockout mice has not always been consistent with pharmacology studies described above.

Considering the potent orexigenic effects of ICV NPY, and the major orexigenic role postulated for NPY neurons in the hypothalamus, the NPY KO

mice, on a mixed genetic background, had normal weight and a normal response to fasting and refeeding [60]. When the mice were backcrossed to the C57BL/6 background, the *Npy*$^{-/-}$ mice had a mild deficit in fasting induced refeeding, but more surprisingly, they were slightly obese by 16 weeks of age [61]. Compensation by other hypothalamic pathways might account for the lack of a lean phenotype in the *Npy*$^{-/-}$ mice. However, *Npy*$^{-/-}$;*Agrp*$^{-/-}$ double knockout mice were not lean either, nor resistant to fasting induced refeeding [62]. More surprisingly, mice deficient in two orexigenic peptides NPY and galanin, were dramatically more sensitive to diet induced obesity, than their littermate controls [63]. Indeed, the only model in which NPY deficiency reduced body weight was the *ob/ob; Npy*$^{-/-}$ double mutant, which was about 30% leaner, with a 42% reduction in hyperphagia, than the *ob/ob* [64].

Since studies suggest hypothalamic NPY acts via the Y1 and Y5 receptors, mice lacking either of those receptors were expected to be hypophagic, resistant to fasting induced refeeding, and lean. One line of *Npy1r*$^{-/-}$ mice did display a marked reduction in fasting induced refeeding, and were marginally hypophagic [37], though another line of *Npy1r*$^{-/-}$ mice had no change in feeding [65]. In contrast, the *Npy5r*$^{-/-}$ mice had a normal refeeding response to fasting but were hyperphagic [36]. Surprisingly, both Y1 and Y5 deficient mice developed moderate obesity [36, 37, 65]. Both receptors appear to mediate the actions of NPY, since ICV injections of NPY into either *Npy1r*$^{-/-}$ or *Npy5r*$^{-/-}$ mice resulted in an acute increase in food intake, compared to vehicle, and chronic ICV injections of NPY led to rapid obesity [8, 60]. As was observed with *ob/ob; Npy*$^{-/-}$ double mutant mice, *ob/ob; Npy1r*$^{-/-}$ mice have a significantly reduced body weight compared to *ob/ob* mice, but *ob/ob; Npy5r*$^{-/-}$ mice do not have reduced weight compared to *ob/ob* mice [36, 66]. Taken together, the mouse genetic data suggests NPY's physiological actions are primarily mediated by Y1.

Compared to the other NPY receptors, the Y2 receptor is located predominantly presynaptically and is thought to control the release of NPY and other neurotransmitters [67]. As noted previously, *Npy2r*$^{-/-}$ mice are resistant to the anorectic effects of i.p. administered PYY3-36, a Y2, Y5 agonist [40]. Thus *Npy2r*$^{-/-}$ mice were predicted to be hyperphagic and obese. However, the phenotype of the two independently constructed lines, on different genetic backgrounds, was quite different [39, 68]. One *Npy2r*$^{-/-}$ line had a normal refeeding response to fasting, but was hyperphagic leading to obesity [68]. However, the other line of *Npy2r*$^{-/-}$ mice was leaner than littermate controls, perhaps because PP was elevated 3–5 fold [39]. Compared to *ob/ob* mice, *ob/ob; Npy2r*$^{-/-}$ mice had no change in food intake or body weight, however the double mutant did have reduced hyperinsulinemia and hyperglycemia [69].

Since PP is selective for the Y4 receptor, deletion of *Npy4r* should provide insights into the role of PP in regulating energy homeostasis. *Npy4r*$^{-/-}$ mice are significantly hypophagic, have lower adiposity and are leaner than their litter mate controls, again somewhat surprising for a deficiency in a gut hormone thought to limit food intake [70]. In addition, the *ob/ob; Npy4r*$^{-/-}$ mice have no

change in food intake or body weight [78]. As might be expected *Npy2r*$^{-/-}$; *Npy4r*$^{-/-}$ which lack the targets for the satiety gut peptides PP and PYY3-36, were hyperphagic, but surprisingly were markedly lean with reduced adiposity, leptin, and insulin [44].

Taken together, the NPY ligand and receptor transgenic mice indicate a significant role for the NPY system in modulating energy homeostasis. However, the phenotypes of mice with alterations in the NPY pathway are clearly more complex than our simple models of PP and PYY3-36 acting as satiety gut hormones, and NPY acting in the CNS via Y1 and Y5 receptors as a major orexigenic pathway (see Fig. 1).

Pharmacological effects of NPY agonism

As discussed previously, NPY is highly concentrated within the hypothalamus and the concentrations of NPY and its mRNA in the hypothalamus are markedly changed by energy status. These observations clearly suggest critical roles of endogenous NPY in energy homeostasis. Central administration of NPY stimulates feeding behaviors in several species including non-human primate [80–85], while chronic administration of NPY into the brain results in obesity accompanied with hyperphagia [7, 76, 77].

ICV studies with selective and non-selective peptides

Several types of selective agonists for Y1 and Y5 receptors have been identified [78–83] and found to be orexigenic. For instance, both a Y1 selective agonist, [D-Arg25]NPY and a Y5 selective agonist, [D-Trp34]NPY cause significant hyperphagia after ICV-injection, and the stimulated feeding is inhibited by Y1 and Y5 antagonists, respectively [79, 84]. However, the Y1 and Y5 receptors seem to have distinct roles. NPY-induced feeding in the Y1 deficient mice is significantly reduced, but the feeding is not changed in the Y5 deficient mice significantly [60].

In contrast to the Y1 and Y5 receptors, central roles for Y4 receptors as an orexigenic receptor is controversial. ICV-injection of rPP, a highly selective Y4 agonist, has little or no significant effect on feeding behavior [60, 85, 86]. However, recent investigation shows that Y4 receptors are expressed in orexin neurons, and direct administration of rPP into lateral hypothalamus causes significant food intake with c-Fos expression in orexin neurons [87]. Thus, the Y4 receptor might play a role as an orexigenic receptor through activation of orexin neurons.

ICV-injection of Y2 selective agonists has almost no effects on feeding in satiated rodents [85, 86]. However, central injection of a Y2 agonist inhibits fasting-induced feeding [40]. It has been reported that the Y2 receptor is predominantly expresses pre-synaptically and works as an auto-receptor [88].

Therefore, the central Y2 receptor could act as an anorectic receptor through the inhibition of NPY release in the hypothalamus.

It is difficult to explain all the orexigenic effects of NPY and related peptides with the known subtypes of NPY receptors. Y5 preferring agonists, PYY3-36, bPP and hPP have similar efficacy for the Y1 and Y5 receptors, while these three peptides have different efficacy to affect feeding behaviors [60]. Although the differences in efficacy might be due to differential peptide stability or receptor heterodimerism, a novel NPY subtype is another possibility. Interestingly, unknown binding sites for [^{125}I]PYY remain in the brain of *Npy1r*$^{-/-}$; *Npy2r*$^{-/-}$; *Npy4r*$^{-/-}$ deficient mice treated with Y5 compounds [89].

Chronic infusion NPY causes obesity with hyperphagia as well as a reduction in energy expenditure [7, 87, 88]. Interestingly, chronic infusion of Y5-selective agonist, [D-Trp34]NPY also causes obesity [84]. Furthermore, there is no significant change between NPY-induced obesity in Y1-deficient and Y5 deficient mice [8]. Future studies using combinations of selective agonists and several receptor deficient mice, could be useful to address the central function of NPY receptors in feeding regulation more precisely.

Peripheral dosing of peptide agonists

As noted above, it has been reported that peripheral injection of Y2 agonist, PYY3-36 and Y4 agonist, hPP cause feeding reduction in humans as well as rodents [40, 43, 58]. These are the first evidence revealing the involvement of the NPY systems in regulation of human appetite. In addition, the anorectic efficacies as well as the anti-obese efficacies of Y2 and Y4 agonists are also observed in rodents [42, 90, 91]. However, abdominal vagotomy abolishes the anorectic effects of PYY3-36 in rats [91]. Furthermore, ICV-injected rPP increased feeding behavior [87]. Therefore, anorectic effects of peripheral PYY3-36 and PP are presumably caused by the activation of peripheral Y2 and Y4 receptors, or a subset of brain receptors, but not all the central NPY receptors.

Opportunities for NPY therapeutics to treat obesity

Extensive physiological, neuroanatomical, genetic, transgenic, and pharmacological data supports a major role for the NPY family of peptides and receptors in the regulation of energy homeostasis. However, most strikingly as detailed in this review, the data sets give conflicting guidance for how best to modulate the NPY pathway to treat obesity. For example, chronic infusion of NPY leads to obesity, but the NPY KO mice appear normal. Similarly, chronic infusion of a Y5 selective agonist leads to obesity, while the Y5 KO is also obese. The Y2/Y5 agonist PYY3-36 injected ICV is a more potent orexigen than NPY, but peripherally it is an anorectic. More puzzling, the two lines of

Y2 KO mice are inconsistent: one is lean and the other obese. Thus, a consistent picture has not developed pointing to a key NPY receptor or molecular target to modulate as a treatment for obesity. However, the various data discussed in this chapter do provide support for Y1 and Y5 brain-penetrant antagonists to treat obesity. Indeed, we have shown that structurally diverse Y1 and Y5 antagonists are effective in several acute food intake models [92–95]. But while one study has confirmed the ability of a Y1 antagonist dosed chronically to reduce obesity, another study has found no effect of a Y5 antagonist dosed chronically [96, 97]. Further studies are needed to confirm these results, but either a centrally acting, Y1 antagonist, or perhaps a dual Y1 and Y5 antagonist, may be an effective anti-obesity agent. In addition, Y2 (such as PYY3-36) and Y4 (such as PP) agonists are potent satiety factors with demonstrated acute anorectic effects in humans suggesting selective single, or perhaps dual, agonists could be effective for treating obesity.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
from Genes to Therapeutics
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Subjugation of hypothalamic NPY and cohorts with central leptin gene therapy alleviates dyslipidemia, insulin resistance, and obesity for life-time

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Summary

An interactive network comprised of neuropeptide Y (NPY) and cohorts is obligatory in the hypothalamic integration of appetite and energy expenditure on a minute-to-minute basis. High or low abundance of NPY and cognate receptors dysregulates the homeostatic milieu engendering hyperphagia, decreased energy expenditure, obesity and attendant metabolic syndrome cluster of dyslipidemia, glucose intolerance, insulin resistance and hyperinsulinemia, risk factors for type II diabetes and cardiovascular diseases. Increasing the supply of the endogenous repressor hormone leptin locally in the hypothalamus with the aid of leptin gene therapy, blocked age-related and dietary obesities, and the sequential development of dyslipidemia, hyperglycemia, and insulin resistance. Thus, sustained repression of NPY signaling with increased leptin selectively in the hypothalamus can avert environmental obesity and the risks of metabolic diseases.

Introduction

A major challenge for the medical and scientific communities worldwide is to decelerate the escalating prevalence of obesity and thus curtail the soaring medical costs of treating obesity-associated afflictions among all segments of the population, regardless of age, race and socio-economic status [1–4]. The obesity burden includes the metabolic syndrome, dyslipidemia, glucose intolerance, insulin resistance and hyperinsulinemia of type II diabetes, and hypertension. An abnormal rate of fat accumulation is also a risk factor in the etiology of neural diseases (e.g. Alzheimer's disease), infertility, sleep apnea, kidney and liver dysfunction, and several forms of cancers [5–10]. Clinical evidence also implicates morbid obesity as a risk factor for shortened life expectancy [4, 8, 11].

Excess energy intake is stored as fat in the white adipose tissue (WAT) for accessible fuel to accommodate the daily needs and to tide over periods of

food-scarcity [1, 3, 4, 7, 12]. Apart from a small percentage of the population predisposed to genetic factors, the current obesity pandemic has been attributed to the technological advances of the 20th century that improve the quality of daily life but simultaneously promote fat storage. This incessant positive energy balance, an outcome of calorie-enriched foods and sedentary lifestyles, precipitates fat storage either gradually as a function of age, or at an accelerated rate due to abrupt life-style modifications [1, 3, 4, 7, 12, 13]. Thus, the two immediate paramount questions are: Is it possible to slow the progression of the age-related and the calorie enriched diet-driven adiposities? Will the incidence of metabolic syndrome disease cluster, e.g., glucose intolerance, hyperinsulinemia and insulin resistance, decrease contemporaneously to contain dyslipidemia and diabetes type II? To address these issues, this article collates novel research endeavors of the bidirectional communication between the hypothalamus and WAT for energy homeostasis [12, 14, 15]. We also elaborate on crosstalk between the hypothalamic effector network of neuropeptide Y (NPY) and cohorts and adipocyte-derived leptin which has been targeted for therapeutic interventions to curb the rate of fat deposition and thereby, alleviate the attendant cluster of metabolic disorders of dyslipidemia, hyperinsulinemia, insulin resistance, and diabetes type II [4, 15–20].

WAT-hypothalamus cross-talk and energy homeostasis

White adipose tissue (WAT)

WAT is an unique endocrine organ that secretes hormones, lipids and proinflammatory cytokines, each of which plays an important role in the regulation of various physiological functions [21, 22]. An imbalance in the circulating levels of these signal molecules with increasing adiposity has been correlated with the genesis of metabolic syndrome, disruption in immune responses, neuroendocrine disorders and diseases of bone, kidney and liver [3–6, 21, 22]. Although leptin is produced primarily by WAT, additional contributions from the stomach and hypothalamus are also important in integration of the bidirectional communication between WAT and hypothalamus for daily meal patterning [3, 12, 15, 21–23]. Leptin is secreted episodically with regularly paced short secretory bursts that are interrupted post-prandially by high amplitude protracted secretion [24–28]. Leptin pulse amplitude increases only gradually with age but rises rapidly in response to a high fat diet [27]. Leptin hypersecretion is an important predicator of metabolic disorders because the onset of metabolic disruptions correlate temporally with the rise in circulating leptin levels and severe hyperleptinemia sustained over extended periods is a common denominator in coalescence of obesity-driven health problems [3–6, 21, 22].

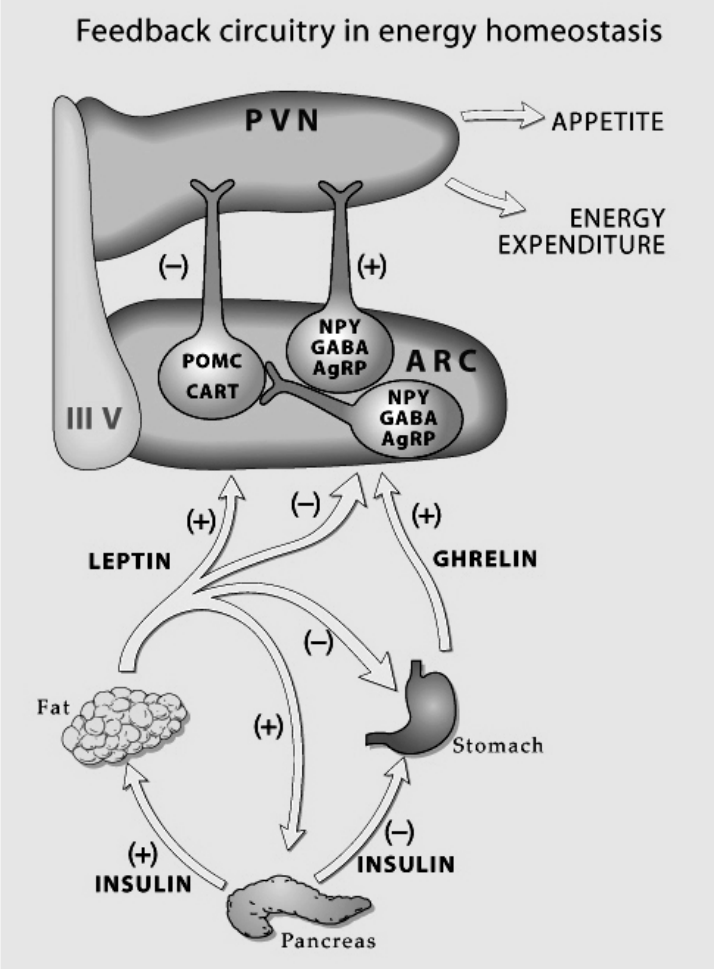

Figure 1. Schematic representation of the feedback circuitry involved in integration of appetite and energy expenditure. The primary components of the appetite regulating network, the orexigenic NPY and co-expressed GABA and AgRP, in an interplay with the anorexigenic pathway compassing the POMC and cocaine- and amphetamine regulating transcript (CART) neurons, stimulate and terminate appetite. The synthesis and release of these peptides in the ARC–PVN axis is regulated by two functionally apposed afferent hormonal signals – leptin from adipocytes (fat tissue) and ghrelin from stomach. Also, depicted in the feedback relationship is the fat–stomach–pancreas axis in the periphery. (+) stimulatory, (–) inhibitory, IIIV = third cerebroventricular (modified with permission from reference [4]).

Hypothalamus

Among the various neural effector pathways identified to date, the network of NPY and cohorts in the hypothalamus is central in integration of energy homeostasis (Fig. 1) [12, 16–19]. NPY is an endogenous appetite transducer

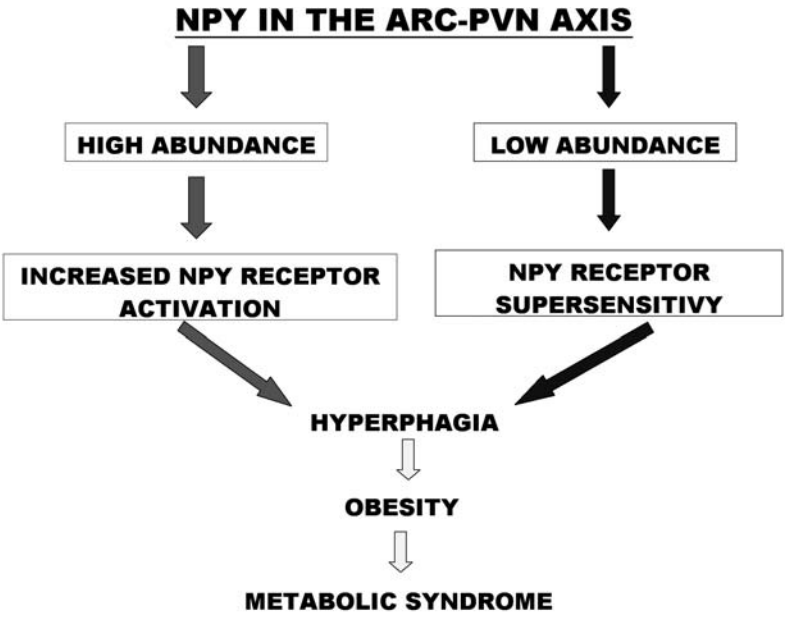

Figure 2. A model showing two disparate ways high and low NPY abundance in the ARC–PVN axis culminates in obesity and metabolic syndrome (with permission from reference [16]).

and participates in daily meal patterning in various ways. Timely release of NPY in the paraventricular nucleus (PVN) from nerve terminals extending from NPY producing perikarya in the hypothalamic arcuate nucleus (ARC) and brainstem, stimulates appetite [12, 16–19]. Enhanced episodic NPY release in the PVN, as that induced by short or long-term food deprivation [29], or observed in genetic and experimental models of obesity in rodents [30, 31], elicits relentless appetite via activation of Y_1/Y_5 receptors in the magnocellular PVN [12, 16–20]. The NPY-induced excess energy intake gradually eventuates in abnormal rates of weight gain [12, 18, 19].

Intriguingly, even low abundance of NPY in the ARC–PVN axis, induced pharmacologically with neurotoxins and lesions, or surgically by interruption of NPY supply in the PVN from ARC or brainstem, evokes hyperphagia (Fig. 2) [16, 18, 32, 33]. Furthermore, a deficiency in NPY Y_1 or Y_5 receptors or an increase in Y_1 receptor abundance induced by diminished supply of NPY at these receptors augments food intake and promotes fat accretion [16, 18, 32, 33]. Thus, both low abundance and high abundance of NPY disrupts NPY signaling in the hypothalamus, disturbs meal patterning and causes hyperphagia, obesity and attendant metabolic disorders [12, 16, 18–20].

Besides stimulating appetite on its own, NPY integrates daily meal patterning in various additional ways (Fig. 1). In the PVN, in concert with the co-expressed orexigenic γ-aminobutyric acid (GABA) and agouti-related peptide (AgRP), NPY synergistically mounts a robust appetitive drive [4, 12, 14].

When released locally in the ARC these three neurotransmitters activate distinct cellular pathways to suppress the anorexigenic melanocortin and initiate feeding [4, 12, 14, 16].

Regulation of NPYergic signaling by leptin

Leptin is a major peripheral afferent hormonal signal that tonically restrains the appetitive drive encoded by NPY and cohorts in the ARC–PVN axis (Fig. 1). The tonic restraint on feeding during inter-meal intervals is a synergistic consequence of suppressed orexigenic NPY, and enhancement of the inhibitory anorexigenic melanocortin signaling in the ARC–PVN axis [4, 12, 15–19]. Leptin has recently been shown to counteract the orexigenic effects of ghrelin, the gastric afferent hormonal signal to the ARC NPY effector network [15, 34, 35]. Leptin inhibits ghrelin induced stimulation of NPY release in the ARC–PVN axis and peripherally it suppresses ghrelin secretion from the stomach [34, 35]. This tonic leptin restraint on the hypothalamic effector pathways is indispensable for energy homeostasis because in the absence of either leptin or leptin receptors in the hypothalamus, orexigenic NPY signaling is upregulated and anorexigenic melanocortin signaling is diminished [4, 12, 16, 19, 30, 31]. These disturbances jointly trigger hyperphagia culminating sequentially in abnormal rate of fat accumulation and metabolic syndrome disease cluster [4, 12, 15].

Regulation of energy expenditure by the NPY and cohorts signal relay

In addition to coordinating the periodic appetitive drive, the communication between hypothalamic NPY and cohorts and leptin reciprocally controls nonshivering thermogenic energy expenditure [4, 36, 37]. Whereas enhanced hypothalamic NPYergic signaling decreases, leptin enhances thermogenic energy expenditure by activating the diffuse sympathetic nervous system brown adipose tissue (SNS-BAT) pathway extending from the rostral medial preoptic area to BAT in the periphery [4, 38–40].

Repression of hypothalamic NPY and cohorts with central leptin gene therapy

The insight that leptin normally exerts a tonic restraint on the hypothalamic network of NPY and cohorts on a minute-to-minute basis and that diminution in this restraint concomitantly promotes increased hyperphagia, decreased energy expenditure and obesity, led us to hypothesize that it is the insufficiency of this powerful tonic leptin restraint on the hypothalamic effector pathways that impels both age- and dietary adiposities. If true, then restoration of leptin

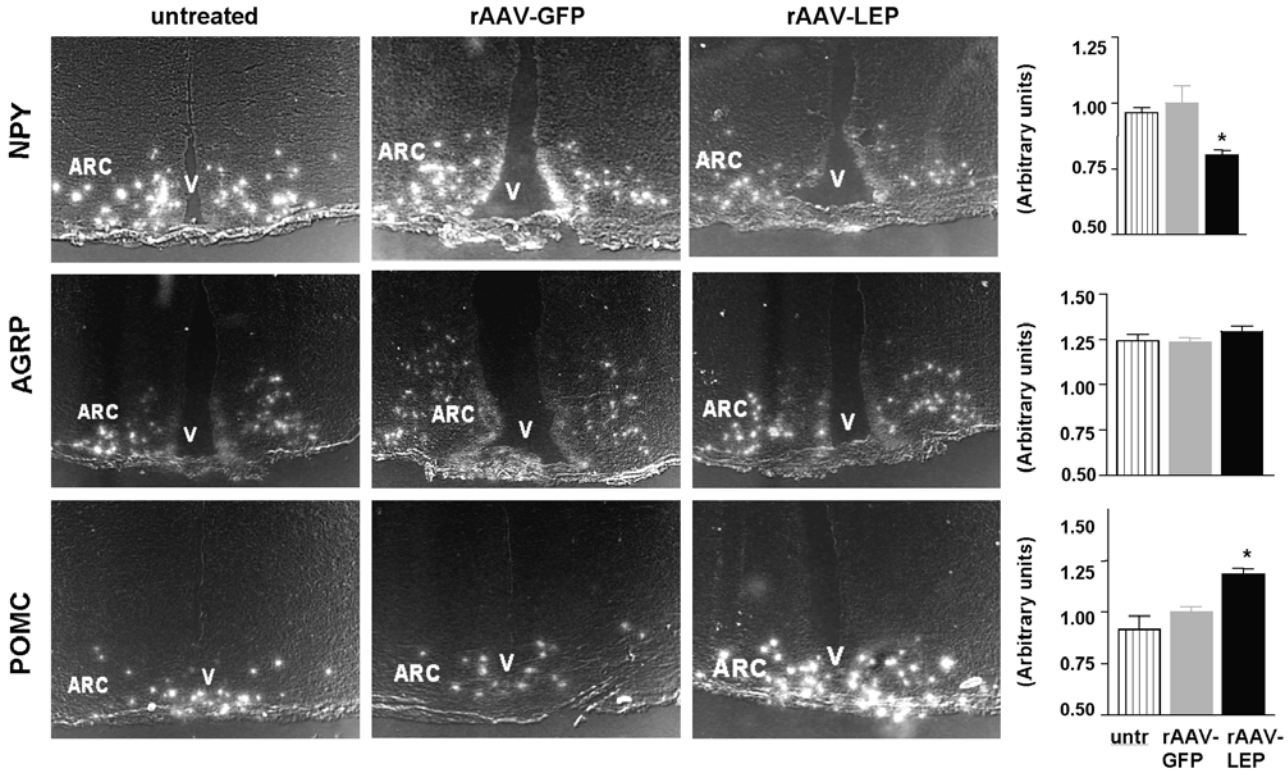

Figure 3. Left: Representative micrographs of NPY, AgRP and POMC mRNA expression in the ARC of untreated, rAAV- green fluorescent protein (GFP), and rAAV-lep at 294 days post-injection in prepubertal rats. Right: Summary of the effects on NPY, AgRP and POMC mRNA expression in the ARC of these three treatment groups at 294 days post-injection. (with permission from reference [44])

restraint should reinstate weight homeostasis and prevent fat accretion and the attendant metabolic syndrome cluster of diseases [3, 4, 15, 36]. To validate this hypothesis, we engineered a replicative deficient, non-immunogenic and non-pathogenic recombinant adeno-associated virus vector encoding the leptin gene (rAAV-lep) for stable expression of leptin at focal neural sites [13, 41, 42]. In the initial study, an intravenous rAAV-lep injection in leptin-deficient ob/ob mice increased circulating leptin levels, suppressed hyperphagia, and decreased weight and adiposity. This normalization of food intake and weight was conferred by decreased hypothalamic NPYergic and increased melanocortin signalings in these mice [13]. In subsequent experiments, the well-documented deleterious effects of severe hyperleptinemia on various physiological functions in the periphery and concomitant development of central leptin insufficiency were circumvented by increasing the supply of leptin locally in the hypothalamus following either intracerebroventricular injection (icv) or intraparenchymal microinjection of rAAV-lep in various hypothalamic sites [34, 36, 38, 43–51].

Indeed, central rAAV-lep administration rapidly and reliably transduced leptin gene expression that was sustained for the lifetime of rodents [43–51]. Increased transgene expression occurred in various hypothalamic sites innervated by the two networks regulating appetite and energy expenditure. Importantly, ectopic leptin expression was confined to hypothalamic sites as it was detected neither in the central cerebrospinal fluid nor in systemic circula-

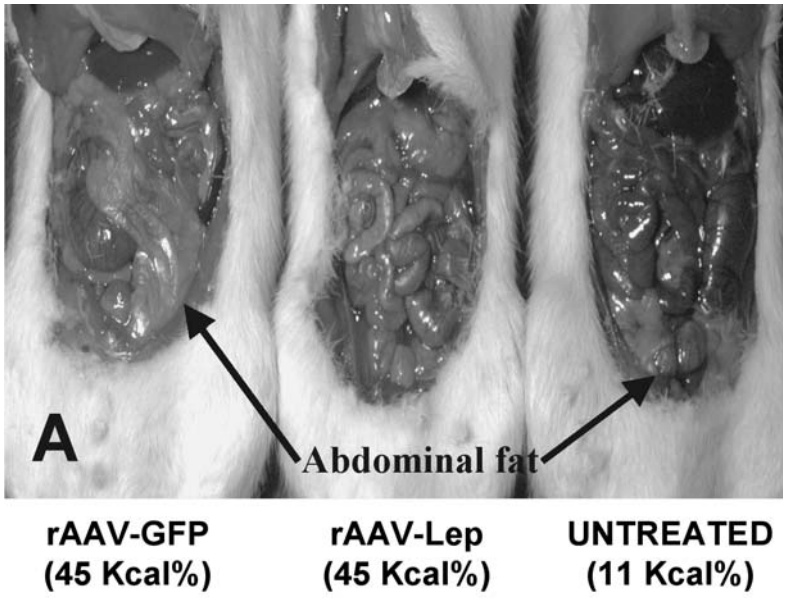

Figure 4. The effects of rAAV-green fluorescent (rAAV-GFP control) or rAAV-lep treatment on abdominal fat deposits in rats maintained on HFD (45% kcal) (with permission from reference [50])

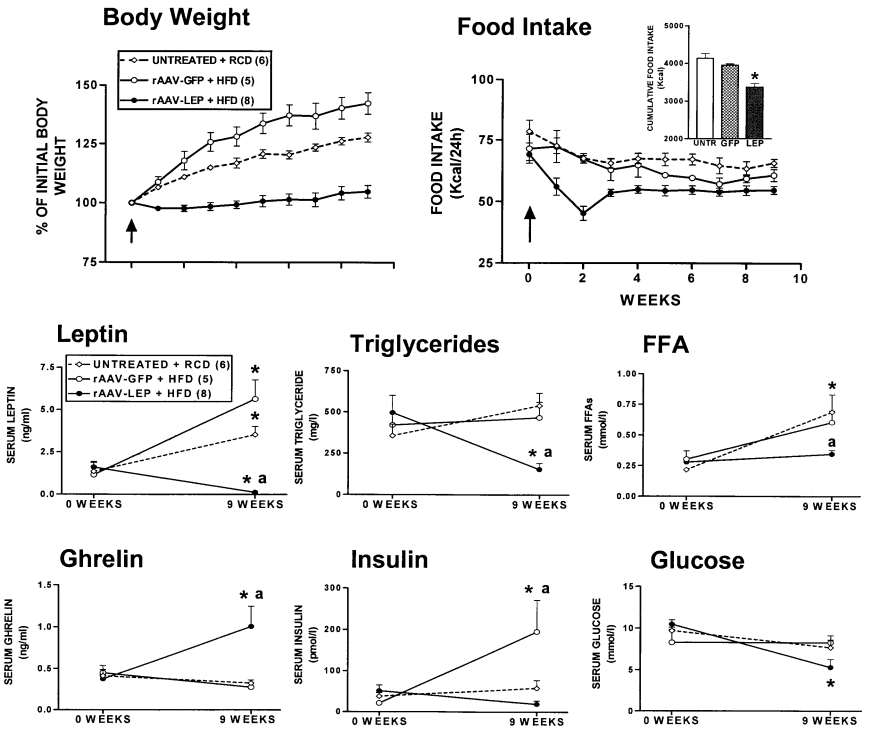

Figure 5. The effects of rAAV-GFP and rAAV-lep administration (arrow) on body weight, food intake, serum leptin, triglycerides, free fatty acids, ghrelin, insulin and glucose levels. For comparison, an additional untreated group consuming rat chow diet (RCD) is also depicted. $P < 0.01$ *versus* controls or "0" week values and a. $p < 0.05$ *versus* other groups (with permission from reference [50]).

tion. The stable expression of leptin in the hypothalamus simultaneously suppressed the orexigenic NPY and enhanced the anorexigenic melanocortin signaling, without affecting AgRP, in the ARC–PVN axis (Fig. 3).

Long-term benefits of repressing NPY signaling with central leptin gene therapy

Obesity

A remarkable long-lasting consequence of the sustained repression of hypothalamic NPY and cohorts by central leptin gene therapy was the complete suppression of gradual age-related as well as rapid high fat diet (HFD)-induced weight gain, adiposity and hyperleptinemia. Suppression of weight resulted solely from deceleration of fat accretion (Figs 4 and 5). This long-term efficacy was seen in prepubertal, young adult and old wild-type rodents

and leptin-deficient obese *ob/ob* mice [34, 36, 38, 43–51]. Thus, central leptin gene therapy re-orchestrated with marked facility the neurochemical sequalae that averted leptin insufficiency. Evidently, stable ectopic leptin expression in physiological amounts can reinstate weight homeostasis for the life-time of rodents by selectively repressing the hypothalamic NPY and cohorts signal relay and enhancing energy expenditures.

Dyslipidemia

Compelling clinical and experimental evidence links increased visceral adiposity with the syndrome of dyslipidemia characterized by triglyceridemia, increased free fatty acids (FFA), cholesterol and other circulating lipids [3–6, 13, 21, 22, 34, 36, 38, 43–51]. Repression of hypothalamic NPY and cohorts signal relay in rodents expressing ectopic leptin in the hypothalamus was accompanied by a severe suppression of circulating levels of triglycerides, FFA and other adipokines (Fig. 5) [34, 38, 43–51].

Hyperinsulinemia and diabetes type II

Glucose intolerance, hyperglycemia, hyperinsulinemia and insulin resistance invariably accompany the increased rate of fat accumulation [5, 6, 21, 22]. In most obese patients, these pathophysiological derangements of the adipoinsular axis on a long-term basis increase the risk of diabetes type II [5, 21, 22]. Indeed, current clinical surveys show a close correlation between the increasing prevalence of obesity and diabetes type II in adolescents and adults [1–7, 22]. The results of our long-term gene therapy investigations demonstrated that central leptin expression completely suppressed the age-related and HFD-induced hyperinsulinemia and restored normoglycemia by reinstating insulin sensitivity (Fig. 5) [13, 34, 36, 43–51]. Further, the prevalence of hypoleptinemia in these rodents supports the hypothesis that hyperleptinemia not only disrupts the adipoinsular axis in the periphery but it also causes leptin insufficiency in the hypothalamus. Additionally, our demonstration that hyperglycemia and hyperinsulinemia in insulin-2 gene mutant Akita mice also were abrogated by central leptin gene therapy extends our postulate that leptin, independent of its central action on weight homeostasis, engages the SNS and/or yet to be identified neural pathways, to impose glucose–pancreatic insulin homeostasis in the periphery [4, 34, 51].

Lifespan

Recent clinical surveys also link overt obesity and the attending metabolic syndrome and degenerative diseases with reduced lifespan [1–11]. Given the

Table 1. Beneficial effects of repression of the NPY and cohorts signal relay with central leptin gene therapy on metabolic and endocrine biomarkers of age-related and dietary obesities

	Obesity		NPY Repression with Leptin Gene Therapy
	Aging	Dietary	
Weight	↑	↑	↓
Adiposity	↑	↑	↓
Energy Intake	↑	↑	↓
Energy Expenditure	↓	↓	↑
Leptin	↑	↑	↓
IGF-1	↑	↑	↓
Insulin	↑	↑	↓
Glucose	↑	↑	↓
Triglycerides	↑	↑	↓
Free Fatty Acids	↑	↑	↓

↑ = increased, ↓ = decreased

fact that repression of the NPY and cohorts signal relay with central leptin gene therapy suppressed the rate of weight gain, adiposity and endocrine and metabolic markers of metabolic syndrome (Tab. 1), we assessed the impact of these beneficial effects of central leptin gene therapy on the lifespan of overtly obese ob/ob mice. A single icv injection of rAAV-lep in ob/ob mice normalized food intake, weight and adiposity, augmented energy expenditure and suppressed hyperinsulinemia and hyperglycemia and the adipocyte-derived hormones – leptin, adiponectin and tumor necrosis factor-α, the etiologic factors in the pathophysiology of insulin resistance and diabetes type II [34, 51]. A remarkable consequence of the long-term amelioration of these hormonal and metabolic markers was decreased mortality and increased life expectancy [52]. Seemingly, optimal leptin restraint on NPY and cohorts in the hypothalamus can avert the adverse effects of obesity and metabolic syndrome on longevity.

Concluding remarks

The hypothalamic network of NPY and cohorts integrates neural, hormonal and metabolic signals by coordinating energy intake and expenditure on a moment-to-moment basis (Fig. 1). The expanding knowledge of the precise working of NPY and cohorts has uncovered two underlying causes of the current pandemic of obesity and metabolic syndrome. 1) progressive disturbances in the afferent metabolic signaling that impels both high abundance and low abundance of hypothalamic NPY and cohorts, accelerates fat accretion and genesis of a constellation of metabolic risk factors. 2) These very derangements in internal metabolic factors engender central insufficiency of leptin

restraint thus disrupting the dynamics of the bidirectional crosstalk between WAT and hypothalamus. Implementation of leptin gene transfer technology was highly successful in ameliorating the adverse impacts of central leptin insufficiency on NPY and cohorts signal relay in the hypothalamus. Repression of NPY signaling on a long-term basis reversed the life-shortening effects of obesity and metabolic ailments. Finally, attempts to reinforce leptin restraint with gene therapy have unraveled a novel role of leptin in the hypothalamus in regulating ultradian secretion of leptin from adipocytes and insulin from pancreatic β-cells. Apparently, efferent inhibitory signals from the hypothalamus traverse a distinct pathway to regulate rhythmic hormonal discharge from peripheral endocrine glands. This novel insight has yielded a new vulnerable locus in the hypothalamus for designing therapeutic interventions to stem the rising tide of the metabolic syndrome cluster of diseases.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
from Genes to Therapeutics
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NPY and bone

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Introduction

The classic role of Neuropeptide Y (NPY) is as a major regulator of food consumption and energy homeostasis, however, recent analysis of Y receptor knockout models has revealed a novel and important role for NPY receptors in the regulation of bone metabolism.

There are five Y receptors known to mediate the actions of NPY and its two other family members, peptide YY (PYY) and pancreatic polypeptide (PP). The large number of Y receptors and their similar pharmacological profiles has made it difficult to delineate their individual contributions to the numerous physiological processes regulated by members of this family. However, recent studies analyzing Y receptor knockout models have started to unravel some of the individual functions of these Y receptors. In particular the use of conditional knockout models has made it possible to pinpoint a specific functional contribution of an individual Y receptor in a particular location. From these studies the predominantly pre-synaptically-expressed Y2 receptor in the arcuate nucleus has emerged as a prime candidate for mediating the regulation of bone formation.

The skeleton is a major contributor to body weight, with bone tissue representing approximately 15% of the human body weight. The loss of bone mass resulting from an imbalance in the process of bone resorption and formation, can have severe consequences. Osteoporosis is particularly prevalent in postmenopausal women and in the elderly, and is characterized by low bone density and increased risk of fracture.

Recently we have shown that specific deletion of hypothalamic Y2 receptors results in a two-fold increase in cancellous bone volume, resulting from an elevation in the rate of bone formation [1]. These findings, therefore, have revealed a novel anabolic bone response, resulting from a central signal processed within the hypothalamus, and as such, opens up exciting new possibilities for osteoporosis treatment.

A second anabolic pathway also mediated through a hypothalamic relay was revealed in the *ob/ob* and *db/db* mice [2], which are deficient in functional leptin, and its receptor, respectively. Leptin and Y2 receptors on hypothala-

mic NPY neurons mediate leptin's effects on energy homeostasis, suggesting these two systems may also interact in the modulation of bone formation. Similarities in the bone phenotypes and neuropeptide expression profiles of the *ob/ob* and Y2 knockout models also suggests a mechanistic link between leptin and Y2 receptors in the regulation of bone physiology. This chapter summarizes the current body of knowledge on the central antiosteogenic pathways mediated by leptin and the Y receptor systems, and assesses the possibility that their activity in the regulation of bone formation occurs by functionally distinct pathways.

The central control of bone metabolism

In the normal skeleton, bone is not a static tissue, but is rather constantly remodeled with the removal of small volumes of bone by bone-resorbing osteoclasts, then replaced by bone-forming osteoblasts. This process of remodeling maintains both mechanical integrity and mineral homeostasis. The balance between these two processes, and the rate at which they occur, is tightly coupled, such that the rate of bone resorption equals the rate of bone formation, ensuring that a constant volume of bone remains present during adult life.

Disturbances in the balance between resorption and formation results in a change in net bone turnover and is responsible for many bone diseases including osteoporosis. Osteoporosis is a common disease in which low bone mass and damage to bone microarchitecture leads to increased risk of fracture with minimal trauma. Following menopause in women, loss of the protective effects of estrogen leads to increased osteoclastic activity, resulting in greater bone resorption [3]. In addition, osteoblast function decreases with age in both women and men [4]. These factors destabilize the coupling of bone remodeling resulting in a net loss of bone and contributing to the development of osteoporosis [5–7]. Currently available treatments for osteoporosis act only to inhibit further bone resorption. These agents, therefore, are of somewhat limited use for treating severe osteoporotic patients already suffering from a major loss of bone scaffolding, or in male osteoporotic patients, whose loss of bone is generally not the result of altered osteoclastic activity, but rather due to a loss of osteoblast activity.

The mechanisms controlling the regulation of bone remodeling have been extensively studied. It has long been thought that the regulation of bone turnover is primarily controlled by endocrine, autocrine and paracrine factors such as vitamin D, calcitonin, parathyroid hormone or cytokines. However, recently it has become apparent that the regulation of bone formation is also controlled by centrally-mediated neural pathways.

It is well established that both sensory and autonomic nerve fibers are present in bone tissues, predominantly associated with blood vessels [8–12]. A number of neuropeptides have also been identified in bone, including substance P (SP), calcitonin-gene related peptide (CGRP), vasoactive intestinal

peptide (VIP), and neuropeptide Y (NPY), as well as neurotransmitters such as serotonin (5-HT), noradrenaline, and glutamate [13]. More recent studies have proposed that such signaling molecules within the nervous system may actually participate in the control of bone metabolism, through the existence of a neuro-osteogenic signaling network [14].

Receptors for CGRP have been identified on osteoblasts [15–17], and their activation increases bone colony formation *in vitro* [18]. Transgenic mice overexpressing osteoblast-derived CGRP have a high bone mass phenotype with increased indices of bone formation, indicating a possible anabolic role for CGRP in bone [19].

Receptors for SP have been identified on osteoclasts, while receptors for VIP have been identified on both osteoblasts and osteoclasts [17, 20]. VIP may have a pleiotropic role, stimulating bone formation *in vitro* possibly through a cAMP-mediated mechanism, in addition to regulating osteoclast activity [14, 21]. Stimulation of SP receptors has been shown to stimulate calcium influx and bone resorption *in vitro* [22].

Transporters for neurotransmitters are also expressed in bone, for example, the glutamate/aspartate transporter (GLAST) is expressed by osteoblasts and embedded osteocytes [23]. Glutamate receptors are also expressed in bone cells and a role for glutamate in the regulation of osteoclast activity has been proposed [24, 25]. Serotonin transporters and receptors have also been identified in osteoblasts, and may be involved in the process of bone formation and mineralization [26, 27].

The presence of these neural components suggest there may be direct signaling between the brain and bone, and furthermore, that these signals may be involved in the regulation of bone remodeling. The presence of neurotransmitter transporters and receptors would enable the osteoblast to both respond to, and regulate neurotransmitter activity. However, as there is not much known of potential synapses between nerve and bone cells, it is uncertain how these transporters would operate.

The Y receptor knockout models

Recently, an important role for the Neuropeptide Y (NPY) receptors in the regulation of bone formation was revealed. NPY is one of the most abundant neuropeptides in the central and peripheral nervous system and in addition to its strong stimulatory effect on food consumption it also regulates numerous other physiological functions. At least five different Y receptors (Y1, Y2, Y4, Y5, and in the mouse y6), are known to mediate the action of NPY and its two other family members, peptide YY (PYY) and pancreatic polypeptide (PP) [28–33]. NPY and PYY have identical affinity for all Y receptors, with PP preferring the Y4 receptor. The lack of a full complement of selective pharmacological tools for the Y receptors has made it difficult to determine which Y receptors are responsible for the different effects of NPY. In order to investi-

gate the specific roles of each of the receptors, and to further understand the mechanisms and pathways involved in these diverse physiological effects, germline knockouts have been generated for each of the Y-receptors.

A potential role for NPY in the regulation of bone formation was first revealed in germline Y2 receptor knockout (KO) mice. These mice displayed a two-fold increase in cancellous bone volume, due to an elevated bone formation and mineralization rate (Fig. 1) [1]. These increases occurred despite no change in the actual number of osteoblasts present, indicating that the change in bone volume was the result of altered osteoblast activity. The absence of detectable levels of Y2 receptor mRNAs in bone tissue suggests that this effect of Y2 deletion was occurring via an indirect, and potentially centrally-mediated, mechanism [1].

Conditional Y2 receptor deletion studies, whereby hypothalamic Y2 receptors were specifically deleted in adult mice, demonstrated that this two-fold

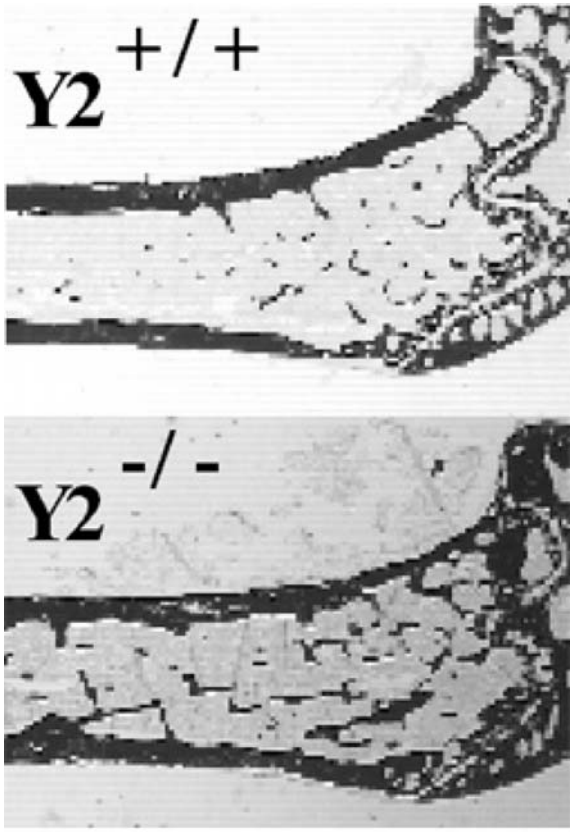

Figure 1. Sagital micrographs of the distal femoral metaphysis of control ($Y2^{+/+}$) and germline Y2 receptor knockout ($Y2^{-/-}$) mice. Each micrograph is a representative sample.

increase in bone volume could be achieved within only 5 weeks [1], illustrating the Y2 receptor pathway to be a potent and rapid-acting bone anabolic pathway, acting via a centrally controlled mechanism. Thus deletion of hypothalamic Y2 receptors acts to release a tonic inhibition of trabecular osteoblast activity, increasing the rate of bone mineralization and formation, and resulting in an increase in trabecular bone volume.

The specificity of this modulation of osteoblast activity by the Y-receptor system has been demonstrated by analysis of the other germline Y receptor knockout mice. Unpublished data from our group shows that deletion of Y1 receptors results in a similar high trabecular bone mass phenotype as the Y2 knockout model. However, while the increase in bone volume in the Y2 knockout model is solely attributed to increased bone formation, the increase in bone volume in Y1 knockout mice is the result of altered bone turnover, with increased indices of both osteoblast and osteoclast activity. Therefore, while the net result of both the Y1 and the Y2 knockout models is an increase in bone formation, the differences in bone cell activity between these two models suggests they may be acting via alternate pathways.

In contrast to the Y1 and Y2 knockout models, germline deletion of the Y4 receptor does not result in a bone phenotype, but rather similar levels of bone volume to wild type mice. Interestingly, double deletion of the Y2 and Y4 receptors results in a gender-specific synergistic three-fold increase in bone volume in male mice [34]. This will be discussed in further detail later.

Relationship between obesity and bone

The principle role of NPY is in the regulation of food intake and the maintenance of energy homeostasis. It has long been known that a link or relationship exists between body weight and bone density. Obesity, caused by an imbalance in energy homeostasis, can protect against osteoporosis [35, 36]. This protective effect of obesity is partially explained by mechanical (weight-bearing) effects, but also by the fact that the sex steroid hormone estrogen, which has protective effects on bone, is produced in adipose tissue. More recently, a potential role for adipose-secreted factors in the regulation of bone turnover has been a subject of increasing interest.

Leptin is one such hormone secreted by adipocytes, which is then transported to the hypothalamus where it acts on specific receptors to regulate energy homeostasis. Serum leptin levels are positively correlated to fat mass, and deficiency of leptin or its receptor, as seen in the natural occurring mouse mutants, the *ob/ob* and the *db/db* mouse, respectively, leads to massive obesity [37, 38]. Hypothalamic levels of NPY mRNA and the secretion of NPY are strongly elevated in these mice [39], and contribute to the massive obesity, hypercortism, and reproductive defects characteristic of this model [40]. NPY ablation in *ob/ob* mice attenuates all these defects [41], further demonstrating that NPY is a major downstream mediator of leptin's central effects.

Surprisingly, *ob/ob* and *db/db* mice display a similar increase in cancellous bone mass as the Y1 and Y2 receptor knockout models [2], despite the hypogonadism and the hypercortisolism associated with leptin deficiency; both situations which would normally favor a reduction in bone. Investigation of other mouse models of obesity which are not related to leptin signaling, and the use of heterozygous *ob*+/– mice, or young *ob/ob* mice fed a low fat diet, have demonstrated this increase to be independent of the presence of fat, indicating that obesity is not the effector involved. In line with this, the A-ZIP/F-1 mouse, which has no white adipose tissue and thus has reduced levels of leptin, also has the high bone mass phenotype, further supporting the concept that the increase in bone volume is due to an absence of leptin signaling, and not due to fat mass [2].

Hypothalamic regulation of energy homeostasis and bone formation

Although leptin receptors have been identified in cultured osteoblasts from rat [42], and human [43, 44], there are no leptin receptors detectable on mouse osteoblasts [45] inconsistent with any possible autocrine, paracrine, or endocrine mechanism of regulation in the ob/ob model. The majority of leptin receptors are found in the arcuate nucleus of the hypothalamus, suggesting that leptin might control bone formation in these mice via the same central mechanism as it regulates body weight. The arcuate nucleus is located in an area with a permeable blood brain barrier and is therefore accessible to circulating hormones such as insulin and leptin [46]. Furthermore, intracerebroventricular (icv) infusions of leptin into both *ob/ob* and wildtype mice, despite causing significant weight loss, also promotes a rapid reduction in bone mass [2]. Taken together, these results suggest that the regulation of energy homeostasis is an integral process not limited to controlling fat and muscle tissue mass, but also includes bone tissue as a major storage component, with leptin acting via the central nervous system as an inhibitor of bone formation.

NPY synthesis is particularly high in neurons of the arcuate nucleus, with many of these neurons also expressing leptin receptors [48]. A high percentage of these arcuate NPY-ergic neurons co-express the Y2 receptor [49] thought to act as an auto-receptor which can modulate the expression and secretion of NPY and other neurotransmitters [50]. It is possible that the putative action of the co-localized pre-synaptic Y2-receptors to inhibit NPY release is augmented by leptin. Since leptin receptors and Y2 receptors are present on NPY-expressing neurons of the arcuate nucleus and are likely to share some common signaling pathways [39, 47–50], the Y2 receptor is the prime candidate to be involved in the central regulation of energy homeostasis.

It is not clear whether the increased hypothalamic NPY expression common to mice deficient in leptin action or Y2 receptors is causally related to the high bone density, because 28-day ICV NPY infusion (albeit, probably resulting in hyperleptinemia) actually decreases bone density [2].

Evidence for a distinct mechanism in leptin and Y receptor antiosteogenic pathways

The similar bone anabolic phenotype present in both the Y2 receptor knockout and *ob/ob* models, both resulting from signals processed within the hypothalamus, suggest a mechanistic link between these two pathways in the regulation of bone. This hypothesis is supported by comparable changes in the expression of certain neuropeptides, in particular, strongly elevated levels of hypothalamic NPY, present in both models [1, 2]. It is known that leptin and Y2 receptors interact in the regulation of adipose tissue, with the deletion of Y2 receptors attenuating the obese phenotype of the *ob/ob* model [51, 52]. The known interactions of the NPY and leptin systems in the regulation of energy balance, and the co-localization of leptin and Y2 receptors on NPY-ergic neurons within the hypothalamus suggest these two models may share a common mediator in their regulation of the bone anabolic response. Recent studies have investigated the extent to which the leptin and the Y receptor systems interact in the regulation of bone.

Several lines of evidence suggest the leptin deficient and Y2 receptor bone anabolic pathways to be somewhat independent.

Firstly, despite the similar bone mass phenotypes of the *ob/ob* and Y2 receptor knockout models, there are differences in the bone cell activity of these models. Leptin deficient *ob/ob* mice exhibit a marked increase in osteoclast number as a result of their hypogonadism [2], while in contrast, osteoclast number in the Y2 receptor knockout model is reduced [1], suggesting a difference in the regulation of the osteoclast in the two models. However, osteoclast surface is not affected by Y2 deletion, suggesting an increase in osteoclast size in these knockouts. A change in osteoclast morphology as seen in these knockouts is consistent with an increase in resorptive activity per cell [53, 54].

Secondly, chemical ablation studies of particular brain regions indicate that leptin's control of antiosteogenic and anorexigenic networks differ, with different neuropeptide populations responsible for the regulation of anorexic function and bone [45]. The specificity of chemical ablation studies makes them difficult to interpret, however, these findings suggest that leptin may not work through the classic NPY pathway to regulate its bone anabolic response.

More recently, genetic studies have been utilized to investigate the degree of commonality between the leptin deficient and Y receptor anabolic pathways, by investigating the effects of specific Y receptor deletion on a leptin deficient background. This was achieved by crossing the different Y receptor knockout mice onto the *ob/ob* background, to determine whether leptin and Y receptor deficiency have additive effects. Interestingly, both the Y1ob and the Y2ob double knockout models showed a decrease in bone volume relative to the single Y1 knockout or Y2 knockout models, respectively, suggesting there may be some degree of interaction or interdependence between the leptin and Y receptor pathways (unpublished observations and [56]). Interestingly, analysis of the bone cell activity of these models also provided supporting evidence

for differential regulation of the Y1 and Y2 receptor anabolic pathways from each other. In the case of the Y2ob model, the reduction in bone volume was attributed to an increase in osteoclast activity, likely due to the hypogonadism associated with leptin deficiency [56]. However, in the case of the Y1ob double knockout model, there was no alteration in osteoclast surface. Rather, the reduction in bone volume in this model actually resulted from a reduction in the rate of bone mineralization. That is, deletion of leptin signaling actually abolished the anabolic bone formation activity of the Y1 receptor knockout model. While these alterations in the bone cell activity of the Y receptor and *ob/ob* double knockout models suggest possible interaction between the two pathways, evidence for an independent component of these pathways also exists. The interaction between leptin excess and Y receptor deletion was examined by central administration of recombinant viral NPY to produce weight gain and thus leptin excess in adult Y receptor knockout mice. Both wild type and Y2 knockout mice exhibited a marked elevation in adipose tissue accumulation, and hence leptin expression. The antiosteogenic effects of leptin acted to reduce osteoblast activity in both wild type and the Y2 knockout model. However, despite an overall reduction in bone volume, the Y2 receptor knockout model maintained it's two-fold elevation in bone formation activity, thereby acting in an opposite direction to the antiosteogenic effects of leptin, and providing strong evidence for an independent or functionally distinct component of the Y2 receptor and leptin deficient anabolic pathways [56] (Fig. 2).

Further evidence of distinct leptin and Y2 pathways stems from the Y2Y4 double knockout model. The synergistic increase in bone volume observed in male mice is associated with a significant reduction in serum leptin compared with wild type or Y2 knockout mice [34]. Both the synergistic effect on bone and the reduced leptin levels are absent in female mice, suggesting that the gender specificity of the bone response may arise from a stimulation of bone formation, due to both reduced levels of leptin, as well as that induced by Y2 receptor deletion.

While the leptin antiosteogenic pathway has been linked to alterations in sympathetic nervous system (SNS) activity [45], possibly by direct actions though β_2-adrenergic receptors expressed on osteoblast cells, the mechanisms behind the anabolic activity of the Y2 receptor knockout pathway remains to be determined. The above studies suggest that the Y2 receptor anabolic pathway is distinct from the leptin response, indicating that alternate regulatory mechanisms may be involved.

Germline or hypothalamus-specific Y2 receptor knockout does not induce any obvious endocrine imbalances that would impact on bone homeostasis [1]. No significant changes from controls were detected in the plasma concentrations of total calcium, leptin, free T4, IGF-1, and testosterone. Furthermore, fertility is not impaired [55]. These findings suggest that Y2 receptor deficiency does not influence bone formation via modulation of humoral factors. However, it is as of yet unknown how a central nervous system mechanism

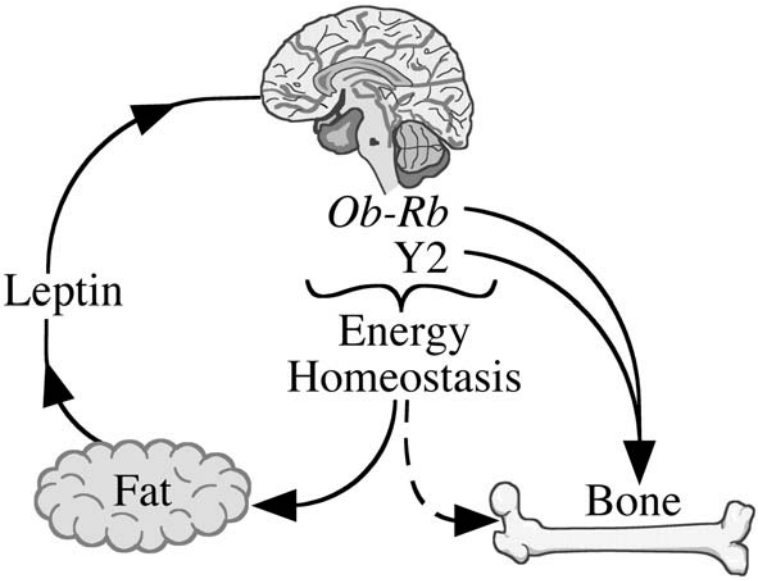

Figure 2. Proposed pathways for the central regulation of bone metabolism by Y2 receptors and leptin. Leptin is secreted by adipocytes and transported to the hypothalamus where it binds its receptor (Ob-Rb). Leptin and Y2 receptors are co-expressed on NPY neurons of the hypothalamus, and the Y2 receptor is likely to be involved in leptin's regulation of energy homeostasis, and thus adipose deposition, acting to modulate NPY levels. Leptin and Y2 receptors also regulate bone formation. A component of this regulation may be via a common pathway shared by the leptin and Y2 receptors. However, another component of this regulation utilizes distinct mechanisms, whereby the actions of leptin and the Y2 receptors can be separated.

would interact with autocrine/paracrine or other factors to regulate bone remodeling and adapt to the mechanical stresses of the environment.

Concluding remarks

Genetic models such as Y receptor knockout and leptin knockout mice have begun to reveal some of the individual functions of the different Y receptors. The finding that some of these receptors appear to be involved in the regulation of bone formation via a hypothalamic relay, has revealed not only a previously unknown and novel function of the Y receptors, but also a novel example of the regulation of bone formation by a very potent, centrally-mediated mechanism.

The rapid increase in bone mass in adult mice following central deletion of Y2 receptor function suggests new possibilities for the prevention and anabolic treatment of osteoporosis. The Y2 receptor pathway appears to be distinct from the antiosteogenic pathway regulated by leptin, and therefore supports

the Y2 regulated pathway as a novel target for anabolic bone therapy. Furthermore, the area of the arcuate nucleus where the Y2 receptors are located is accessible without the need to cross the blood brain barrier, and is therefore potentially an ideal target for drug intervention. The additional advantage of this particular sub-population of arcuate Y2 receptors is that their specific inhibition will not influence any other central functions of the Y2 receptor such as effects on seizure susceptibility, anxiety, or memory, therefore limiting the possibility of side effects associated with such a treatment for osteoporosis.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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NPY in alcoholism and psychiatric disorders

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NPY in affective disorders

Symptoms of anxiety and depression commonly co-exist and both disorders are thought to reflect maladaptive changes in stress-responsive systems [1]. Known genetic factors increase vulnerability for both anxiety and depression [2]. It has furthermore been suggested that the present classification of depressive and anxiety disorders may be artificial, and that for a large proportion of subjects with affective symptoms, a more appropriate categorization would be "major depression – generalized anxiety disorder" [3]. Thus, the role of NPY in these two conditions is dealt with jointly.

In rodent models, injections of nanomolar doses of NPY have been shown to decrease anxiety-like responses in a variety of tasks, including the elevated plus-maze [4, 5], social interaction task [6], fear potentiated startle and fear conditioned responses [4, 5, 7, 8]. In addition, intracerebroventricular (icv) administration of NPY to a large extent prevented gastric ulceration induced by water restraint, a strong stressor [9]. Mutant mice lacking NPY show increased anxiety-like behavior [10]. Although a transgenic mouse overexpressing NPY has been developed, only a limited phenotypic characterization for this line is available [11]. However, transgenic rats overexpressing NPY in hippocampus were shown to be resistant to stress-induced increases in anxiety-like behavior [12, 13].

These studies together indicate that pharmacological or transgenic activation of NPY signaling is stress reducing. The physiological involvement of endogenous NPY in mediation of stress responses and anxiety related behavior was demonstrated in two studies showing that NPY gene expression in amygdala and cortex is regulated by stress. Acute stress downregulates NPY-IR and NPY mRNA expression within 1 h, with mRNA levels returning to normal levels within 10 h and peptide levels within 2 h [14]. This stress has been shown to be anxiogenic on the elevated plus-maze. Interestingly, with repeated stress exposure, leading to a behavioral habituation, this effect is reversed. Under these conditions, NPY expression is instead upregulated [15]. On the basis of these pharmacological and expression studies, it was proposed that an upregulation of NPY expression may contribute to successful behavioral adap-

tation to stress. This extends a previously introduced hypothesis that NPY may act to "buffer" behavioral effects of stress-promoting signals such as CRF [16].

For depression, a differential NPY expression has been detected in a genetic animal model, the Flinders Sensitive rats (FSL) [17–20]. This is in agreement with the finding that chronic cocaine reduces NPY expression in the prefrontal cortex [21], since clinical hallmarks of cocaine withdrawal and dependence are symptoms of depression. Treatment with clinically effective antidepressants was early reported to increase NPY expression in several brain regions in rats, with frontal cortex being the most consistent region [22]. Initial attempts to replicate the effects of chronic antidepressant treatment and extend them to mRNA level were unsuccessful [23, 24] for reasons which remain unclear but may be related to assay specificity or, more likely, the half life of the drugs used being insufficient to maintain adequate plasma concentrations. Subsequently, a region-specific regulation of NPY and Y1 receptor expression was reported following chronic treatment with the serotonin-selective reuptake inhibitor (SSRI) fluoxetine, both in the "depressed" (FSL) line and the corresponding control line (FRL) [17, 25]. In these studies, fluoxetine elevated NPY-IR in the hypothalamic arcuate nucleus and anterior cingulate cortex, and increased Y1 binding sites in the medial amygdala and occipital cortex in both lines. In agreement with these findings, an increase in the NPY mRNA was found in the arcuate nucleus in both lines. In other brain regions, fluoxetine treatment caused a differential effect on the induction of NPY-related genes in these two rat strains: in hippocampus, NPY mRNA expression was increased in the "depressed" (FSL) subjects, but decreased in the "non-depressed" (FRL) line. In contrast, Y1 mRNA levels tended to decrease by fluoxetine in the nucleus accumbens of the FSL rats, but increased in the FRL. On the basis of these findings, an involvement of NPY was suggested in the antidepressant effect of fluoxetine.

Another established and effective antidepressive treatment, electroconvulsive shock (ECS), has been much more consistent in upregulating brain NPY-levels, with hippocampus as a seemingly central target. An elevated NPY level was demonstrated after repeated, but not single ECS, paralleling the requirements for clinical effect in depressed subjects [26–28]. These data has been both replicated and extended [29–31] and this effect seems robust in both "normal" laboratory rats and in the genetically selected FSL and FRLs. The mechanism is an upregulation of preproNPY expression which leads to an increased extracellular availability of the NPY peptide. Against the background of our behavioral finding in the transgenic rat model [12], upregulated hippocampal NPY-expression might be of importance both for therapeutic and amnesic effects of ECS.

The anti-anxiety and anti-depressive actions of NPY appear to be predominantly mediated via the Y1 receptor system. This was initially based on the observation that full length NPY peptide produced an anti-anxiety effect in elevated plus-maze, Vogel test [5] and Geller-Seifter test [7], while the C-terminal, presumably Y2-selective fragment, $NPY_{13–36}$, did not generate this

action. The *in vivo* use of intracerebral antisense oligonucleotides targeting Y1 receptor transcript made it possible to demonstrate a selectively lowered density of Y1 binding sites, with an outcome of decreased behavioral effects on the elevated plus-maze [32]. With the development of more selective pharmacological tools, Y1 mediation of anti-stress effects of NPY appears to have been confirmed.

Y2 receptors may also play a role in the regulation of emotionality. NPY-Y2 receptors are located presynaptically on NPY-ergic neurons, and control the release of endogenous NPY [33, 34]. Antagonizing this receptor is expected to potentiate the release of NPY and through this mechanism offer an "NPY-mimetic" effect without developing an Y1 agonist. This mechanism would therefore be an attractive target in the drug development efforts. Studies of NPY Y2 receptor knockout mice have supported this idea [35, 36] and results are consistent with the anxiogenic-like effects of intra-amygdala treatment of Y2-preferring agonists in the rat social interaction test [37, 38]. Another more direct involvement of Y2 receptors has been suggested within the locus coeruleus, where an anxiolytic-like effect was detected after a 10 pmol NPY microinjection into this structure, mimicked by $NPY_{13–36}$ but not by [Leu31, Pro34]NPY, a "non-Y2" ligand [39].

The amygdala has so far been the most prominent region of interest with regard to emotionality. Central amygdala was initially suggested to be the mediating site of anxiolytic NPY actions [40]. However, subsequent microinjection studies using smaller injection volumes have prompted a re-evaluation of the data, suggesting that the lateral/basolateral complex in fact mediates anti-stress effects of NPY within the amygdala [6]. Periaqueductal grey matter (PAG) is involved in the behavioral output of fear responses, with subcompartments that are differentially involved in defensive behaviors [41, 42]. Its dorsolateral compartment (DPAG) has been suggested to tonically inhibit the amygdala. Microinjections of Y1 antagonists within DPAG produced an anxiogenic effect in elevated plus-maze [43] and social interaction task [44].

Septum has been implicated to be part of another important "behavioral inhibition system" but septal lesion that studies demonstrated effects on anxiety-like behaviors most likely reflected effects on fibers passing through this structure, probably belonging to hippocampal output through fornix fimbriae [45]. Hippocampus is an important component of neuronal circuitry controlling anxiety-related behaviors and stress responses, and in particularly dorsal hippocampus [46, 47], and septo-hippocampal circuits are likely to be important for fear related behaviors. NPY microinjections into lateral septum reproduced anxiolytic-like actions of intracerebroventricular administration of NPY, and reversed the anxiogenic action of corticotrophin releasing factor (CRF). This was clearly mediated by the Y1 receptor, since a highly selective Y1 receptor antagonist, BIBO 3304, blocked this anxiolytic-like action [48].

Human studies support an involvement of NPY in depression and anxiety disorders. An early study stated decreased levels of NPY in the cerebrospinal fluid (CSF) of patients with major depression [49], which could reflect a

decrease in central availability of NPY. Low levels of NPY in brain tissue were also reported in suicide victims [50]. These studies were followed by reports which failed to replicate their results [51, 52], although issues of assay specificity are particularly likely to complicate matters in this case. In a recent re-examination of this issue in a large number of therapy-refractory depressed patients, a highly significant, 30% reduction of CSF NPY was found [53]. Interestingly, postmortem studies have meanwhile shown a decreased NPY mRNA expression which is most prominent in bipolar disorder [54]. It is a well-known fact that a proportion of patients diagnosed with unipolar disorder in fact has a genetic vulnerability for bipolar disorder, but has not yet presented with their first manic episode, and may never do so. It is therefore possible that the involvement of NPY is primarily related to bipolar traits and that the discrepant CSF results are partly due to varying proportion of this patient category in the different clinical populations.

In summary, compelling evidence exists for a role of NPY as an endogenous anti-stress compound, which is physiologically recruited to cope with prolonged stress. Dysfunction of this system seems to be present in affective illness. Targeting the NPY system, possibly through antagonism at presynaptic Y2 autoreceptors, offers an attractive strategy to develop novel antidepressant and anti-anxiety treatments.

NPY in alcoholism

In addition to involvement in mood disorders such as depression and anxiety syndromes, NPY has been demonstrated to have a role in alcohol intake, dependence, and withdrawal. The effect profile of NPY shows numerous similarities with not only that of established anti-anxiety compounds, but also that of alcohol. Furthermore, in clinical studies of alcohol dependence a correlation between initial anxiety and subsequent alcohol abuse, possibly due to the anxiolytic action of alcohol, has been demonstrated [55, 56]. While this may only be true for a subgroup of alcoholics, it may partially explain some of the changes and effects seen for NPY in alcoholism.

A direct link between NPY signaling and regulation of alcohol consumption was first shown in a study where mice with a transgenic overexpression of NPY consumed less alcohol, while mice with a null-mutation, i.e. inactivation, in the NPY gene had an increased alcohol consumption [11]. Genetic studies in both experimental animals and humans provide tentative support for a role of NPY in regulation of ethanol intake. Within the genome of a genetic rat model of high alcohol drinking state, the P-rat (see below), a quantitative trait locus was identified which spans the locus for the NPY gene [57, 58]. Furthermore, associations between alcoholism and polymorphisms within the NPY gene have been suggested. A substitution (Pro7 for Leu7) in the signal peptide region of the NPY precursor, prepro-NPY, leads to increased plasma NPY in response to stress compared to control subjects without the substitu-

tion [59, 60]. A 34% higher average alcohol consumption was reported in Finnish men with this substitution compared to matched control subjects [59]. Another report showed alcoholic European/American men had a 5–5.5% Pro7 allele frequency while the frequency in the non-alcoholic control group was 2% [61]. However, this polymorphism has also been reported to be of lower frequency in alcoholics or to not be significantly different between alcoholics and controls [61, 62]. We have recently reanalyzed this issue by reconstructing the haplotype structure of the preproNPY – gene using five polymorphic markers. This has yielded two preliminary insights: The coding Leu7Pro 1128 SNP is in strong linkage disequilibrium with a novel promoter polymorphism, and is present almost exclusively on a common haplotype. The frequency of this haplotype differs significantly between alcohol dependent subjects and normals. All of this difference can be attributed to type I alcoholics, i.e., patients with late onset of alcohol problems. Interestingly, this clinical subtype is characterized by high trait anxiety, which makes the association of particular interest considering the established role of NPY in anxiety. Finally, another polymorphism, a C-to-T substitution at the 5671 locus of the NPY gene, was reported to be more frequent in a Japanese alcoholic patient population [63].

In animals, selective breeding for ethanol consumption or preference has created several lines of mice and rats which have been well characterized with regards to numerous behavioral, pharmacological, and biological traits. Mouse lines include the high-alcohol-preference (HAP) and low-alcohol-preference (LAP) line, and rat lines include the Sardinian preferring (SP) and non-preferring (SNP) lines, the Indiana alcohol-preferring (P) and non-preferring (NP) lines, the Alko alcohol (AA) and Alko non-alcohol (ANA) lines, as well as the high-alcohol drinking (HAD) and low alcohol drinking (LAD) lines [64]. Each high drinking/preferring line consumes sufficient amounts of alcohol to achieve pharmacologically significant blood levels (50–250 mg%), is motivated by ethanol's pharmacological properties rather than smell, taste, or caloric content, and develops physiological tolerance after long term access to alcohol. NPY and NPY receptor expression patterns have been examined in these 'genetic models of alcohol dependence'. For example, P rats have been shown to have low levels of NPY in amygdala, frontal cortex, and hippocampus compared to the non-preferring NP-line, but higher levels in the paraventricular nucleus, arcuate nucleus, and cingulate cortex [65, 66]. In the HAD line NPY-IR was decreased in central nucleus of the amygdala, paraventricular nucleus of the hypothalamus, and the arcuate nucleus as compared to LAD rats [67]. In the AA/ANA, a different pattern was seen, with lower hippocampal NPY mRNA expression compared to the non-preferring line [68]. The NPY Y2 receptor subtype was also found to be reduced in the medial amygdala of the AA line as compared to the ANA line.

The effect of NPY on alcohol consumption appears to be in part dependent on the individual's history and state of alcohol consumption. In animal studies, central administration of NPY into the lateral ventricles, central nucleus of the amygdala, or the third ventricle leaves level of ethanol intake unaffected in

normal, out-bred rat strains [69–72]. However, a significant suppression of alcohol intake was found in the P-line as compared to NP and normal Wistar rats, and in the HAD rat line [73, 71]. The lack of effect in states of low intake but efficacy in the preferring lines which consume ethanol for its pharmacological properties is key to understanding a basic distinction, which is further highlighted by experiments in animals with or without a history of dependence. Thus, a basal component of ethanol consumption seems to be unrelated to the pharmacological/rewarding actions of ethanol, but might instead be related to its properties as caloric nutrient, regulated by factors modulating appetite. This component is not suppressed by NPY; in contrary, it is stimulated by hypothalamic NPY injections, as would be expected from NPYs well established effect to stimulate appetite [74]. In contrast to the suppressive effects of NPY on ethanol intake in high-preferring animals, the modulation of the low level intake component appears to be the same in rats genetically selected for low and high preference, making it further unlikely that it is related to the addictive properties of ethanol [75].

Further evidence for the dichotomy between effects of NPY on ethanol consumption related to addictive properties of this drug, *versus* effects on low level intake, has been provided using animals in which dependence and high alcohol preference was induced using 8 weeks exposure to intermittent ethanol vapor (14 h on/10 h off per day; target BAL 200 mg%). This models chronic alcohol consumption and leads to similar clinical manifestations as well as long-term changes in neurochemistry and increases in alcohol intake [76]. In this model, NPY was shown to significantly suppress alcohol intake in exposed animals as compared to saline treatment. Notably, consumption was reduced back to but not below pre-vapor exposure levels [77].

Thus, the NPY system may offer an attractive target for developing novel therapies for alcohol dependence. The likelihood of this has been strengthened by recent findings that mice in which the Y1 receptor gene as been inactivated consume increased amounts of ethanol [78]. Furthermore, icv administration of the selective Y2 antagonist BIIE0246 lead to decreased ethanol intake in non-dependent rats, and a sensitization to this effect was shown in post-dependent (vapor exposed) rats [79, 80].

Conclusion

The NPY system may well be one of the most interesting target systems for development of treatments for alcohol dependence as well as mood disorders such as depression and anxiety syndromes. NPY is an endogenous anxiolytic compound, functions as an antidepressant, and is effective in modifying alcohol intake in high drinking states. Through receptor subtype specific compounds, the NPY system offers an interesting and innovative future approach for treatment designs. Selective Y2 receptor antagonists and/or Y1 agonists that are peripherally available and effectively penetrate the CNS are possible

candidates. In conclusion, the NPY system offers attractive targets for development of future treatments for depression, anxiety, and alcohol dependence.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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Plasticity of neuropeptide Y in the dentate gyrus after seizures, and its relevance to seizure-induced neurogenesis

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Introduction

Neuropeptide Y (NPY) has a multitude of functions in peripheral organs, so it is no surprise that the same appears to be the case in the brain. One of the reasons why NPY potentially contributes to so many functions may be due to its plasticity, meaning the ability to change its level of expression and pattern of expression. This makes NPY a potentially useful tool to modulate the behavior of an organism in response to the challenges of the external environment. Thus, NPY mRNA, protein, and receptors can change dramatically not only in their level of expression but also their location in different types of neurons and neuronal processes. This occurs in response to perturbations of the environment, and also in pathological conditions, suggesting a role not only in the normal condition, but also in disease.

This plasticity is well exemplified in the dentate gyrus of rat hippocampus, and this review will focus on NPY in the dentate gyrus to illustrate the striking plasticity of NPY in the brain. We will specifically address the changes that occur after seizures because the changes in NPY and its receptors after seizures are robust and dramatic. Furthermore, they could have important functional implications. Thus, the excitability of neurons in the hippocampus change after seizures, and these changes may be initiated, or at the very least influenced, by the alterations in NPY and its receptors. In addition, there are additional functional implications for dentate granule cell neurogenesis, because NPY appears to be a key regulator of dentate granule cell neurogenesis. This chapter first explains the pattern of expression of NPY and its receptors in the normal rat dentate gyrus, and then describes the changes in expression after seizures. Functional implications are subsequently considered, including effects on excitability, as well as neurogenesis.

Overview of the cell types and circuitry in the normal rat dentate gyrus

The adult rat dentate gyrus consists of a dense layer of granule cells, and many other types of neurons and glia that are dispersed throughout all strata (Fig. 1). The somata of granule cells are tightly packed, and form the so-called granule cell layer (also referred to as stratum granulosum). Granule cells are oriented in a stereotypical fashion, with a spiny arbor of dendrites that begins at the cell body and extends unilaterally to the hippocampal fissure (Fig. 1). The area where the dendrites are located also contains scattered non-granule cells, and is referred to as the molecular layer (stratum moleculare). It is divided into three sublayers: the inner, middle and outer molecular layers (Fig. 1). These layers contain numerous afferents from nuclei extrinsic to the hippocampus, and axons from neurons that lie within the dentate gyrus such as various GABAergic neurons (inhibitory interneurons) and the glutamatergic "mossy" cells. The middle and outer molecular layer are the primary sites of termination for the major cortical input to the dentate gyrus from the medial and lateral entorhinal cortex, respectively. A hallmark of the extrinsic afferents is the specificity for each particular sublayer, with axons from one system rarely crossing the borders to adjacent substrata.

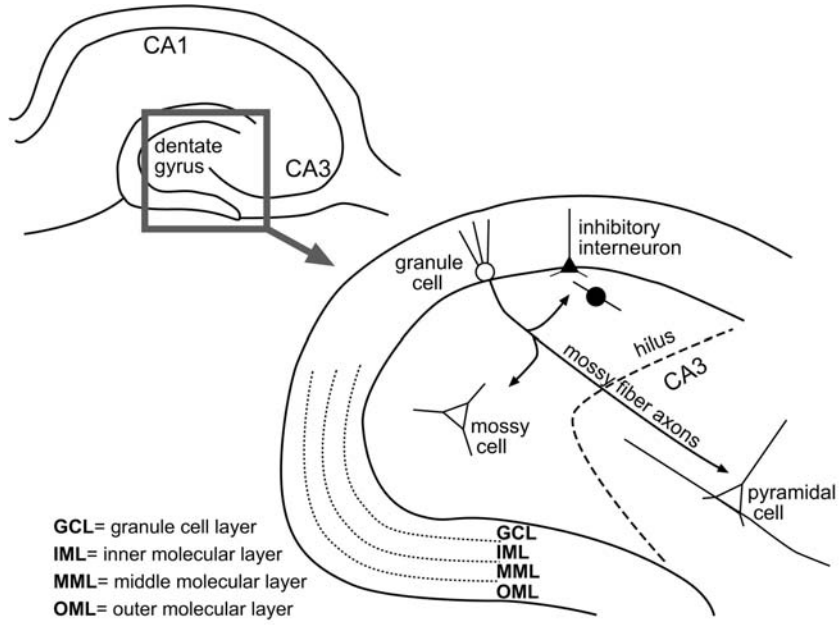


Figure 1. Schematic of the adult rat dentate gyrus. A transverse section through the rat dentate gyrus in the horizontal plane illustrates its location in the hippocampus. The inset diagrams the locations of the different cell types and lamination of the dentate gyrus.

On the opposite pole of the granule cells from their dendrites lie the granule cell axons or "mossy fibers." These axons are a major component of the large region just beneath the granule cell layer, called the hilar region or hilus (Fig. 1). The mossy fibers primarily target the proximal apical dendrites of CA3 pyramidal cells of hippocampus, but also form a complex array of collaterals that terminate on hilar cells. These hilar cells can be divided into the mossy cells, which innervate the inner third of the ipsilateral and contralateral granule cell layer, and also have local collaterals. There also are hilar cells that are GABAergic, and these neurons are highly diverse in morphology and projection. The somata and axons of GABAergic neurons are located throughout the dentate gyrus.

All of the cells in the dentate gyrus, both glutamatergic and GABAergic, contain secondary chemicals and proteins that are thought to be neuromodulators. Different cell types are often selective for the expression of distinct neuromodulators. For example, granule cells contain several peptides such as enkephalin, the calcium binding protein calbindin D28K, as well as other substances such as zinc, but these are only weakly expressed, if at all, in other types of dentate gyrus neurons [1–3]. Mossy cells preferentially express calcitonin gene-regulated peptide (CGRP; [4]). GABAergic neurons contain a variety of unique substances that are not normally expressed in other dentate gyrus neurons, such as somatostatin, cholecystokinin, the calcium binding protein parvalbumin, and vasoactive intestinal polypeptide, to name a few [5].

Normal localization of NPY and its receptors in the rat dentate gyrus

The normal expression pattern of NPY protein and its receptors in the rat dentate gyrus are shown in Figures 2 and 3. NPY is normally expressed very specifically in GABAergic neurons and not other cell types [6, 7]. Fiber systems that innervate the dentate gyrus do not appear to express NPY.

Only a subset of the GABAergic neurons in the dentate gyrus express NPY. Many of these cells co-localize NPY with other peptides, including somatostatin and cholecystokinin, but this is not always the case. Thus, immunocytochemistry reveals some neurons that are NPY-immunoreactive but lack somatostatin-immunoreactivity, whereas in others the two peptides are co-localized. Depending on the antibodies, laboratory, and other variables, the percentages of GABAergic neurons that are NPY-ergic can vary substantially. However, there are some general rules that can be concluded. First, most of the cell bodies are located in the hilus and in the granule cell layer [7]. In addition, these neurons appear mostly to be "local circuit neurons" because their axon typically arborizes in the hippocampal lamella where the cell body is located. There is only one report of NPY-immunoreactive neurons that have a contralateral projection, and they were estimated to be only 2% of the total population of NPY containing neurons [6]. The axon contributes to the innervation of granule cells, hilar cells, and also contributes to fibers innervating process-

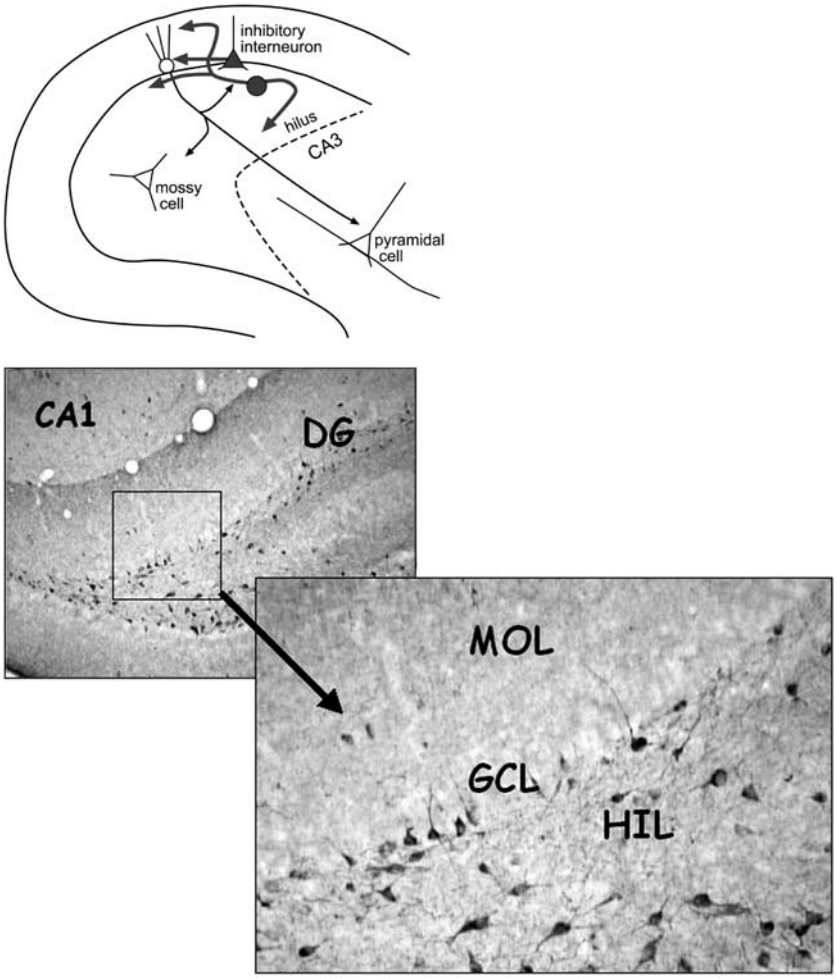


Figure 2. Normal NPY expression in the adult dentate gyrus. TOP: A schematic shows the normal distribution of NPY protein is in various inhibitory neurons. BOTTOM: A micrograph showing expression of NPY in a normal male adult rat. The neurons that are NPY-immunoreactive have somata mainly in the granule cell layer (GCL) and hilus (HIL), and co-express GABA. DG = dentate gyrus. MOL = molecular layer.

es in the outer two-thirds of the molecular layer [6–8]. Most NPY-immunoreactive terminals appose molecular layer dendrites, including dendrites of granule cells as well as other non-granule cells [6, 8]. NPY-immunoreactive terminals do appear to appose other terminals in the outer molecular layer [8], but there does not appear to be a strong influence of exogenous NPY on the perforant path input to the molecular layer [9, 10]. NPY terminals also appose astrocytic processes [11], suggesting an interaction with glia [12].

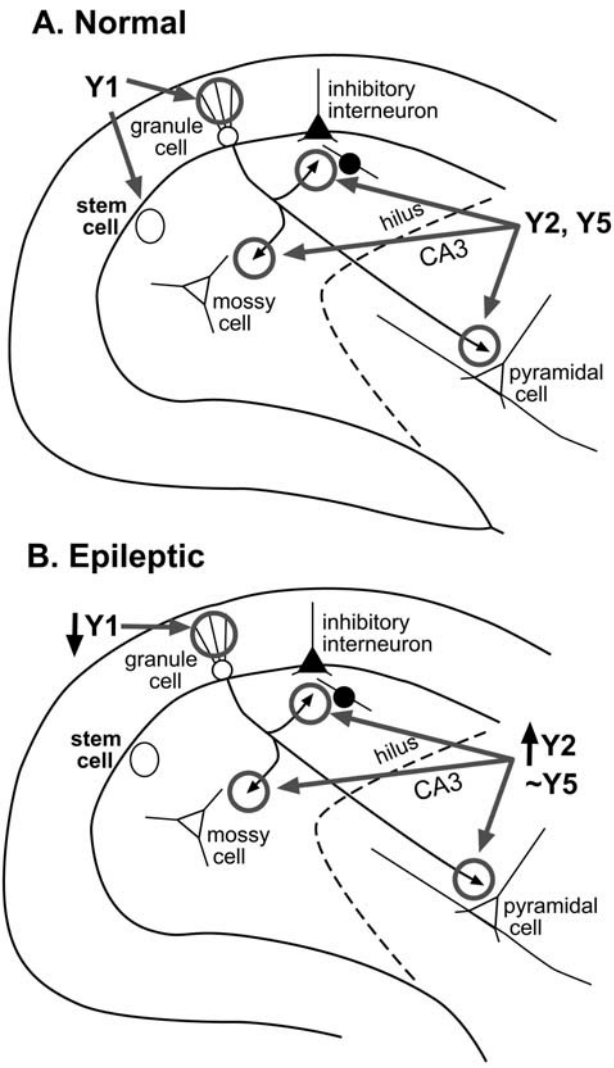


Figure 3. The distribution of NPY receptors in the normal and epileptic rat dentate gyrus. NPY receptor distribution of a normal adult rat (A) and an adult rat that has had severe continuous seizures (status epilepticus; B) are shown. The distributions appears similar regardless of the methods to induce status, and seizures last for 1 or more hours. Methods that are commonly used to induce status include the convulsant pilocarpine or kainic acid, or electrical stimulation. After status epilepticus, spontaneous repetitive seizures develop within weeks, and persist for the life of the animal (i.e., epileptogenesis occurs). For further explanation and references, see text.

Of the five NPY receptors known to be expressed in the brain, Y3 and Y4 are relatively weakly expressed in the hippocampus. In the adult rat dentate gyrus, Y3 mRNA is not detectable in the normal adult rat, and Y4 mRNA lev-

els are low [13]. In contrast, Y1, Y2, and Y5 mRNA is robust [13]. mRNA for Y1, Y2, and especially Y5 receptors appear to be expressed primarily on hilar neurons and in granule cells [13]. Receptor protein appears to have a different expression pattern, with Y2 and Y5 receptors primarily on mossy fibers and and Y1 receptors primarily in the molecular layer ([14]; Fig. 3A). The localization of Y2 and Y5 receptors to mossy fibers suggests a presynaptic action of NPY. This is also suggested by electrophysiological studies, which have shown that NPY influences mossy fiber transmission by a presynaptic mechanism [10]. Both Y2 and Y5 receptors have been implicated in these effects [15, 16].

The role of the molecular layer Y1 receptor is less clear. Studies of Y1 receptor agonists reveal inhibitory effects on Ca^{2+} entry into dendrites of granule cells [16]. Other studies suggest a potentially proconvulsant effect [17]. Studies in mouse slices indicate that Y1 receptors regulate the G-protein inwardly rectifying potassium (GIRK) potassium channel [18]. To date, no studies have examined the hypothesis that transmission from the entorhinal cortex is modulated by Y1 receptors, although such actions are suggested by the evidence that boutons of NPY-immunoreactive neurons appear to innervate non-NPY-immunoreactive boutons in the outer two-thirds of the molecular layer [8]. However, these could be other boutons besides those of the perforant path, because NPY itself seems to do little to perforant path transmission (see above). Interestingly, another potential role of Y1 receptors is regulation of neurogenesis, because granule cell progenitors express Y1 receptors, and neuroproliferation increases in response to NPY (see below).

NPY and dentate gyrus neurogenesis

Neurogenesis occurs primarily in three locations in the adult brain, the olfactory bulb, the subventricular zone, and the dentate gyrus. In the dentate gyrus, there is a 50–100 μm layer in the hilus just beneath the granule cell layer called the subgranular zone (SGZ), where it is thought that the precursors of granule cells are located (Fig. 4). These progenitors appear to spontaneously divide at a slow rate throughout life, and their progeny develop into neurons (neurogenesis). Primarily the new neurons become granule cells, but there are reports that other neuronal types, such as GABAergic neurons, may also develop [19]. In addition, these precursors may develop into non-neuronal cells. Indeed, their development may arise entirely from radial glia, dividing into one or more daughter cells that become neurons, as well as additional cells that ultimately become mature astrocytes [20, 21].

The regulation of adult neurogenesis has become a topic of substantial interest because several studies have implicated dentate gyrus neurogenesis in important functions, such as learning [22, 23]. Thus, it is possible that new neurons must be continually supplied to the dentate gyrus granule cell layer to maintain the ability of the hippocampus to mediate or modulate learning and memory. In addition, it has been observed that many factors that influence

behavior also influence neurogenesis in the dentate gyrus, such as the hormones estrogen and prolactin, exercise, administration of growth factors, etc. [24]. Increased neurogenesis in the adult dentate gyrus has typically been associated with beneficial effects, and decreased neurogenesis with the opposite, such as psychiatric disorders and learning deficits. For example, decreased neurogenesis in the dentate gyrus has been suggested to underlie depression [25]. Indeed, stress and glucocorticoids, which are elevated in depressed individuals, decrease neurogenesis in laboratory animals [26, 27]. Neurological disorders such as Alzheimer's disease, autism, or schizophrenia may be a result of altered neurogenesis in the adult brain also [28–31].

Interestingly, it has been shown that various types of insults to the brain, as well as seizures, lead to a rapid increase in the rate of dentate gyrus neuroge-

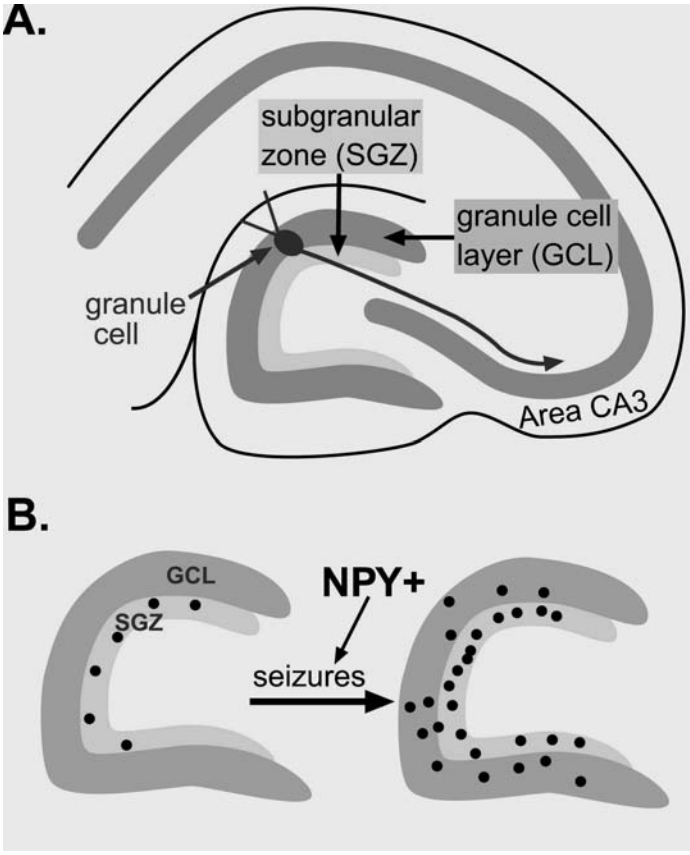


Figure 4. Seizure-induced neurogenesis in the rat dentate gyrus. A. A schematic illustrates the area of the dentate gyrus where progenitors are located in the adult rat, the subgranular zone. B. A schematic illustrates the increase in progenitors, labeled by the mitotic marker bromodeoxyuridine (BrdU), after seizures. As discussed in the text, NPY appears to facilitate this process.

nesis (Fig. 4). Remarkably, this period of increased neurogenesis can be long lived, lasting weeks in the case of some of the most severe seizures, such as status epilepticus [32–34]. This is remarkable because status epilepticus typically leads to substantial neuronal cell death. This phenomenon raises the possibility that neurogenesis may increase when the organism is threatened, injured, or damage occurs. In other words, a regulatory mechanism is present that provides compensation for the damage, and part of that mechanism includes increasing dentate gyrus neurogenesis. This is consistent with the general consensus that injury and seizures may be followed by a period when the brain begins to express proteins similar to those that are produced during development, a so-called "recapitulation of development" that is an effort by the brain to re-grow and hence repair itself. Interestingly, the increase in neurogenesis after status epilepticus may not necessarily be beneficial, because some of the new neurons appear to migrate to abnormal locations and disrupt the normal circuitry of the hippocampus [34, 35].

A role for NPY in dentate gyrus neurogenesis has only recently been identified. Initial studies hinted at such a role by showing that NPY was implicated in neurogenesis in the olfactory bulb [36]. This led to studies in the dentate gyrus, and it has now been shown that NPY facilitates dentate gyrus neurogenesis also. These studies have identified that Y1 receptors are present on the progenitors of the dentate gyrus that are located in the SGZ. This may explain part of the role of the Y1 receptor. It also suggests a potential "division of labor" among NPY receptors: Y2 and Y5 may primarily influence synaptic transmission, whereas Y1 may be dedicated to other types of functions, such as neurogenesis. The actions of Y1 receptors on the regulation of calcium entry may actually work in part to modulate neurogenesis and proliferation indirectly, since it is likely that intracellular calcium will influence the cell cycle and associated events [37, 38].

NPY protein and receptor expression after seizures

It was first shown in the 1990s that NPY expression in the rat dentate gyrus dramatically increases after seizures. Subsequent studies from many different laboratories showed the reproducibility and robust nature of these changes [39–41], and the upregulation of NPY in the dentate gyrus has become an accepted marker of seizure activity.

One of the reasons why NPY expression after seizures has been studied so much is that it changes in a very interesting and yet robust manner, depending on the degree and duration of seizures. After acute seizures (lasting minutes), there is an elevation of expression in many non-granule cells, particularly those in the hilus and granule cell layer (Fig. 5). Acute studies have used, for example, pentylenetetrazol-induced seizures [42], electroconvulsive shock [43], and kindled seizures [44]. After more severe seizures, such as status epilepticus following electrical stimulation, kainic acid or pilocarpine admin-

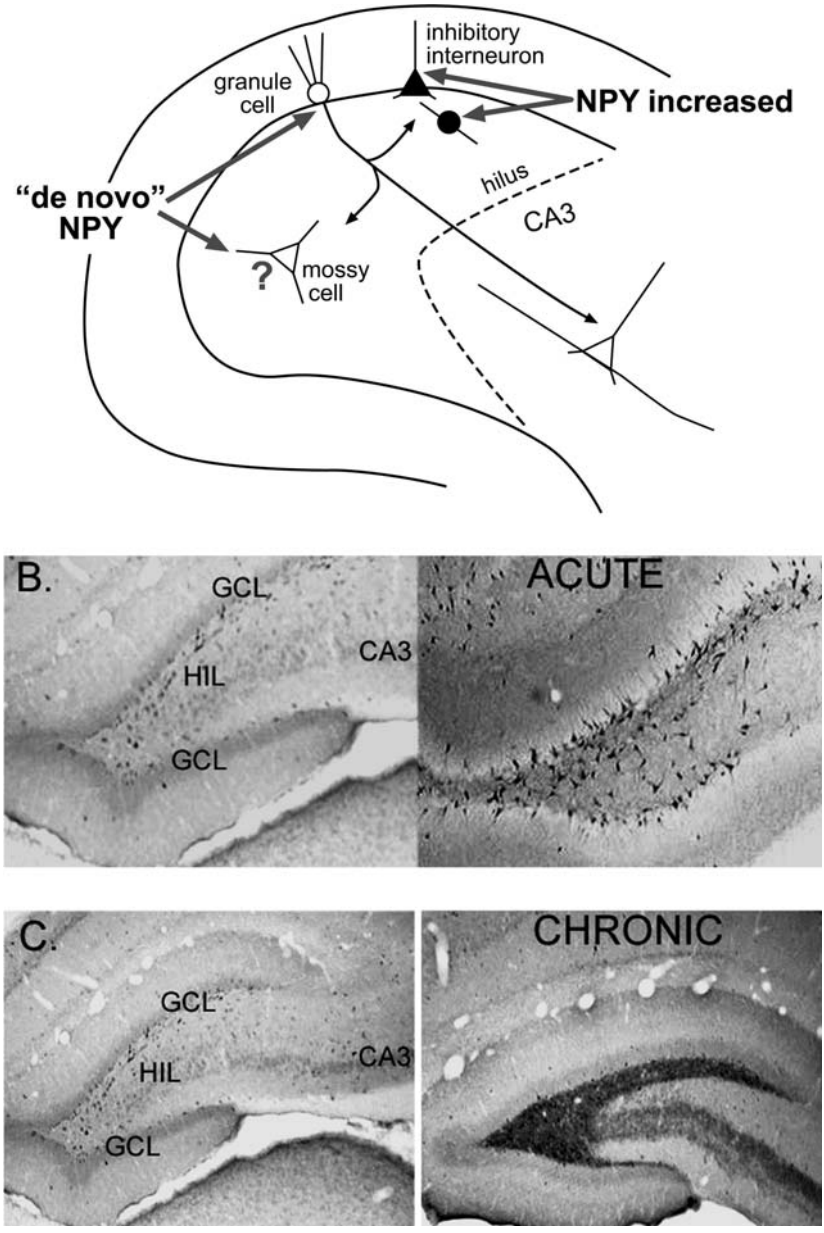


Figure 5. Changes in NPY expression after seizures. A. A diagram is used to illustrate where NPY protein increases in the adult rat dentate gyrus after seizures. It appears to increase in inhibitory neurons, and also develop in neurons such as granule cells and mossy cells that do not normally express the protein (*de novo* expression). B. The increase in NPY protein in the dentate gyrus is shown using an antibody to NPY. Left: Saline control. Right: 1 day after status epilepticus. C. Increased NPY in mossy fibers is illustrated after chronic seizures. Left: Saline control. Right: 2 months after status epilepticus. Spontaneous seizures were observed in the animal that had status epilepticus.

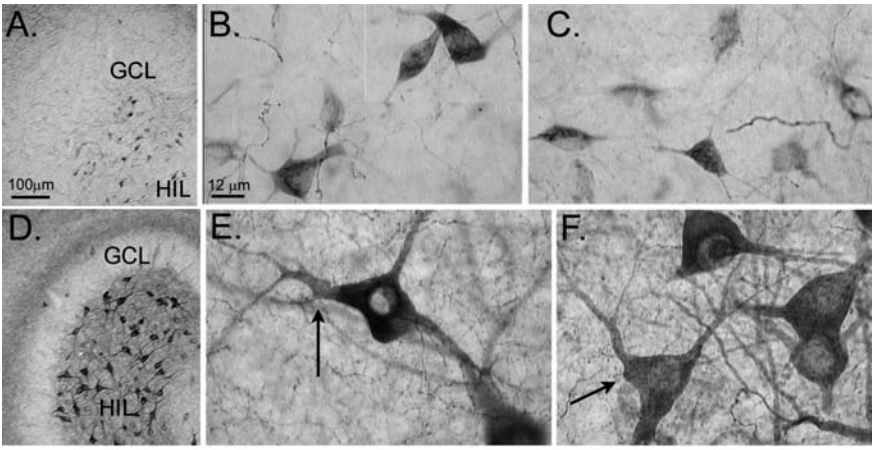


Figure 6. *De novo* expression of NPY in hilar cells after seizures. NPY immunoreactivity in the dentate gyrus of a pilocarpine-treated rat that had no behavioral seizures (A-C) compared to a pilocarpine-treated rat that had 1 h of status epilepticus and subsequently had spontaneous seizures (D-F). Both animals were killed 2 months after pilocarpine treatment. A-C. The normal pattern of NPY immunoreactivity includes NPY expression in many hilar cells and fibers, as well as fibers in the outer molecular layer (A). B and C show NPY-immunoreactive hilar cells at higher magnification. GCL = granule cell layer; HIL = hilus. D-F. NPY immunoreactivity in the epileptic rat shows increased immunoreactivity in hilar cells and in fibers (D; same magnification as A). In addition, a novel band of staining in the inner molecular layer is present, a reflection of mossy fiber sprouting. E and F show higher magnification of hilar cells that are NPY-immunoreactive. Note the large size of these cells and irregular, large primary dendrites (arrows) relative to the normal NPY-immunoreactive cells shown in E and F (magnification is the same in B, C, E, F). It is assumed from these changes in immunoreactivity that the cells in the normal hilus die due to seizure-induced neuronal death, and the residual surviving cells, including some mossy cells, develop NPY immunoreactivity.

istration [39], this acute upregulation also occurs, but in addition some of the NPY cells die due to excitotoxicity [45].

After status epilepticus, it appears that NPY expression can also develop in some of the hilar neurons that normally do not express the protein (Fig. 6). This can be appreciated by comparing the immunoreactivity of sections from animals that had status and those that did not (Fig. 6). The normal, small hilar cells that are NPY-immunoreactive appear to be lost after status, but the hilus does not appear devoid of cells as a result. Instead, other cells that are quite large appear to express NPY. These large NPY-immunoreactive cells are not apparent in the normal tissue, so it is likely that they have developed *de novo* expression. They appear to be either large GABAergic neurons or the glutamatergic mossy cells. The latter is surprising, because mossy cells are not thought to express NPY normally or after seizures. However, this may be due to the fact that most periods of status epilepticus induce substantial mossy cell loss. We have found that if status epilepticus is abbreviated by diazepam administration after 1 h, mossy cells can survive [46]. When our tissue is examined for NPY expression, cells with the morphology of mossy cells are

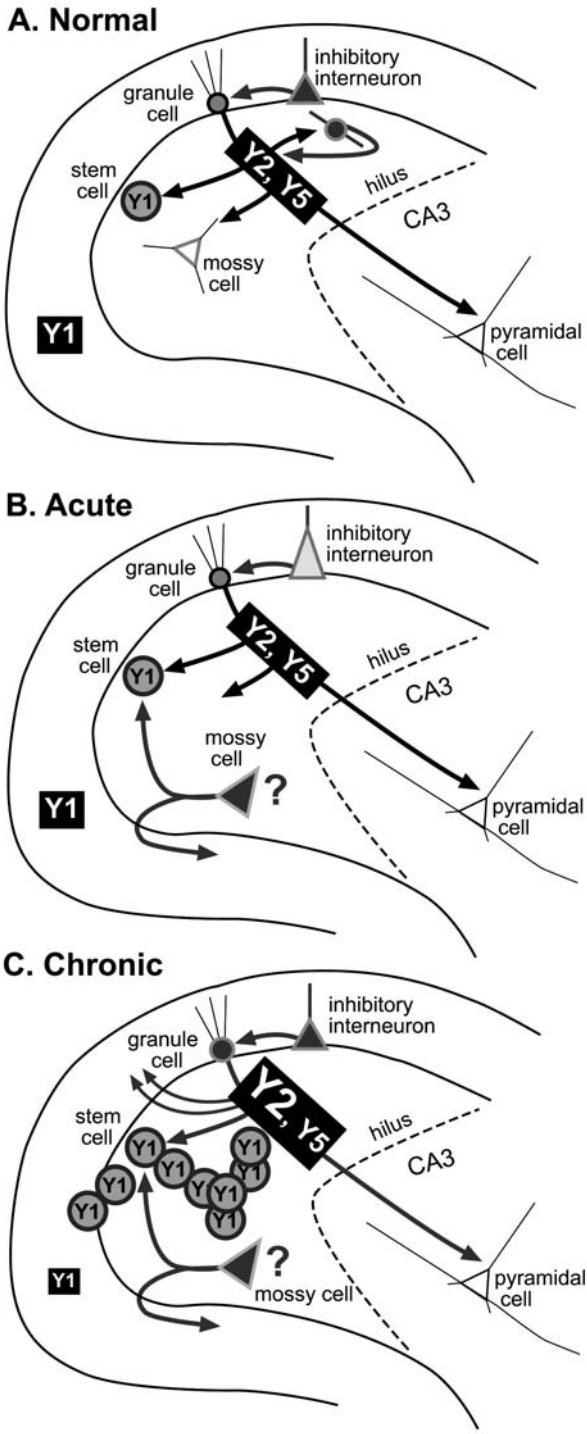
immunoreactive (Fig. 6). Thus, they are large, multipolar, and have large-diameter primary dendrites. Furthermore, the area where the primary dendrites join the soma is uneven or "ruffled", rather than smooth. One would expect this characteristic of mossy cells, which have complexes of large spines (thorny excrescences) on their primary dendrites, especially at the junction with the cell body. Other hilar neurons do not have thorny excrescences, and the initial portion of their primary dendrites is relatively smooth.

When seizures occur chronically, for example in animals that have status epilepticus, and then are examined months later, NPY expression is also abnormal, but the changes are distinct from the pattern observed after acute seizures (Fig. 5C) [46–48]. There continues to be an increase in NPY expression in non-granule cells, but in addition, NPY is apparent in the granule cells and their axons [42, 49]. NPY immunolabeling becomes distributed throughout the mossy fiber axon plexus. Another change that occurs in many GABAergic neurons that survive seizures is sprouting of their axons [50]. This sprouting also appears to occur for the NPY-immunoreactive GABAergic neurons that survive seizures [51]. It may allow compensation for the loss of some of the original NPY-containing neurons.

Another interesting change in expression of NPY after seizures relates to the presence of NPY in mossy fibers, and the fact that many chronically seizing animals develop collateralization of mossy fibers into the inner molecular layer. This "mossy fiber sprouting" may serve to increase recurrent excitation, because the new collaterals innervate granule cells [52–55]. But the new collaterals also innervate GABAergic neurons, which would potentially negate any increase in recurrent excitation [56, 57]. What is significant for the present discussion is that the parent and sprouted mossy fibers are indistinguishable in NPY immunoreactivity; the new fibers appear identical in NPY expression as the parent axons. Thus, epileptic animals demonstrate a novel band of NPY immunoreactive fibers in the inner molecular layer that is not present in normal rats (see Fig. 6).

There are substantial changes in NPY receptor expression after seizures (Fig. 3B), although no evidence of a distinct pattern of expression, as appears to be the case for NPY protein. After acute and chronic seizures, Y2 receptors increase expression in mossy fibers, but do not appear to change elsewhere [57]. The data for Y5 receptors are less clear [59], but it appears that they also change in mossy fibers [60]. Regarding Y1 receptors, it appears that molecular layer expression of Y1 receptors diminishes after seizures [61].

In summary, acute seizures increase NPY expression of many non-granule cells. There also may be cell death of NPY-expressing neurons, especially if seizure activity is severe. After chronic seizures, additional NPY expression develops in the mossy fiber axons of dentate granule cells, and possibly surviving mossy cells. Regarding receptors, Y2/Y5 receptors mainly increase and Y1 receptors appear to decrease. These changes are summarized in Figure 7.


A. Normal
inhibitory interneuron
granule cell
stem cell
Y1
Y2, Y5
hilus
CA3
mossy cell
Y1
pyramidal cell
B. Acute
inhibitory interneuron
granule cell
stem cell
Y1
Y2, Y5
hilus
CA3
mossy cell
?
Y1
pyramidal cell
C. Chronic
inhibitory interneuron
granule cell
stem cell
Y1
Y2, Y5
hilus
CA3
mossy cell
?
Y1
pyramidal cell

Functional implications of seizure-induced changes in NPY and its receptors

Synaptic transmission

Why might the changes in NPY, and its receptors, occur in the epileptic brain? The increased expression in non-granule cells, as well as mossy fiber NPY expression, is an inherent compensatory reaction that serves to decrease the possibility of subsequent seizure activity. This follows logically from studies showing that NPY depresses mossy fiber transmission. This would blunt any ability of seizures to propagate through the major pathway that seizures take through the hippocampus, the trisynaptic circuit. Depression of excitatory mossy fiber transmission might also be neuroprotective because the transmitter is glutamate, and the targets of mossy fibers (hilar cells, pyramidal cells) are quite vulnerable to seizure-induced damage [51, 62].

Consistent with the hypothesis that the changes in NPY protein after seizures may suppress further seizures, there is an increase in the receptors that mediate actions of NPY to depress synaptic transmission of the mossy fibers. Taken together, the increased mossy fiber NPY, coupled with the increased Y2/Y5 receptors, should serve to dampen excitability very effectively in the epileptic rat dentate gyrus.

However, the anticonvulsant and neuroprotective effects discussed above assume that NPY receptor-mediated actions are equivalent in the normal and epileptic brain. One cannot necessarily assume they are identical, and in fact there appears to be little effect of NPY on perforant path-evoked transmission to granule cells in normal rat brain [9, 10], but a potent inhibitory effect in epileptic human brain [63].

There are at least a few other reasons to suspect that the increase in NPY and NPY receptors after seizures may not necessarily have the same effects on synaptic transmission as it does in the normal brain. One of the factors that could be complicating is that other peptides and receptors of the mossy fibers change after seizures. They may interact with NPY in a way that does not occur normally. Metabotropic glutamate receptors are a prime candidate,

Figure 7. Summary of NPY plasticity in the rat dentate gyrus after seizures. A summary of the changes that occur in protein and receptor expression after acute seizures and further changes after chronic seizures. A. Normal condition B. After acute seizures, NPY protein increases in inhibitory neurons, although some may also be lost due to seizure-induced neuronal death, and some may sprout collaterals, making the new NPY interneurons network potentially novel. In addition, some hilar cells that do not normally express NPY protein may begin to do so, such as surviving mossy cells. C. After chronic seizures, granule cells and their axons, the mossy fibers, express NPY and sprout into the inner molecular layer. Y2, and possibly Y5 receptors, increase in mossy fibers, and Y1 receptors in the molecular layer appear to decrease. In addition, seizures increase the proliferation of granule cells from progenitors in the subgranular zone which express the Y1 receptor; this is likely to occur during a window between day 3–4 and 30 after status epilepticus, at least in the case of pilocarpine-induced status epilepticus [32, 34]. This coincides with the period when spontaneous seizures are beginning to occur, suggesting a role for seizure-induced neurogenesis in epileptogenesis [33].

because they normally modulate mossy fiber transmission, these receptors change after seizures, and they influence NPY [64]. Another complication is that the expression of GABA increases in mossy fibers after seizures [65]. It appears that this pool of GABA can be released and inhibit the target cells of mossy fibers [66], although the net effect is not clear, because GABA and glutamate are released at similar times from mossy fiber boutons. Nevertheless, if NPY depresses mossy fiber transmission, it may not only depress glutamatergic transmission (as has been assumed) but also GABAergic components of mossy fiber transmission. Indeed, in thalamus, NPY does depress GABA release [67]. If this is the case, NPY might actually have a partially disinhibitory effect by blocking the GABAergic component of mossy fiber transmission. This potential disinhibition could be greater after seizures, because under these conditions the GABAergic component of mossy fiber transmission appears to increase greatly.

Neurogenesis

One of the aspects of seizures that is perhaps as robust as the induction of NPY expression is the ability to increase neurogenesis of granule cells [33, 35]. This may not be a coincidence: it is possible that the increase in NPY mediates the increase in neurogenesis of granule cells. Thus, NPY may serve to protect the epileptic brain from further seizures by modulating synaptic transmission (see above), and at the same time contribute to its repair by promoting the genesis of new granule cells.

What is the evidence for the hypothesis that increased NPY after seizures mediates seizure-induced neurogenesis? First of all, experimental seizure models which have demonstrated that seizures increase neurogenesis are models that have also shown an increase in NPY. Second, the increase in NPY is rapid, and occurs before neurogenesis begins. Such timing would be necessary if neurogenesis is dependent on NPY. Third, NPY facilitates neuroproliferation in the olfactory bulb [36], providing a precedent.

Much more direct evidence has been obtained recently by studying the influence of NPY exposure on cultures of granule cell precursor [68]. NPY facilitated neuroproliferation, and did so at very low concentrations, similar to what would be expected to occur *in situ*. In addition, mice that lacked NPY had a lower basal level of neurogenesis [69]. Interestingly, the effect appeared to be due to the Y1 receptor, based on pharmacology [68]. In addition, cultures from mice that lack the Y1 receptor failed to demonstrate an effect of NPY on neurogenesis [70]. Furthermore, the precursors appear to be immunoreactive to a Y1 receptor antibody [68]. Thus, NPY appears to modulate neurogenesis and does so by acting at Y1 receptors.

How NPY modulates neurogenesis *in vivo* is still somewhat unclear. One question is how NPY from NPYergic neurons is made available to precursors in the SGZ. This is an issue because most of the axon projection of NPY con-

taining cells is thought to project far from the SGZ, in the outer molecular layer. Only a small number of collaterals are thought to terminate in the SGZ. If the role of NPY is to modulate neurogenesis, why would the axons not terminate entirely in the SGZ? Perhaps the answer is that Y1 receptors have two functions in the dentate gyrus, one to modulate excitability by actions of ion channels (in the molecular layer and hilar neurons) and the other to influence progenitor division (in the SGZ). The innervation of the molecular layer would allow NPY neurons to influence dendritic function, and the innervation of the SGZ would allow them to influence neurogenesis.

Another puzzle is that the Y1 receptors that appear to mediate the actions of NPY on neurogenesis exist primarily in the molecular layer, and furthermore, they decrease after seizures. This is not logical if a function of NPY is to contribute to seizure-induced neurogenesis in the SGZ. One would expect that NPY receptors, and specifically Y1 receptors, would be necessary in the SGZ. However, it is likely that the Y1 receptors are located in the SGZ, but are sparse enough not to generate a large signal, at least relative to other lamella. This is because physiological evidence indicates they are present [17]. And some Y1 binding is apparent in hilus, although it may be associated with mossy fibers [71]. And perhaps the SGZ Y1 receptors do not decrease after seizures, although those in the molecular layer do. Indeed, there was a transient increase in Y1 receptor binding in the dentate gyrus molecular layer after seizures, but this was not significant [61]. Higher resolution techniques will be needed to clarify these issues. It is important to add that it is not yet clear how NPY-containing cells would influence SGZ progenitors specifically, because synapses have not been revealed, and release onto progenitors has not yet been examined. One possibility is that there is diffusion from the processes of NPY-expressing neurons, analogous to the diffusion of dynorphin from granule cell dendrites to its target receptors in the molecular layer [72]. Although long range diffusion would be unlikely in light of the ability of proteases to cleave NPY extracellularly, there have been suggestions that NPY could potentially function over long distances nevertheless [73].

Summary

In summary, NPY is clearly an important peptide in the adult rat dentate gyrus because it has the potential to influence synaptic transmission and neurogenesis. It may even have other functions, as yet undiscovered, mediated by glia or vasculature [74]. The remarkable plasticity of NPY puts it in a position to allow dentate gyrus function to be modified in a changing environment. The importance of this plasticity in the context of epilepsy cannot be emphasized enough. It could help explain a range of observations about epilepsy that currently is poorly understood. For example, rapid increases in NPY could mediate postictal depression, the period of depression that can last for several hours after generalized seizures. It may mediate the “priming effect,” which is a

reduction in seizure threshold following an initial period of seizures [75–77]. Finally, it could contribute to the resistance of dentate granule cells to degeneration after seizures [78]. However, despite the focus in this review on seizure-induced changes, the changes described here also appear to occur after other types of manipulations [79–82], which considerably broadens the scope of NPY's role in the brain.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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NPY and extreme stress: lessons learned from posttraumatic stress disorder

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Introduction

Posttraumatic stress disorder (PTSD) is a psychiatric condition that results from exposure to an extreme traumatic stressor event or experience [1]. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [1], the traumatic stressor must meet two criteria: 1) the person experienced, witnessed, or was confronted by an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others; 2) the person's response involved intense fear, helplessness, or horror [1]. Frequently studied types of traumatic stressors include military combat [2], rape and domestic violence [3], natural disasters [4, 5] and childhood abuse [6]. PTSD symptoms are defined by three categories: 1) intrusions such as nightmares and flashbacks; 2) avoidance symptoms including both emotional numbing and behavioral[a]; and 3) hyperarousal[b]. PTSD is a relatively common disorder in the general population. The National Comorbidity Survey (NCS) showed that lifetime PTSD occurred in 7.8% of the population [7], with a current (12 month) rate of 3.85% [8].

This chapter presents a brief review of the literature concerning psychosocial and biological aspects of PTSD, and then discusses an emerging evidence for the role of NPY in this condition (Fig. 1).

Psychological aspects of PTSD

PTSD can substantially impair various aspects of both psychological and biological functioning [9]. Trauma takes a heavy toll on mental health, resulting in

[a] e.g., feeling detached, restricted range of affect or emotion (numbing) and efforts to avoid thoughts, feelings, or conversations associated with the trauma (avoidance)

[b] e.g., hypervigilance, exaggerated startle response

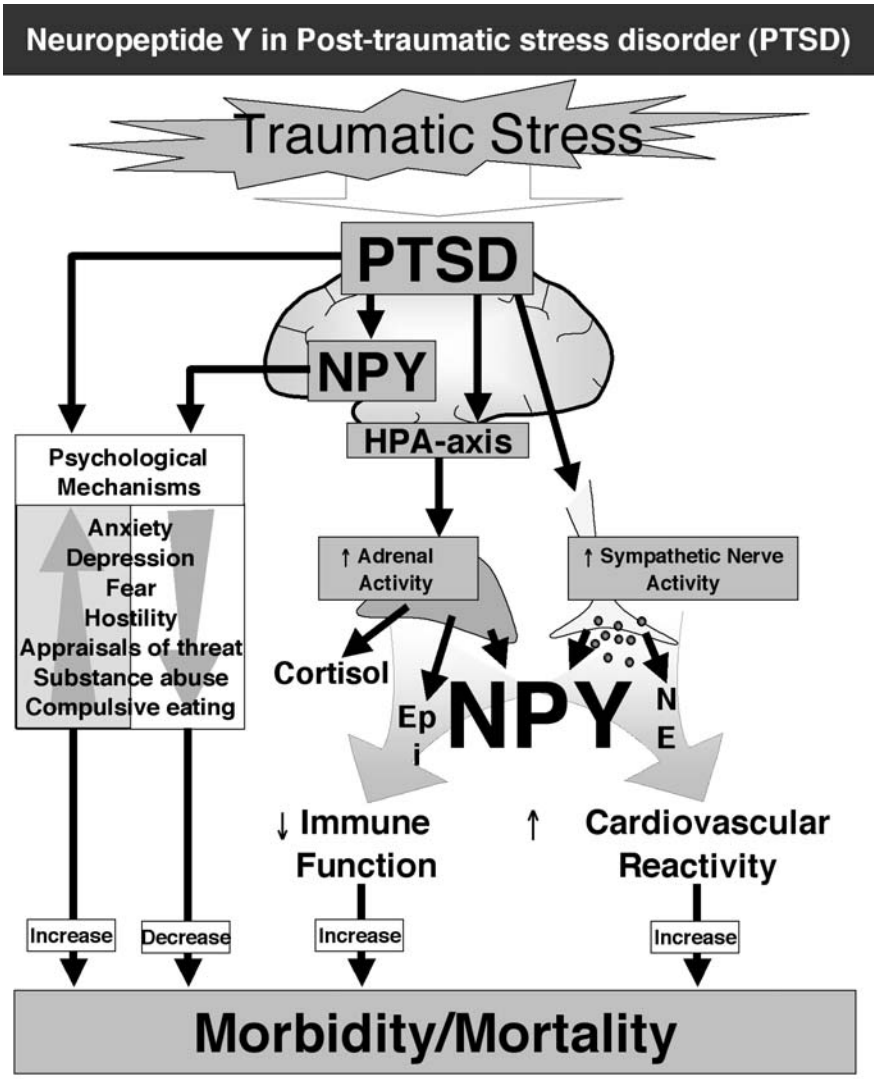

Figure 1. Interactions between central and peripheral NPY with cortisol and catecholamines in elaboration of stress-induced cardiovascular, immune and behavioral/psychological changes. Adapted from Schnurr PP, Green BL (2004) Understanding relationships among trauma, PTSD, and health outcomes. In: PP Schnurr, BL Green (eds): *Trauma and Health: Physical health consequences of exposure to extreme stress*. American Psychological Association, Washington, DC

a wide variety of mental and physical health problems. For example, the likelihood of an episode of depression is significantly increased among trauma-exposed individuals who develop PTSD, relative to those who do not [10]. Nearly all of the veterans with PTSD in the National Vietnam Veterans Readjustment Study had other lifetime disorders [11]. Breslau [10] concluded

from data based on a large sample of young adults in Michigan that these disorders do not arise from separate vulnerabilities. Yet, research has yet to address adequately the specificity of PTSD as a reaction to trauma exposure. For example, biological studies of PTSD have tended to compare individuals with PTSD to those who have experienced trauma but do not have a psychiatric disorder. Thus, findings in these studies are not necessarily specific to PTSD and may reflect outcomes for other disorders or combinations of disorders.

An increased cognitive appraisal of threat following exposure to violent traumatic experiences is also associated with PTSD. In one study of New York City workers exposed to violence in the September 11 attacks [12], greater worry about future attacks (threat appraisal) was associated with higher levels of PTSD. Increased appraisals of threat [13] are hypothesized to predict intense fear and anxiety characteristic of PTSD.

Traumatic exposure also affects the way victims think about themselves and others. In a study of victims of intimate partner violence [14], individuals with comorbid PTSD and Major Depressive Disorder had more depressogenic cognitive styles than those without PTSD. However, these maladaptive schemas did not contribute to the identification of psychiatric co-morbidity caseness. A study of journalists [15] found an association between a higher level of work-related traumatic exposure and PTSD symptoms including negative cognitive schemas. In this study, negative cognitive schemas only partially mediated the relationship between trauma exposure and PTSD. Hostility is another cognitive correlate of PTSD [16].

Finally, PTSD and related trauma disorders have been associated with high-risk health behaviors, such as substance abuse [17] and compulsive eating [18].

Biological aspects of PTSD

A growing research literature has emphasized the impact of PTSD on human pathophysiology, including its association with a number of non-psychiatric diseases. Patients with the history of PTSD have been shown to have higher rates of a heart disease, osteoarthritis, diabetes, comorbid depression, obesity, and elevated lipid levels [19]. This is likely related to significant alterations observed in the two primary neurobiological systems of the stress response pathways have been documented in patients with PTSD: the locus coeruleus/norepinephrine-sympathetic (LC/NE) system and the hypothalamic-pituitary-adrenal (HPA) axis [20].

The HPA axis

The HPA axis is one of the major endocrine components of the stress-response system [20, 21]. Chronic stressors are associated with sustained abnormalities in the HPA axis [22]. Individuals with PTSD also appear to have dysregulation

of the HPA axis, altered cortisol levels, increased number of glucocorticoid receptors and increased receptor sensitivity. Findings pertaining to increased levels of cortisol among those with PTSD have been variable, however. For example, greater elevations in evening salivary cortisol of a community sample with PTSD compared to trauma-exposed and no PTSD groups [23] were no longer found when lifetime comorbidity with Major Depressive Disorder was included in the analyses. A recent study examined the HPA response to stress in PTSD among individuals with a history of childhood sexual abuse [24] suggesting that persons with PTSD appeared to have an increased cortisol response in anticipation of a cognitive challenge relative to controls. In a recent review [25], authors suggested that an adrenal neurosteroid dehydroepiandrosterone (DHEA) serves as a mediator of HPA axis adaptation – specifically upregulation – to extreme stress and the psychiatric symptoms associated with PTSD, and thus explains some of this variability.

The sympatho-adrenomedullary system (SAS): catecholamines and neuropeptide Y (NPY)

The other arm of stress reactions is activation of the sympatho-adrenomedullary system (SAS), which release catecholamines, norepinephrine (NE) and epinephrine (EPI), and a co-transmitter/hormone, NPY. A large body of evidence confirms that stress, both acute and chronic, elevates plasma catecholamine levels in a stress-intensity-dependent manner [26]. Adrenergic activation is also known to be a major determinant of vascular tone and cardiac function, responsible for vasoconstriction, high blood pressure, and tachycardia during stress. Individuals with PTSD appear to also have altered adrenergic activity demonstrated by elevations in basal catecholamine levels and adrenergic reactivity in some individuals [27].

Evidence for the role of the non-adrenergic neurotransmitter, NPY, in PTSD is only recently emerging but its role in stress is well supported by both experimental and human studies. NPY is a 36-amino acid peptide found both in peripheral sympathetic nerves and in brain structures where it is co-localized with norepinephrine [28–30]. It is a potent vasoconstrictor [28–30]. By activating its Y1 receptors, the peptide causes a prolonged vasoconstriction and a hypertensive response, particularly in males (humans and rats), due to androgen-driven regulation of NPY expression [29, 30]. Although NPY is often co-released with NE, the regulation of its release differs from that of catecholamines. While even a mild acute stressor can elevate plasma catecholamines, more intense and prolonged stress is required to release NPY into the circulation [29, 30]. Increased plasma NPY levels may also persist longer than that of NE, which is quickly metabolized, and thus, be a better marker of chronic stress. In addition, our recent experimental data suggests that platelets may take up the peptide from the bloodstream and store it for prolonged period of time, as a kind of “memory” of stress experienced in the past [31].

Interactions of HPA axis and SAS in stress and PTSD

Each of these HPA- and SAS-derived stress mediators exerts multiple physiological and pathological consequences, some of which are synergistic, other antagonistic. For example, all three classes of mediators, catecholamines, NPY and cortisol, synergize to elevate blood pressure but have contrasting effects on the immune system [29]. Different stressors have differential effects on the release of these mediators and hence cause differential effects on the body. These varied neurohormonal cocktails often persist in both HPA and SAS as "memory" of previous stressors and set a stage for exaggerated or pathological responses to new stressors. In addition, the predominant type of the released stress mediators and their physiological effects depend also on duration and intensity of stress. For example, acute stress enhances immune functions of the organism, while chronic, physiologically exhausting stress results in immunosuppression [32]. Although the primary function of stress mediators is protection of the organism, their excessive and prolonged release can also lead to development of other stress-related conditions, such as cardiovascular diseases.

The stress mediators described above – cortisol, NE, EPI and NPY – are commonly used as markers of stress and an index of its intensity [33], with NPY indicating chronicity and severity of stress. For example, when compared with non-abused control women, women exposed to physical and psychological IPV had higher levels of evening cortisol as measured with saliva samples [34]. This relationship remained significant even when factors such as age, smoking status, pharmacological treatment, and lifetime trauma history were controlled. Importantly, biological alterations associated with chronic stress have been implicated in adverse health consequences [20, 21].

Compared to trauma-exposed individuals without PTSD, those with a current diagnosis of PTSD have been found to produce larger physiological responses, including heart rate, skin conductance, and facial electromyogram, in response to reminders of their traumatic event(s) (e.g., personalized trauma scripts) as well as standardized aversive stimuli (e.g., startling tones, distressing pictures) [35]. This heightened responsiveness has been demonstrated among individuals with PTSD resulting from a wide range of traumatic events, including combat veterans [36–38], women with a history of childhood sexual abuse [39], female veterans witnessing injury or death while serving as a military nurse [40], and motor vehicle accident survivors [41]. In fact, a number of these studies have demonstrated that psychophysiological responsiveness accurately classifies 60% to 90% (sensitivity) of individuals who meet DSM criteria (see Posttraumatic Stress Disorder, Definition and Prevalence above) for current PTSD and 80–100% (specificity) of those who never had PTSD [35]. In addition to elevated reactivity, PTSD has been implicated in heightened tonic/baseline levels of psychophysiological activity. In their meta-analysis of 34 studies that measured baseline or ambulatory cardiovascular activity, Buckley and Kaloupek [42] found that relative to individuals without PTSD, those diagnosed with PTSD have elevated resting heart rate and diastolic blood

pressure levels. They also found evidence to suggest that basal heart rate may be highest among individuals with chronic, as compared to acute PTSD, indicating that elevated psychophysiological activity may result from cardiovascular adaptation to repeated stress responses over many years [35].

PTSD and NPY

NPY is of particular interest in the study of PTSD for two reasons. As a sympathetic neurotransmitter and a stress mediator, it can mediate some of the biological symptoms of PTSD, and be a marker of chronic and extreme stress, as described above. However, NPY, derived from the brain, can play an opposite role – anxiolytic and enhancing stress resilience in humans. Experimental data clearly show that *central* NPY is an *anti*-stress system, although it uses the same type of NPY receptors, the Y1, which in the *periphery* are *pro*-stress and vasoconstrictive [43] (see chapter by Thorsell et al. in this book). Unfortunately, there are limitations on how to study the central NPY system since plasma peptide levels may not reflect changes in activity of the brain NPY-ergic neurons.

In humans, plasma NPY levels were shown to increase in response to standard stress tests such as cold pressor test and treadmill exercise [30, 44]. Exercise-induced NPY increases in plasma were also augmented by hypoxia [45] and by an α_2 antagonist, yohimbine [46] suggesting inhibitory influence of the presynaptic adrenergic system. The reciprocal interaction of NPY on NE release also exists, as shown in rats overexpressing NPY gene which have reduced plasma catecholamine and pressor responses to stress [47]. Interestingly, these rats also have insensitivity to anxiogenic stress, supporting major role central NPY plays in anxiolysis [43].

Few studies have examined peripheral plasma NPY levels in relation to naturally occurring stress in humans and most of these are by a single group of investigators. To test the hypothesis that NPY may buffer the effects of stress, Morgan and colleagues [48] compared plasma NPY levels in active duty male soldiers at baseline *versus* 24 h following survival training in one group and baseline *versus* during exposure to a military interrogation in a second group. Increases in NPY were associated with a robust increases in both salivary and serum cortisol. NPY was significantly increased by the acute stress of military interrogation, as previously shown in laboratory stress tests. Interestingly, plasma NPY levels were significantly reduced in a subset of individuals (non-Special Forces) 24 h after the cessation of stress. The investigators suggest that this reduction of NPY may illustrate the effect of prolonged exposure to high-intensity stress in these individuals and reflect mal-adaptation. Interestingly, this study found that "stress hardy" individuals (i.e., Special Forces) had higher NPY levels immediately after stress exposure and that their NPY levels had returned to baseline within 24 h compared to non-Special Force soldiers. This suggests individual differences in ability to upregulate NPY, suggesting the

importance of examining genetic factors and prior history of trauma exposure as potentially important in understanding the role of NPY in stress reactions. Further, it is worth noting that the stressor experience involved primarily acute psychological, rather than physical, stress, thus pointing to the important role of threat appraisal for understanding the human stress response. Finally, higher levels of NPY were associated with lower levels of psychological symptoms of dissociation during stress. These data were replicated with a sample of soldiers in the US Navy [33] suggesting that NPY, presumably derived from the brain but reflected in the plasma levels, played an anti-stress, anxiolytic effect in "hardy" individuals.

One of the first studies to examine the relationship between NPY and PTSD [49] found that combat veterans who met diagnostic criteria for PTSD had lower baseline plasma NPY levels and blunted yohimbine-stimulated increases in plasma NPY compared to healthy controls. This study was designed to investigate the possibility that NPY may contribute to sympathetic system hyperreactivity in PTSD, including elevation in heart rate, blood pressure, and plasma catecholamines in response to stimuli associated with prior traumatic events. Interestingly, there was no effect of depression on plasma NPY level within the PTSD group. However, in another study of depressed patients [45] without PTSD, platelet-poor- plasma levels of NPY were also decreased although they were actually higher in platelet fraction of plasma. This intriguing observation may suggest that in humans like in rodents [29, 30], NPY may be taken up from the blood stream, at sites of increased NPY release, and buffer the changes in NPY activity over a longer period of time, i.e., the life cycle of the platelets. Thus, platelet NPY in addition to its plasma levels may be a better marker of chronic stress.

The finding of a dose-response in terms of the negative relationship between level of traumatic exposure and baseline plasma NPY levels suggest that stress-induced alterations in NPY may be one biologic mechanism that contributes to the development of PTSD. Results also showed that stress-induced elevations of NPY were associated with elevations in systolic blood pressure in individuals with PTSD, consistent with peptide's role in the cardiovascular system [29, 30]. The authors suggest that this may result in more persistent ischemic risk to the heart and brain and thus account for increased rates of stroke and other circulatory diseases in persons with trauma histories.

While virtually all previous studies have involved soldiers, one recent study focused on a small sample of female victims of intimate partner violence (IPV) with and without lifetime/current PTSD and healthy, non-abused controls [50]. Results found no significant differences in NPY levels with and without PTSD, although mean cortisol levels were lower in those with IPV exposure compared to controls. Authors suggest that NPY and cortisol may be markers of exposure to stress, rather than PTSD *per se*.

Indeed, one of the most recent studies of NPY and PTSD tested the hypothesis that baseline plasma NPY and NPY response to yohimbine might be associated with trauma exposure rather than PTSD [33]. Results showed that plas-

ma NPY was negatively associated with trauma exposure, but not PTSD. Baseline plasma NPY levels were lower for combat veterans both with and without PTSD, compared to non-traumatized individuals. Further, a dose effect was found in that the greater number of life-threatening traumatic events was negatively associated with baseline plasma NPY levels. This study left unanswered the question of whether a stress-induced release or baseline levels of NPY play a role in the maladaptive responses to stress.

Conclusions

NPY is a mediator and a marker of chronic stress in humans, including extreme trauma and PTSD, but its actions are complex. In the periphery, as a sympathetic neurotransmitter, it exerts excitatory effects on the cardiovascular system and modulates immune responses. In contrast, in the central nervous system, NPY-ergic neurons are powerful inhibitory, *anti*-stress and anxiolytic system. In PTSD, stress-induced elevations of plasma NPY are associated with increases in cortisol, catecholamines and blood pressure, consistent with peptide's actions in the cardiovascular system. However, *lower* plasma NPY levels, baseline, stress-induced, and/or post-stress are associated with *poorer* behavioral performance under stress – suggesting that reduced activity of the central NPY system diminishes individual's stress resilience. Many of the peripheral *pro*-stress and central *anti*-stress actions of NPY appears to be mediated by the same subtype of receptors, the Y1, which complicates the case of using Y1 antagonists for treatment of stress-related cardiovascular and immune symptoms, unless drugs which do not penetrate the blood–brain-barrier are used. Future studies should determine more precise mechanisms of NPY's actions and type of receptors involved in psychological, behavioral, cardiovascular and immune consequences of chronic stress, particularly PTSD, where peptide's role appears to be quite compelling.

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NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
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NPY and chronic neurodegenerative disease

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Introduction

The role of NPY in chronic neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson disease (PD) and Huntington's disease (HD) has not been well defined. After a flurry of initial studies on NPY levels in brain sections and in cerebral spinal fluid (CSF) from individuals with AD in the 1980s, the research into NPY and its involvement in chronic neurodegeneration has slowed. However, the intense expression of NPY and NPY receptors in those central nervous system (CNS) regions associated with AD or PD and the potential importance of NPY as a neuromodulator of synaptic events suggest that NPY pathophysiology may be an under explored, but basic component of the degenerative disease process. In this review, we will examine the specific expression patterns of NPY and NPY receptors in those regions of the CNS that are closely related to the main pathological outcomes of neurodegenerative diseases. We will summarize the current knowledge on changes in NPY in AD, PD and HD and examine potential roles for NPY in the pathophysiology associated with chronic neurodegenerative disease.

NPY in the CNS

NPY is one of the most common peptides in the central nervous system (CNS) [1, 2] and is widely distributed throughout the brain where it serves as a neurotransmitter and/or a modulator of neuronal function. The functional outcomes which are regulated by NPY include food and water intake, blood pressure, the innate immune response to infection, motor activity, learning and memory and the emotional and physical response to stress [3–9]. As predicted by these functional outcomes, NPY is readily observed in the cortex, hippocampus, amgydala, basal ganglia and the hypothalamus [10–15]. In this review, however, we will focus on those CNS areas of particular importance to chronic neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). For AD, although multiple brain regions are ultimately affected by the neurodegenerative

process, the earliest stages (Braak stages I, II) are confined to the transentorhinal region of the cortex [16, 17]. Neural degeneration then spreads to the adjacent limbic regions which include the hippocampus (Braak stages III, IV) and eventually extend into association areas of the neocortex (Braak stages V, VI). By stage IV, significant damage to the hippocampal CA1 region is observed and is commonly associated with memory loss and dementia [16, 18]. For neurodegenerative diseases like PD and HD that are associated with movement disorders as well as cognitive changes, primary neuropathological lesions are observed in the striatum and substantia nigra. Loss or dysregulation of NPY in the hippocampus or striatum, thus, may directly impact the chronic neurodegenerative disease processes observed in AD, PD and/or HD.

NPY in the hippocampus and entorhinal cortex

A number of studies have examined the distribution of NPY containing neurons in the hippocampus using *in situ* hybridization to detect NPY mRNA and immunocytochemistry to detect NPY protein [14, 19–23]. In general, the levels of mRNA expression for NPY parallel the level of NPY protein immunoreactivity [24]. As depicted in Figure 1A, NPY expression in the hippocampus is found within the soma and processes of interneurons and is distributed in a relatively regional specific manner. The greatest density of NPY-immunoreactive (NPY+) neurons in the normal adult hippocampus is observed in the hilus of the dentate gyrus and in the stratum oriens of the CA1 and CA2 region. Other regions such as the molecular layer of the dentate also demonstrate strong immunoreactivity for NPY in both cell bodies and in fibers. Adjacent cortical areas such as the subiculum and the entorhinal cortex demonstrate a low density (approximately 1–2% of the total cells) of NPY neurons. This value is typical of most regions in the cortex [14, 25, 26]. Similar staining patterns are observed in rodent (mouse and rat), primate and human brain, although scattered regional differences in the distribution and density of NPY neurons and NPY receptors between primate and rodent brain are observed [13, 27, 28].

At least four different subtypes of NPY+ interneurons have been described and are differentiated by morphology and by their co-transmitter profile [14, 19, 20, 29]. NPY containing interneurons in the hippocampus (and throughout the CNS) express multiple neurotransmitter/neuromodulator substances including GABA, other peptidergic neurotransmitters such as somatostatin (also known as somatotropin release inhibitory factor, SRIF), nitric oxide synthase (the enzyme that generates NO) and various calcium binding proteins such as calbindin [3, 30]. The pattern of co-transmitter expression or other characteristics of NPY interneuron subpopulations for each hippocampal region has not been fully characterized. It is clear, however, that specific subtypes of NPY neurons are more or less vulnerable to pathological insults. For example, a subpopulation of NPY neurons in the central hilar region is lost with age or with chemical lesion of the cholinergic afferent innervation using

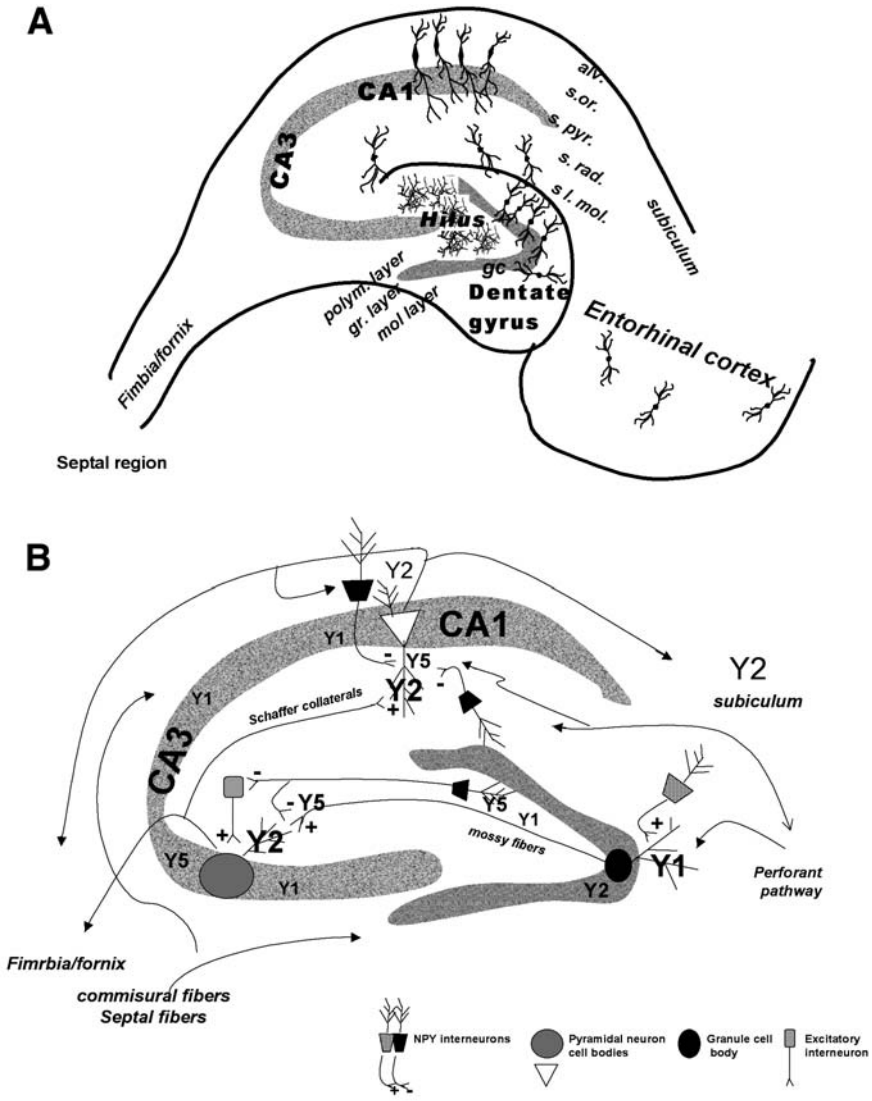

Figure 1. Location of NPY interneurons and NPY receptors in the hippocampus and entorhinal cortex. A: NPY interneurons are located throughout the hippocampus and entorhinal cortex but are most dense in the hilar and molecular layer of the dentate gyrus and in the stratum oriens (s.or) layer of the CA1 and CA3 regions. B. Interconnections between NPY interneurons and afferent or efferent pathways. Also shown are the locations of Y1, Y2 and Y5 NPY receptors. The larger type reflects the larger density of the receptor compared to other NPY receptors. Abbreviations: alv = alveus; s. or = stratum oriens; s. pyr = stratum pyramidale; s. rad. = stratum radiate; s.l. mol = stratum lacunousum moleculare; gc = granule cells; polym = polymorphic; gr = granule; mol = molecular

192 IgG saporin, a specific cholinergic neuron toxin [19]. Other subtypes of NPY immunoreactive neurons are apparently resistant to lesioning and to dis-

ease processes in the hippocampus [31]. Differences in experimental outcomes that are observed throughout the NPY literature may be due to the distinct differences between individual NPY subpopulations. This may explain why studies, using similar lesioning of the septohippocampal cholinergic afferents to the hippocampus, have not observed changes in hippocampal NPY levels as determined by radioimmunoassay on hippocampal lysates [32].

For NPY interneuons in the dentate gyrus, afferent input arises from three main sources; the perforant path from the entorhinal cortex, commissural afferents via the fimbria/fornix and mossy fiber collaterals [20]. In turn, the NPY interneurons located in the molecular layer of the dentate gyrus form extensive synaptic contacts with the granule cell dentritic trees in the molecular layer and with the soma of granule cells (Fig. 1B). NPY interneurons located in the polymorphic layer of the dentate gyrus make synaptic contacts with axon terminals of mossy fibers emanating from the granular cells. They also form synapses with the soma and dendrites of CA3 pyramidal cells and with excitatory interneurons located in the hilar/CA3 region. As predicted, receptors for NPY are found in the corresponding synaptic regions (Fig. 1B). Five NPY receptors have been identified by cloning to date (Y1, Y2, Y4, Y5 and Y6). Of these, the Y1 receptor is the most common receptor expressed by the granule cell and is located on dendrites in the molecular layer. Y5 receptors, and to a lesser extent, Y2 receptors, have also been observed in the granule cell layer [33–35]. The Y2 receptor is predominantly found in the dense fiber network associated with the mossy fiber-pyramidal cell dentritic synapses and on the cell bodies of the pyramidal neurons. Y5 receptors are also widely expressed in the CA3 region while Y1 receptors appear to be more sparsely represented and are more localized to cell bodies in that region [34–36]. Wolak et al. [35] have reported frequent co-expression of Y1 and Y5 receptors within the same neuron in the pyramidal cell layer of CA1-CA3.

In the CA1 region of the hippocampus, NPY interneurons in the stratum lacunosum-moleculare (s.l-mol.) receive input from the entorhinal cortex and collaterals from commissural and other afferent paths while interneurons in the stratum oriens receive input from numerous collaterals arising from both efferent and afferent neurons passing through the alveus and stratum oriens (Fig. 1B). The synapse between the Schaffer collateral axon and the pyramidal neuron apical dendrite is a primary target of NPY interneurons in this region. Numerous NPY-immunoreactive dense core vesicles are localized to axon terminals at this synapse [29]. In addition, the NPY immunoreactive axon terminals impinge upon unlabeled axons, providing the anatomical basis for a presynaptic action of NPY in this region. Strong electrophysiological data further support the idea that NPY released from interneurons acts to alter pre-synaptic release of transmitter at its target cell [37, 38]. The distribution and subtype of NPY receptors in CA1 are similar to those observed in CA3. Y2 and Y5 receptors are expressed in the fiber network formed by the Schaffer collateral axon terminals and the pyramidal cell dendrites [7, 35, 36, 39]. Immunoreactivity for Y1 receptors is generally low in this region of the hippocampus [35].

NPY in memory and learning

The expression of NPY in interneurons and the localization of NPY-immunoreactive cells and NPY receptors in the entorhinal cortex and hippocampus suggest that NPY may regulate hippocampal function. However, unlike seizures where a direct role for NPY has been observed [22, 23, 36, 40], the involvement of NPY in hippocampal-based learning and memory processes has been suggested, but not fully substantiated. Behavioral studies on rodents support a role for NPY in memory. Early experiments by Flood et al. [41, 42] demonstrated that injection of NPY into the 3rd ventricle or the rostral hippocampus of mice promoted retention of memories. In addition to enhanced performance on learning and memory behavioral assays, NPY also blocked the amnesic effect of a protein synthesis inhibitor. Furthermore, NPY mRNA expression in the hippocampus of adult rats was increased in association with the induction of long term potentiation (LTP) in CA3 pyramidal neurons [43]. The strongest data supporting a role for NPY in memory and learning is derived from studies on Y2 receptor knockout mice. NPY $Y2^{-/-}$ mice were tested using an object recognition test which assesses the differential exploration of familiar or novel objects. Normal mice recognize objects placed into their environment and retain memory of those objects [44, 45]. However, NPY Y2 knockout mice were unable to retain the memory of objects for the same duration of time as control mice, indicating that genetic deletion of the Y2 receptor altered memory [45]. These data clearly support the initial experiments by Flood showing enhanced memory retention with NPY injection and suggest that NPY and interneuron function may be intimately involved in memory processes.

A number of other experiments have provided conflicting results. For example, studies using cholinergic lesioning have questioned the role of NPY in memory. It is well known that lesions to the basal forebrain/septal cholinergic neurons induced either experimentally or by diseases such as AD result in impairment to memory processes. These experiments demonstrate that the cholinergic input to the hippocampus is important for memory formation and retention [46–48]. As mentioned above, NPY levels in the hippocampus were not changed from the contralateral sham lesioned controls when the basal forebrain cholinergic neurons were destroyed by placing the immunotoxin, 192-IgG-saporin in the lateral ventricle [49]. Both cortical and hippocampal choline acetyltransferase (ChAT) levels were significantly reduced, however, indicating loss of cholinergic neurons. In addition, significant impairment in Morris water maze performance was observed and demonstrated that learning and memory was severely impaired in the IgG-saporin-lesioned rats. Although alternative explanations exist for the above data, dissociation between NPY levels and learning and memory is further supported by studies on aged rats. Cadiacio et al. [19] have demonstrated that hippocampal NPY interneurons in the hilus of the dentate gyrus decreased in number with age but these changes were not correlated with learning index scores. These data suggest that, at least

this subpopulation of NPY interneurons, may not be directly associated with the cholinergic control of cognition.

Tests for memory acquisition also do not show a significant difference between NPY $Y2^{-/-}$ and control mice. In this case, mice were placed into a Morris water maze with a hidden fixed platform followed by a probe trial with the platform removed. The fixed platform section of the Morris water maze tests the ability of the mouse to move toward and to find a hidden platform on repeated trials. This test is used to detect changes in working memory and is trial dependent [50, 51]. Knockout mice were not significantly different from the control mice in the hidden platform task, indicating that acquisition and spatial reference memory that is used to detect the location of the hidden platform is unaffected by the loss of the Y2 receptor [45]. However, the probe trial which tests the spatial accuracy of the mouse when searching for a platform after its removal resulted in a significant difference between the NPY Y2 knockout and control mice. Thus rather than a direct role, NPY's effects on memory and learning may be more subtle. Overexpression of NPY in rats also have complex effects on memory. In this case, young rats demonstrated a reduction in both acquisition and spatial memory while NPY transgenic mice aged to 52 weeks did not show any defects in learning and memory [8, 52]. Interestingly, both ages of the NPY-overexpressing rat showed a decreased response to stress, indicating that the anxiolytic effect of NPY is independent of effects on learning and memory [52].

NPY in Alzheimer's disease

With over 4,000,000 individuals affected currently in the United States, Alzheimer's Disease (AD) is the leading cause of dementia and a leading cause of death in the aging population [53]. Over a typical 10–20 year course, AD is characterized by progressive memory loss and the eventual death of the patient [18, 54]. The associated dementia caused by damage to key hippocampal and cortical regions of the brain has been linked to the formation of neurofibrillary tangles (NFTs) and senile (amyloid) plaques. NFTs are intraneuronal accumulations of insoluble paired helical filaments of tau, a microtubule-associated protein that helps to stabilize the cytoskeleton. Extracellular amyloid plaques are composed of fibrillar deposits of Aβ peptides, the proteolytic cleavage products of amyloid precursor protein (APP), dystrophic neurites and activated glia. The formation of tangled neurons in the entorhinal cortex and the perforant path of the hippocampus is significantly associated with the cognitive decline of individuals with AD [16, 55].

Numerous biochemical mechanisms have been proposed to explain the changes in neuronal function that lead to the deficits in memory observed in AD. Clearly, physiological mechanisms or cytoactive factors that affect learning and memory are of great interest. From the conflicting data presented above on NPY's potential roles in learning and memory, it is difficult to pre-

dict if alteration of NPY is a factor in the hippocampal damage observed in Alzheimer's disease (AD). Opposing data and viewpoints are prevalent. A number of studies have assessed the level of NPY in cerebral spinal fluid (CSF) as a surrogate marker of NPY levels within the brain. These experiments have used antibodies to screen CSF samples from healthy aged match control individuals and individuals diagnosed with AD. The results from these studies show that CSF NPY levels were significantly reduced [56, 57] or remained unchanged [58–60]. The large variation observed in these experimental studies hinders a clear interpretation of the results. Part of the problem is the small number of individuals in each study. In addition, CSF proteolysis of peptides such as NPY may have also contributed to the varied results observed in the analysis of CSF [61]. The large experimental variation has reduced the potential usefulness of CSF levels of NPY or other neuropeptides as a diagnostic indicator of AD.

Immunocytochemistry on brain sections has provided a more direct approach to examine changes in NPY in AD. Chan-Palay et al. [31, 62] have characterized NPY immunoreactive neurons and fiber networks in selected regions of autopsied brain from individuals with AD and normal aged matched controls. Hippocampal NPY –immunoreactive neurons were severely reduced in number in the hilus, CA1, subiculum and entorhinal cortex. Both the morphology of the NPY interneuron was altered and the total cell number was reduced in AD sections, suggesting that NPY interneuron function is damaged [63]. However, when radioimmunoassays were used to detect NPY in cortical or whole brain lysates from postmortem AD brain, NPY levels were not changed compared to aged matched brains from unaffected individuals [11, 13, 64]. Somatostatin levels in the same samples were significantly decreased.

Transgenic mice overexpressing amyloid precursor protein (APP) and that demonstrate amyloid plaques have also been used in studies on NPY [21, 65]. To date, two different mouse models have been used; the PDAPP-109 line that overexpresses a mutated APP (APP717V $\rightarrow$ F) driven by a platelet derived growth factor-b (PDGF-b) promoter [66] and the APP23 line that overexpresses human APP751 with the Swedish double mutation (K670M/N671L) driven by a murine Thy-1 promoter fragment [67]. Essentially a similar pattern of changes in NPY neurons was observed in both models. Increased immunoreactivity for NPY was observed in the hippocampus and entorhinal cortex in aged (18–26 months) mice. Strongly immunoreactive fibers were observed in the *stratum lacunosum moleculare* and in the polymorph layer of the dentate gyrus. In addition, NPY as well as dynorphin and enkaphalin staining was increased in the mossy fibers. NPY immunoreactivity was also observed in aberrant, swollen fibers in close apposition to amyloid plaques. Double staining demonstrated that NPY-immunoreactive cell bodies or fibers frequently co-localized with amyloid deposits. Since both mouse models demonstrated similar upregulation of NPY immunoreactivity, the changes are likely due to Abeta production, the toxic peptide cleavage product of APP, and/or amyloid deposition.

The striking differences between the data derived from human AD brain and the data derived from mouse models of AD are interesting and unresolved. Clearly there are multiple potential reasons why NPY is consistently decreased in human AD brain but is consistently increased in brains from mouse models of AD. Severity of disease is one likely critical difference between humans and mice. Also, mouse models of AD generated by overexpression of mutated APP do not faithfully recapitulate the neuropathology associated with human AD brain. For example, neuronal loss is minimal in mouse models although the APP23 mouse model is one of the few AD models to demonstrate moderate neuronal death [67–69]. Importantly, tau pathology is limited to regions surrounding plaques and neurofibrillary tangles, one of the hallmark neuropathological lesions of AD, are not observed in APP overexpressing mice [67, 69–72]. Newer mouse models that co-express amyloid plaques and neurofibrillary tangles such as those developed by Oddo et al. [73, 74] may demonstrate altered NPY levels that are more consistent with AD in humans.

NPY in the basal ganglia-striatum and substantia nigra

NPY neuronal distribution, the type and location of synaptic contacts and the axonal targets have been analyzed in detail for the basal ganglia (see Parent et al. for an extensive review) [28, 75, 76]. NPY immunoreactivity and/or mRNA expression is observed in the striatum (caudate nucleus and putamen) where it is localized to a discrete subset of neurons [28, 75, 77]. Parent et al. [28] have clearly demonstrated that NPY neurons form contacts only within the striatum and are thus, interneurons (Fig. 2). Based on morphology, NPY neurons have been termed medium aspiny interneurons and are one of four different subsets of interneurons in the striatum [75, 78, 79]. NPY+ neurons comprise 1–2% of all neurons in this region and are characterized by a small cell body (approximately 15 microns diameter), low dendritic spine density and short, highly branched axons [30, 75]. In rat striatum, interneurons that express NPY invariably co-express neuronal nitric oxide synthase (nNOS) and somatostatin [76, 80]. As discussed previously, co-localization of NPY, nNOS and somatostatin is found in other regions of the human and rodent CNS such as the cortex and hippocampus [63, 81, 82]. Although frequently observed, it is not yet clear if these three modulators are always expressed together. In a large number of studies, co-localization experiments have not evaluated the presence of all three and instead have examined two of the three. From these studies it is likely that NPY is co-expressed in the CNS with other peptide neuromodulators such as vasoactive intestinal peptide (VIP) and may not be expressed with nNOS in certain regions of the hippocampus and cortex [25]. The co-localization of NPY and NOS is of interest because of the regulation of NPY by NO. Recent studies by Espey (unpublished data) have demonstrated that NPY activity can be changed if critical tyrosines within NPY's structure are nitrated. Nitrotyrosine formation is a typical reaction for NO [83]. These data sug-

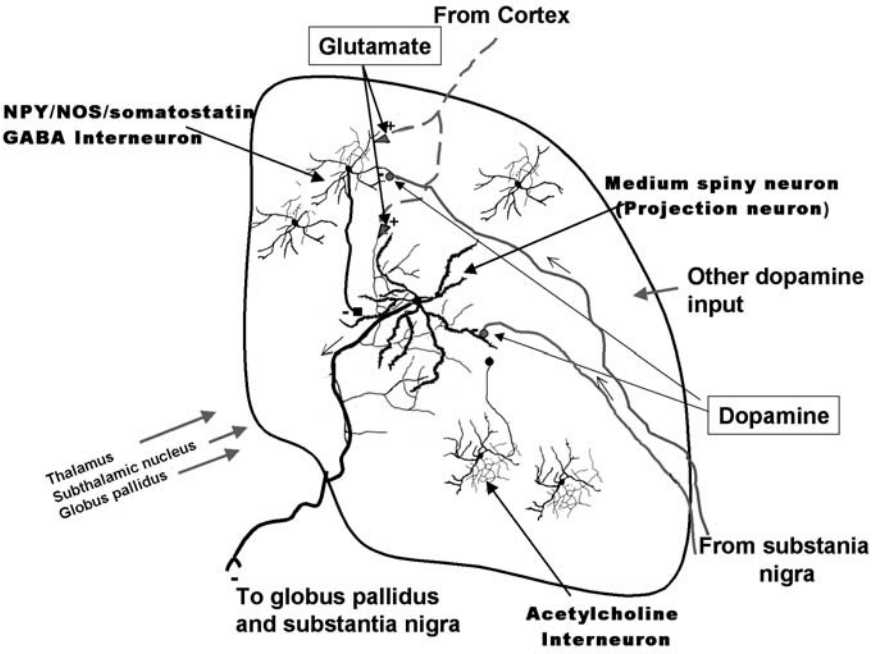

Figure 2. Interconnections of NPY interneurons in the striatum. + = excitatory input; – = inhibitory input

gest that the production and outcomes of NPY's activity may be modified if NO is present at the same time and in the same location.

Similar to hippocampal NPY interneurons, striatal NPY neurons inhibit their target neurons. In contrast to hippocampal NPY+ neurons, however, it is not clear if GABA is commonly co-expressed with NPY in striatal interneurons [30]. Immunoreactivity for glutamic acid decarboxylase (GAD), the enzyme that catalyzes the production of GABA, is difficult to detect within the cell bodies of NPY+ interneurons. However, GABA has been localized to the nerve terminals, indicating that the NPY+ interneurons most likely produce and release GABA [75]. NPY Y1, Y2 and Y3 receptors are expressed in the localized target fields of the NPY interneurons within the striatum. Recent studies using highly specific antagonists also indicate the presence of Y5 receptors [84]. NPY immunoreactivity or NPY receptor binding is not observed in other regions of the basal ganglia such as in the globus palladius or is only expressed at low levels such as in the substantia nigra or subthalamic nuclei [28, 34]. These structures are the target areas for the major efferent outflows from the striatum.

The afferent input to the striatum in general, and to the NPY interneurons in the striatum is extensive. As shown diagrammatically in Figure 2, neurons from the cortex, the substantia nigra, the thalamus, the subthalamic nucleus

and the globus pallidus send afferent fibers to the striatum, many of which synapse with NPY interneurons [26, 28, 75, 77, 79, 85]. Direct input from the cortex and substantia nigra afferent neurons to NPY interneurons has been determined using selective deafferentation techniques. These experiments demonstrated that the loss of the cortical or nigral input produced characteristic degenerative nerve terminal boutons close to the NPY immunoreactive interneurons [79]. In addition, a specialized subset of acetylcholine-secreting neurons localized within the striatum and local interneurons that release GABA also form synaptic contacts with NPY neurons [28, 30, 86].

The importance of dopaminergic regulation of NPY interneurons in the striatum was recognized a number of years ago. Kerkerian et al. [87] and more recently, Obuchowicz et al. [86] demonstrated that lesioning and the subsequent loss of the dopaminergic input to striatal interneurons increased NPY expression. Thus, dopamine tonically downregulates the expression level of NPY. In turn, NPY regulates activity of the dopamine neurons. Adewale et al. [84] have used highly selective Y2 (BIIE0246), Y1 (BIBO3304) and Y5 (CGP71683) antagonists to dissect NPY's effects on dopamine production in the striatum. Essentially, they showed that KCl-mediated-dopamine production is inhibited by NPY via Y1 and Y5 receptors and stimulated via Y2 receptor activation. This study confirms and extends previous findings on KCl-mediated dopamine accumulation and electrically-stimulated dopamine release [88, 89]. Thus, NPY produced by interneurons in the striatum differentially modulates dopamine synthesis and/or dopamine input to the striatum.

NPY in HD and PD

Huntington's disease (HD) and Parkinson's disease (PD) are two of the chronic neurodegenerative diseases that are largely associated with deficits in motor function and neuronal loss in the basal ganglia. Huntington's disease (HD) is an autosomal dominant, fatal neurodegenerative disease that displays characteristic abnormal motor activity in addition to behavioral changes and loss of cognition [90]. HD is one of at least eight neurodegenerative diseases that are based on the expression of a pathological protein within cells in which a CAG repeat within the coding region of the gene is translated to a polyglutamine repeat domain within that specific protein [90]. For HD, the affected protein is huntingtin (Htt) and the pathological repeat length varies from 36–120 glutamines. Medium spiny neurons in the striatum are the most susceptible to the damage caused by overexpression of the pathological length Huntingtin protein [91, 92]. Loss of these projection neurons that are the main outflow of the striatum (Fig. 2) is a direct component of the motor abnormalities of HD. Importantly, NPY-expressing interneurons have been reported to be the *least* vulnerable of the striatal neurons in HD [28, 76, 93, 94]. Consistent with the retention of NPY in the striatum, levels of striatal immunoreactive NPY were

found to increase in both mild and severe cases of HD compared to aged matched control brain [95]. A similar increase was observed in the cortex [96]. However, analysis of a key mouse model for HD yields different results. Gene array experiments have identified genes that are downregulated or upregulated in specific brain regions from the R6/2 HD model compared to a wild type control mouse [97]. In the R6/2 mouse striatum, NPY mRNA, as well as mRNA for somatostatin and enkephalin, is significantly lower compared to wild type control mice. Other rodent models for HD that involve quinolinic acid-mediated induction of HD pathology demonstrate both increased and decreased vulnerability of NPY-expressing interneurons [80, 94, 98, 99].

Parkinson's disease is the most common neurodegenerative disease associated with motor function and is also characterized by the loss of a specific population of cells. In PD, dopamine neurons in the substantia nigra (A9 dopamine cells) are severely damaged by the disease process. The loss of dopamine input to the striatum (Fig. 2) and other regions of the basal ganglia directly leads to the hypoactivity (loss of motor function) observed in PD [28, 100, 101]. In contrast to HD where a single gene defect is central to the disease, multiple genes and multiple environmental factors have been associated with PD [101, 102]. Protein inclusions composed of alpha-synculein are found within affected neurons and are increased by dominant mutations of the gene encoding alpha-synculein. Recently other genes which appear to be independent of alpha-synuclein- based inclusions have been implicated in the nigral dopamine neuronal loss [102].

Analysis of cerebral spinal fluid and basal ganglia levels of NPY immunoreactivity have failed to produce a consensus on the changes in NPY associated with PD. Either no change or an increase in NPY mRNA expression or immunoreactivity has been observed using *in situ* hybridization or immunochemistry in tissue lysates from PD brain [77, 103]. CSF levels of NPY decreased in HD individuals compared to unaffected individuals [56]. But, as discussed previously for AD, the reliability of data acquired from radioimmunoassay of human CSF samples may be questionable. Since no unequivocal genetic mouse model for PD currently exists, studies using model systems have focused primarily on lesioning of the substantia nigra using 1 methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone or 6-hydroxydopamine. Using radioimmunoassay, Obuchowicz et al. [86] measured striatal NPY-like immunoreactivity in lysates from MPTP- treated mice. NPY levels increased after 2 weeks and remained high for 6 weeks after MPTP treatment. However, the increase in NPY was delayed in time with respect to the MPTP-mediated fall in striatal dopamine levels. To establish a direct connection between the increased striatal NPY levels and the loss of dopamine induced by MPTP, Obuchozicw et al. also treated the mice with deprenyl for 30 min before MPTP lesioning. Deprenyl, a monoamine oxidase B (MAO-b) inhibitor, blocked the loss of dopamine and prevented the increase in NPY. A similar increase in NPY immunoreactivity was shown in 6-hydroxy dopamine lesioned rats [104]. Thus although controversial, the loss of dopamine input

from the loss of substantia nigral dopamine neurons in PD, results in increased NPY. These data suggest that NPY expression in the striatum is generally down-regulated by dopamine and that this down-regulation of NPY may be lost in PD. Alternatively, independent factors such as inflammatory signals override dopamine regulation of NPY.

Table 1 summarizes the changes in NPY that are observed in autopsied brain samples derived from individuals with AD, HD or PD. In general, the data suggest that NPY levels more commonly decrease in brain regions of individuals with AD and either don't change or increase in specific brain regions of individuals with HD or PD. These findings, however, remain controversial because of the discrepancies between experimental findings and the lack of clear confirmation in genetic mouse models that mimic chronic neurodegenerative diseases such as AD. The data also demonstrate that NPY levels may change independently of other neuropeptides, even those known to co-localize with NPY. For example, long term cerebroventricular infusion of Abeta peptide in rats has been shown to decrease the overall levels of somatostatin immunoreactivity but does not change NPY immunoreactivity [49]. A similar dissociation between somatostatin and NPY levels was shown in AD brain [57, 105]. However, the fact that some areas of the brain demonstrate an associated decrease in NPY and somatostatin levels in AD while other areas do not underscores the uniqueness of the interneuron populations. Careful immunocytochemical analysis using double labeling techniques unequivocally demonstrate that cortical somatostatin neurons that also express NPY in AD brain do not show similar atrophy or cell loss as those cortical neurons that are immunoreactive for somatostatin or NPY alone [81, 106]. This is also true if the somatostatin neuron co-expresses nNOS compared to neurons expressing somatostatin alone. Thus, the specific neuropeptide profile of interneuron subpopulations is a critical factor in determining the functional integrity and survival of NPY-immunoreactive interneurons in the hippocampus and cortex during AD. A similar relationship exists for NPY interneurons in the basal ganglia.

Table 1. Summary of the changes in NPY immunoreactivity in CSF, brain sections or tissue lysates in AD, PD, and HD. ↓ = increased levels; ↑ = decreased levels

	Human	Rodent models
Alzheimer's disease	In CSF- no change or ↓ In brain tissue- no change or ↓ Number of NPY+ neurons – ↓ Altered morphology of NPY neurons- yes	APP23 – ↑ PDAPP-109 – ↑
Parkinson's disease	In CSF – ↓ In brain tissue – ↑	MPTP lesioned – ↑ 6-OH DOPA-lesioned- ↑
Huntington's disease	In brain tissue – ↑ Number of NPY+ neurons – no change	R6/2 – ↓

Potential mechanisms of action of NPY in chronic neurodegenerative diseases

The expression of NPY mRNA and protein within many interneurons clearly implies that NPY participates in the complex regulation of brain circuitry that is associated with interneuron function. In general, NPY serves as an inhibitory neurotransmitter and reduces neuronal excitation. However, experiments using the NPY knockout mouse ($NPY^{-/-}$) provide strong evidence that NPY is not a classical neurotransmitter such as glutamate or GABA. Genetic removal of NPY does not produce any physiological differences in synaptic activity at two different regions in the hippocampus where NPY-immunoreactive neurons are known to exist, specifically CA1- Schaffer collateral synapses and perforant pathway-granule cell synapses. When compared to wild type, mice lacking NPY did not demonstrate any change in population spike amplitude, the input/output relationship, paired pulse facilitation or typical EPSP/IPSP patterns at both low and high stimulation frequencies [33].

Although not a classical neurotransmitter, NPY fulfills the criteria used to identify neurotransmitter substances. NPY is stored within neuronal terminals in dense core vesicles, it is released upon activation of the presynaptic neuron, it acts on specific receptors and alters the response of post-synaptic cells [3, 25, 37, 107]. For example, exogenous application of NPY to hippocampal CA1 pyramidal neurons reduces the amplitude of population spikes independently of GABA or other classical neurotransmitters [37]. The reduction in spike amplitude is also observed in the $NPY^{-/-}$ mouse hippocampus when NPY is exogenously added to the cells [33]. A direct action of endogenous NPY has also been clearly confirmed. Sun et al. [107] have demonstrated that repetitive stimulation of NPY-containing neurons generates a slow IPSP in thalamic slice preparations from wild type mice that is not observed in preparations from $NPY^{-/-}$ mice. Since these experiments were done in the presence of GABA receptor blockade, the inhibitory signal cannot be due to GABA. Despite the above data, the slow release of NPY, it's co-localization with other more well defined neurotransmitters such as GABA and its coupling to G-protein signaling has led to the classification of NPY as a neuromodulator [3]. In fact, the distinction between neurotransmitter and neuromodulator are blurred for NPY as well as for other non-classical neurotransmitters such as nitric oxide.

The outcome of NPY's actions can be neuroprotective. Numerous studies have now shown that NPY inhibits glutamate-mediated synaptic activity in the hippocampus and striatum as well as other brain regions [36, 39]. This action is primarily presynaptic, results in reduction of calcium entry into the nerve terminal and is mediated primarily by the Y2 receptor, although Y1 and Y5 receptors are also implicated in presynaptic inhibition [37, 38, 108–110]. As a consequence of this activity, NPY decreases limbic seizures, reduces kainic acid induced epilepsy and protects against glutamate-mediated neuronal death [33, 36, 40, 111, 112]. Interestingly NPY mRNA and protein expression

increases after seizures, particularly in the mossy fibers of the hippocampus [112]. This ectopic level of NPY is thought to reduce glutamate release, and thus decrease the abnormally high glutamate activity observed during seizures.

For AD, then, the relative lack of NPY or the failure to upregulate NPY in the hippocampus may be a contributing factor to the neurodegenerative disease process. Since NPY promotes memory retention, changes in NPY during AD are likely to impact cognition. Furthermore, the potential failure of the interneuronal network to appropriately regulate glutamate-mediated synaptic transmission is an important factor in the excitotoxic-neuronal loss in AD hippocampus [85, 113, 114]. Loss of NPY's control over glutamate release is likely to further facilitate excitotoxic damage.

A similar case can be made for neuronal loss in the striatum in HD. As mentioned previously, the striatal interneurons and the striatal projection neurons (medium spiny neurons) receive direct glutaminergic input from layers 5 and 6 of the cortex [92]. Both cell types express ionotropic and metabotropic glutamate receptors, although the density of glutamate receptors is less for interneurons compared to the high glutamate receptor density observed on medium spiny neurons [115]. Medium spiny neurons, thus, can be killed by agents that act at glutamate receptors including glutamate itself and glutamate receptor agonists such as quinolinic acid or kainic acid [116]. The idea that excitotoxic neuronal death occurs in HD is supported by chemical lesioning using quinolinic acid. Injection of quinolinic acid into the striatum induces neuropathology that closely resembles HD [117]. Since NPY inhibits glutamate presynaptic release, then the presence of NPY is likely to regulate the overall level of glutamate in the striatum, thus reducing the loss of medium spiny neurons. The observed increase in NPY immunoreactivity in brain lysates from humans with HD are consistent with a neuroprotective action for NPY and is similar to the increase in NPY associated with seizure activity in the hippocampus. However, despite the increased NPY in human striata, the large loss of medium spiny neurons continues unabated in HD. Thus, unless NPY or the NPY receptors have changed their functional characteristics, the effects of NPY in the striatum are more complex than predicted. NPY may not play a dominant role in controlling the level of glutamate activation in the striatum as it does in the hippocampus. Interestingly, nNOS mRNA and protein levels decrease in HD [118, 119]. The subsequent loss of NO and of nitrating species may alter NPY nitrotyrosine formation and hence, its activity. The consistent loss of NPY in mice models of HD underscores the differences in striatal function between humans with HD and rodent models of HD which have yet to be reconciled.

PD is associated with hyopactivity produced by the initial loss of dopamine afferent input into the striatum. The loss of dopamine inhibition over the projection neurons allows unabated activity to occur in the efferent pathway to the globus pallidus. The unabated activity, in turn, is inhibitory to neurons in the globus pallidus and leads to reduction in motor activity [28]. NPY would appear to mimic the loss of dopamine input to the striatum since injection of

NPY directly into the striatum results in decreased motor activity. Since NPY is increased in PD or when the dopamine afferents are removed, this change would be predicted to worsen the outcome of the disease. However, Adewale et al. [84] have described opposing actions of NPY which are dependent on specific subtypes of NPY receptors. Y2 receptor activation leads to facilitation of striatal dopamine synthesis and release whereas Y1 and Y5 receptor activation leads to inhibition of dopamine. If this is true for humans, then activation of Y2 receptors or conversely, inhibition of Y1 and Y5 receptors in individuals with PD could lead to restoration of a more normal motor output in face of the loss of dopamine input to the striatum.

NPY in neuroinflammation

Part of the disease process in chronic neurodegenerative disease is the sequelae initiated by neuroinflammation. In AD, the innate immune response is initiated, at least in part, by the accumulation of amyloid and in HD and PD, by dying cells in the striatum and substantia nigra [120–125]. The cells that mediate the innate immune response in the brain are microglia, the CNS macrophage, and astrocytes [126]. In the CNS microglia play the predominant role in producing and releasing factors that initiate a proinflammatory environment while astrocytes both potentiate and later slow the inflammatory process. Interestingly, an interrelationship between NPY and peripheral macrophage activation has been recently shown and which is likely to apply to microglia in the brain. For example, monocyte derived macrophages isolated from human blood express NPY mRNA when stimulated with phorbol myristate acetate (PMA), a phorbol ester that is commonly used as an induction signal for macrophage activation [127]. Macrophages also respond to NPY. Induction of superoxide anion production, a component of the respiratory burst responsible for the bactericidal action of macrophages, is increased by NPY [4, 5]. Other macrophage functions such as chemotaxis, the directed movement of macrophages toward a target, and phagocytosis are inhibited by NPY [128]. Dimitrijevic et al. [5] have recently shown that NPY induced upregulation of macrophage function is mediated by Y1 and Y2 receptors while the reduction in activation is initiated by Y2 and Y5 receptors. This dual regulation by combinations of NPY receptors provides at least a partial explanation for the opposing actions of NPY on macrophage function. Since NPY receptors have been localized to human and mouse astrocytes [129], it is likely that microglia also express NPY and respond in a similar, if not identical, manner to NPY. Microglia have been shown to produce a respiratory burst and to migrate in a directed fashion [130, 131]. Thus, NPY via Y1, Y2 and Y5 receptors may contribute to the regulation of neuroinflammation during AD, PD and HD. Whether inflammation is increased or decreased by the increased expression of NPY as seen in HD and PD or by the loss of NPY as seen in AD remains to be experimentally resolved.

NPY's "other" effects

NPY's importance to chronic neurodegenerative diseases can extend beyond protection of neurons via regulation of glutamate release and beyond regulation of neuroinflammation. As discussed previously, NPY affects multiple systems in the brain as well as throughout the body. NPY is now well known to alter the secretion of hormones that impact energy homeostasis. Its overall effect is to promote lipoprotein lipase activity thus increasing cellular stores of lipid, to alter cholesterol balance by raising circulating cholesterol levels and to increase appetite and food intake [6, 132, 133]. Although this may adversely affect normal individuals, patients with chronic neurodegenerative disease may benefit from these effects of NPY. Individuals with AD, HD and PD lose significant amounts of weight as the degenerative diseases progress despite robust appetites and large caloric intakes [134, 135]. Failure to supply adequate nutrition reduces the brain's ability to repair or to regenerate. How NPY participates in this aspect of chronic neurodegenerative disease is essentially unknown.

The regulation of energy expenditure in the brain can also occur at the cellular level. Mitochondrial production of ATP by oxidative phosphorylation is critical to the maintenance of normal cellular functions. When compromised, changes in mitochondrial membrane potential, calcium influx and ATP production occur and can lead to induction of cell death pathways [136, 137]. Dysregulation of mitochondria has been implicated for each of the major neurodegenerative diseases and is thought to be a major factor in the death of neurons in AD, HD and PD [138–140]. Interestingly, NPY immunoreactivity has been recently localized to mitochondria [141]. Furthermore, oxygen consumption by mitochondria in brown fat cells is depressed by NPY. This effect, however, is dependent on thyroid hormone (T3). This emerging interrelationship between mitochondria and NPY may lead to a better understanding of how the observed changes in NPY that occur in neurodegenerative disease may lead to either neuronal protection or neuronal loss.

Final thoughts

The fact that NPY is the most abundant peptide in the CNS implicitly implies an importance to the CNS that has not been fully explored. In addition, the wide range of systems affected by NPY further underscores the need to understand how this peptide carries out its functions and how these functions are altered by neurodegenerative disease. As new molecular tools such as NPY knock out and NPY transgenic mice emerge, they can be readily applied to study the role of NPY in AD, PD and HD by crossing the mouse models of disease with mouse models of NPY. These types of experiments as well as the continued development of reliable outcome measures of NPY and its action will provide new inroads to our understanding and potential treatment of these devastating diseases.

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Genetics of the NPY family of peptides and their receptors

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Human *NPY* gene variants in cardiovascular and metabolic diseases

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Introduction

The neuropeptide Y (*NPY*; MIM 162640) gene is located on 7p15.1 and exists as a single copy [1]. The transcription sequence spans approximately 8 kb pairs, containing: one exon with only non-translated DNA, three exons with translated DNA, and three introns ranging approximately from 900 to over 4000 bp [2]. NPY is part of the structurally highly conserved PP-fold peptide family, which are probably evolved by gene duplications of the ancestral gene sequence [3]. NPY itself is the most conserved neuroendocrine peptide throughout evolution [3]. Although mature NPY has a highly conserved peptide sequence, the signal peptide sequence and the carboxy-terminal peptide of NPY (C-PON) has a lower degree of identity [4].

There are many association studies which connect NPY and NPY receptors to a condition or disease. Table 1 lists some of the positive association studies, but it should be remembered that there are usually as many negative as positive association studies published in different populations. Also the publication bias of negative studies distorts the results published. To date, NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) reports only two nonsynonymous sequence variants in the *NPY* gene: GenBank NM_000905.2:c.20T > C (p.L7P; amino acid 7 of the preproNPY, leucine, is changed to proline; rs16139) and c.64C > A (p.L22M; amino acid 22 of the preproNPY, leucine, is changed to methionine; rs5571), which both are located in the signal sequence part of the *NPY* gene. In a recent publication Ding et al. (2005) screened, by direct sequencing, a part of the promoter region, the complete human *NPY* coding sequence, and neighboring intronic sequences in a population of 30 randomly selected Swedes. They found one nonsynonymous (L7P) and two synonymous sequence variants in the coding part in addition to several in the intron or untranslated sequences. For the L22M polymorphism dbSNP database reports an average allele frequency of 0.021 for the A allele (M22), but there are no published reports on this variant in any populations [10]. We have typed a Finnish population according to this sequence variant, but did not find

any carriers (n = 60, Pesonen et al., unpublished results). The dbSNP is known to contain 15–17% false positive alterations [5], so the existence of this non-synonymous sequence variant remains to be confirmed. Additionally, there seems to be a functional sequence variant in the promoter region of the *NPY* gene (GenBank AC004485:154841C > T), which putatively destroys the SP1 consensus motif. This alteration has been reported to change the transcriptional activity of the gene by greater than five-fold in *in vitro* studies [6, 7]. The implication of this polymorphism on cardiovascular and metabolic parameters is not currently studied.

In 1998, we published the identification of a common L7P sequence variant in the signal peptide part of NPY, which is so far the only non-synonymous alteration in the *NPY* gene. The carrier frequency of the P7 allele varies from 6% to 15% in the Caucasian populations, and it seems to be totally absent or extremely low in some populations, including Mexican, African American and oriental populations [8, 9]. Since our original landmark publication, it has been linked to several other cardiovascular and metabolic phenotypes (Tab. 1). In this review, I am going to present current knowledge on the effects of the human *NPY* gene L7P polymorphism and cardiovascular and metabolic diseases and their well known risk factors obesity and lipid balance.

Functional consequences of the L7P alteration

The L7P alteration is a consequence of a single base substitution, c.20T > C, in the signal peptide part of preproNPY. Although this amino acid change causes a significant change in the tertiary structure of the signal peptide, as predicted by molecular modeling programs (Fig. 1), it has been difficult to find any exact functional consequence at the molecular level. *In vitro* translation and subcellular processing studies have so far resulted in negative findings (Pesonen et al., unpublished results, [10]). However, there has been a report on the NPY signal peptide and an action of its own after being cleaved from the nascent NPY. This action seems to be different for the wild type signal peptide than for the mutant signal peptide [10]. This is a totally new concept and goes beyond the current concept of a signal sequence being responsible only for targeting and translocating the secretory proteins and membrane proteins through the ER membrane into the ER lumen to the secretory pathway. At the cellular level, the mutation causes a different distribution of NPY-related immunoreactivity in primary cultured human umbilical vein endothelial cells. With double-labeling techniques, it has been demonstrated that in human endothelial cells carrying the p.P7 allele, the amount of mature NPY without C-PON was prominent. Homozygous [p.L7]+[p.L7] endothelial cells contained almost exclusively not fully processed proNPY, i.e., NPY with C-PON [11]. This strongly suggests a difference in cellular storage and processing of the proNPY between the genotypes.

Table 1. Some examples of the positive association studies on the different disease and conditions on NPY and NPY receptors

Gene	Mutation (dbSNP ID)	Ref seq.	Localization			Disease/condition	Ref.
			genomic	coding (c.)	protein (p.)		
NPY	rs3037354	NT_007819.15	promoter	-968_-969delTG	-	Waist-to-hip-ratio	[79]
	rs17149106	NT_007819.15	promoter	-688G > T	-	Alcohol dependence	[92]
	rs16147	NT_007819.15	promoter	-485T > C	-	Depression	[93]
	rs16147	NT_007819.15	promoter	-485T > C	-	Schizophrenia	[6]
	rs16139	NT_007819.15	coding	20T > C	L7P	Hyperlipidaemia	[14]
	rs16139	NT_007819.15	coding	20T > C	L7P	Higher birth weigh	[16]
	rs16139	NT_007819.15	coding	20T > C	L7P	Increased BMI	[10]
	rs16139	NT_007819.15	coding	20T > C	L7P	Alcohol dependence	[94]
	rs16139	NT_007819.15	coding	20T > C	L7P	Elevated alcohol consumption	[95]
	rs16139	NT_007819.15	coding	20T > C	L7P	Enhanced rate of atherosclerosis	[17, 18]
	rs16139	NT_007819.15	coding	20T > C	L7P	Retinopathy	[70, 73]
	rs16139	NT_007819.15	coding	20T > C	L7P	Depression	[93]
	rs5574	NT_007819.15	coding	204C > T	S68	Alcohol withdrawal	[96]
NPY1R	rs7687423	NT_086656.1	intron1	-3091A > G		IgA nephropathy	[97]
NPY5R	-	NT_016354.17	intron1	62+225G > A*		Decreased TG and increased HDL	[98]
NPY5R	-	NT_016354.17	3' UTR	1523T > C	-	Obesity	[99]

*This marker also part of the intron of the *NPY1R* gene.
The numbering is according to the sequence NT_007819 in GenBank (Homo sapiens chromosome 7 genomic contig, vers. NT_007819.15). The nucleotide +1 is the A of the ATG-translation initiation codon of the preproNPY gene, the nucleotide 5' to nucleotide +1 is numbered –1. Accordingly, the first A of the untranslated exon1 is nucleotide –86. The number of the last nucleotide of the preceding exon is marked IVS1+1

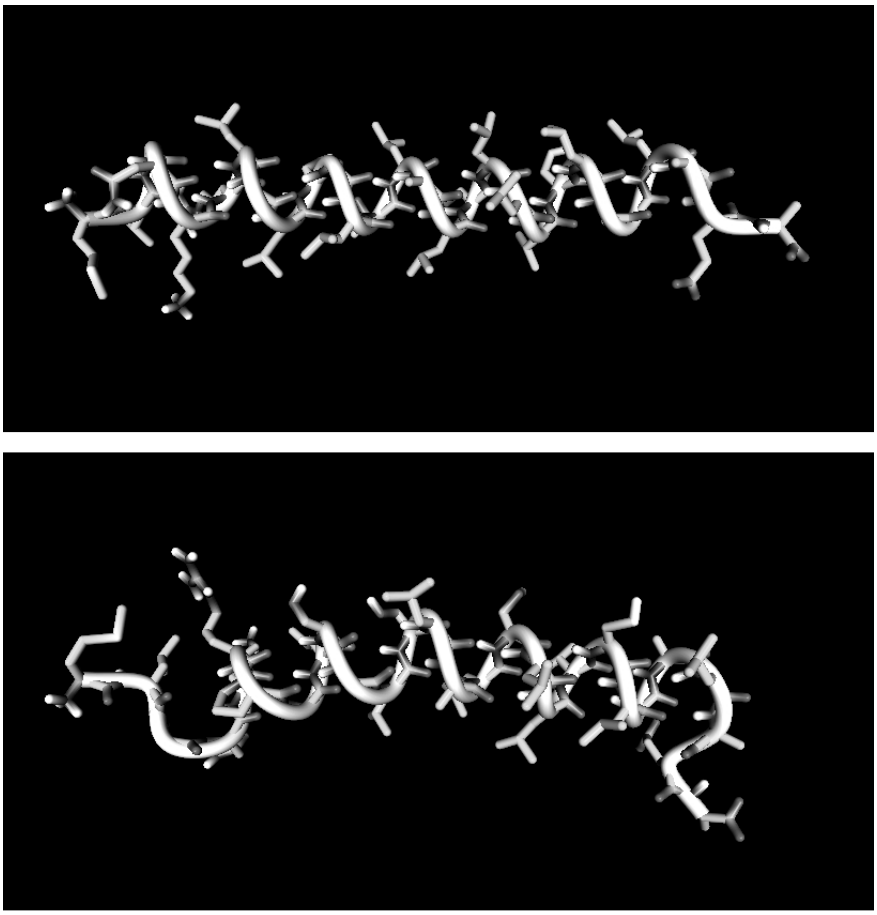

Figure 1. Molecular modeling of L7P variant of human NPY signal peptide. Upper panel represents the 28 amino acid signal peptide of preproNPY with L7, and the lower panel the same signal peptide with the P7 substitution. The consensus sequence for signal peptides is not evident, but they share a central hydrophobic region as a common structural feature. Leucine, which has a hydrophobic aliphatic side chain, is known to favor formation of a-helices. Proline, which has a cyclic structure that considerably influences protein secondary and tertiary architecture, does not favor formation of a-helices. Instead, proline introduces breaks and kinks into a-helical parts of the peptide backbone, as seen in the modeling in the lower panel. The signal peptides are modeled with Composer Program and the graphics with SYBYL 6.7 Program (Courtesy of Professor Antti Poso, University of Kuopio, Kuopio, Finland.

In studies with healthy volunteers who are carefully matched according to their genotype, we have shown that heterozygous [p.L7]+[p.P7] subjects have lower basal NPY secretion at rest, but greatly increased NPY release with sympathetic stimulation [11, 12]. This indicates that the L7P alteration has different effects on the plasma NPY kinetics during rest *versus* exercise. Because circulating NPY is derived mainly from perivascular sympathetic nerve bun-

dles, the mechanisms may be related to changed intraneuronal kinetics of NPY. It is possible that during the resting state the L7P alteration leads to impaired release and intracellular retention of NPY, followed by an exaggerated release of NPY during high-intensity sympathetic stimulation. Also, the elimination of NPY from plasma by degradation or excretion through the kidneys could be affected differently during rest *versus* exercise in subjects carrying the P7 allele. However, changed intracellular kinetics of NPY synthesis and processing seems to be a more plausible mechanism on the basis of known functions of signal peptides and the cellular studies. It has also been proven by others that NPY is regulated at the level of neuronal neurotransmitter content and release [13]. This hypothesis is supported by the cellular studies showing that there is increased intracellular storage of mature NPY, which is the secreted form and more easily released by sympathetic stimulation. There is a smaller mean NA/NPY ratio in plasma from heterozygous [p.L7]+[p.P7] study subjects as compared with the group of homozygous [p.L7]+[p.L7] study subjects during exercise. This may reflect the greater amount of NPY in the storage vesicles and a different ratio of NA to NPY which is released during sympathetic stimulation [11]. In conclusion, it seems that the L7P substitution causes altered synthesis, processing, and release of the active, mature NPY.

Lipid levels and atherosclerosis

In the landmark study in 1998, we associated the P7 allele with high serum cholesterol and low-density lipoprotein (LDL) cholesterol levels in Finnish and Dutch obese non-diabetic persons [14]. Before this study there was only one publication on NPY and serum cholesterol levels [15]. Our work provided the first genetic evidence that NPY may be linked to altered cholesterol metabolism and that the P7 allele in the NPY signaling peptide may be one of the strongest genetic factors influencing serum cholesterol and LDL levels in obese subjects. It also started a new era in NPY research and changed the focus from a systemic sympathetic neurotransmitter to a neuromodulator on a local vascular level.

After the initial study we examined the association in different study populations. Since elevated serum cholesterol and triglyceride (TG) concentrations in youth are considered important determinants for future coronary fatty streak formation and atherosclerosis, we studied pre-school aged children at the ages of 5 and 7 years in the follow-up study [16]. The L7P alteration was not associated with serum total or low density lipoprotein (LDL) cholesterol values in boys or in girls. However, P7 substitution was constantly associated with 14–17% higher mean serum TG values in the boys at the ages of 5 and 7 years ($P = 0.023$). The L7P alteration may thus be linked with serum TG concentrations, but not with serum cholesterol concentrations, in a gender-specific manner in preschoolers. These findings were further evaluated in a large population-based sample of 966 elderly men (aged 42–60 years) in Finland [17]. The

P7 substitution was associated with an accelerated 4-year increase in the mean and maximal common carotid intima-media thickness (IMT). Men with P7 substitution had a 31% greater increase in the mean IMT and a 20% greater increase in the maximal IMT than homozygous [p.L7]+[p.L7] men. In this study, the P7 substitution was also related to increased serum total cholesterol and LDL cholesterol in obese (body mass index (BMI) > 30 kg/m^2) men. The association of the P7 substitution and atherosclerosis was further confirmed in an independent population study where the P7 substitution was associated with increased carotid IMT in type 2 diabetic patients, as well as with the accelerated progression of carotid atherosclerosis during a 4-year observation period in middle aged men [18].These studies suggest that the P7 substitution in preproNPY is an important risk factor for accelerated atherosclerotic progression and increased serum cholesterol in humans.

Most of the above mentioned positive association studies have been done in Finnish populations, but there are also negative reports in different populations. In the Brazilian population, non-diabetic individuals of European descent, P7 carriers had a tendency to have lower total cholesterol values, but the number of subjects with a P7 substitution was very low (n = 18) to reach any statistical power [19]. In recent reports, the researchers could not find associations of plasma TG, total cholesterol, VLDL cholesterol, LDL cholesterol, and HDL cholesterol with the L7P sequence variation in Swedish subjects nor in subgroups of obese and non-obese subjects analyzed separately [10] or in a Polish family study [20]. Some of the later studies on healthy, normal weight Finnish cohorts [11, 21, 22] have also suggested that a P7 substitution might carry a risk factor of elevated plasma lipid levels only in combination with another major metabolic or environmental risk factor, like older age, obesity or a diet rich in dairy products, which is very common in Eastern Finnish populations [23].

Free fatty acids (FFA) are mainly derived from the TG rich lipoprotein particles and their main purpose is to transport energy between the blood circulation and tissues. The liver takes up the released FFAs in the blood, where hepatic lipoprotein lipase (LPL) turns FFA to TGs, causing hypertriglyceridemia. Insulin decreases plasma FFA concentrations by stimulating endothelial LPL in fat tissue to remove FFA from plasma to fat cells, and by inhibiting the hormone-sensitive LPL in fat cells, which inhibits the release of FFAs from the fat cells. Although in physiological states, chronic elevation of FFA is restricted to a number of specific situations, like prolonged fasting, FFAs are considered as a link between lipid metabolism and insulin resistance, which is in turn a risk factor for atherosclerosis [24]. In the study with carefully matched pairs of different L7P genotypes exposed to strenuous exercise, the group with heterozygous [p.L7]+[p.P7] had clearly lower post-exercise FFA values [11]. Generally, during exercise, plasma levels of catecholamines, growth hormone (GH) and FFA increases and insulin decreases. Catecholamines and NPY increases intracellular cyclic adenosine monophosphate (cAMP) concentration, which leads to phosphorylation of hormone-sensitive LPL and raises the plasma FFA con-

centrations. Catecholamines are especially strong stimulants of lipolysis and can overcome the inhibitory effect of insulin on FFA release in humans [25]. The inhibition of lipolysis in human adipocytes by NPY has been shown to be dose-dependent [26], and therefore higher NPY release during exercise in subjects with the [p.L7]+[p.P7] genotype may explain the difference in FFA concentration between the genotypes. Since the catecholamine levels were similar in this study, it is possible that higher NPY secretion is capable of overcoming the stimulatory effect of catecholamines on lipolysis, as shown in *in vitro* studies [27]. Low levels of FFAs in subjects with the P7 variant of preproNPY was observed in the presence of both low and high insulin levels, which indicates that this effect is independent of insulin's anti-lipolytic action. This notion is also supported by our observations that the activities of LPL and hormone sensitive LPL, which are regulated by insulin, are not changed in subjects with the [p.L7]+[p.P7] genotype [28]. Most likely, insulin and NPY, independent of each other, lower cAMP levels which in turn leads to inactivation of hormone-sensitive LPL and lower serum FFAs.

NPY reduction of intracellular cAMP concentrations in human adipocytes can inhibit lipolysis and increase liver and white adipose tissue lipogenesis [29]. It has also been shown that central administration of NPY increases the expression of LPL and white fat LPL enzymatic activity [30, 31]. There is no significant difference between the L7P genotypes in the magnitude of postprandial lipemia induced by an oral fat tolerance test. In homozygous [p.L7]+[p.L7] genotype subjects the hormone sensitive LPL-to-LPL ratio correlates with the area of total plasma VLDL and chylomicron TG, but not in subjects with the heterozygous [p.L7]+[p.P7] genotype [28]. This suggests that there might be a compositional difference in the lipid particles affecting postprandial lipid metabolism and the relationship of hormone sensitive LPL and activities to lipid metabolism may differ between the L7P genotype groups.

Blood pressure, heart rate and sympathetic activity

Since enhanced sympathetic nerve activity seems to play a role in the pathogenesis of hypertension and NPY is one of the most potent vasoconstrictor peptides isolated to date, it is natural to connect NPY to high blood pressure. A major source of circulating NPY is the perivascular sympathetic nerve endings, but a minor fraction of NPY is also derived from endothelial cells. The blood vessels have a dense concentration of nerve fibers containing NPY, especially in the arterial walls [32]. NPY and NA are co-localized in sympathetic neurons both in the peripheral and central nervous system, and are co-released into the circulation during sympathetic activation. NPY has three major actions that are important in the modulation of blood pressure homeostasis. First, when released from sympathetic neurons innervating the cardiovascular system, NPY causes long-lasting vasoconstriction via a direct stimulatory effect on smooth muscle cells (SMC). Second, NPY pre-synaptically inhibits

the release of NA and other neurotransmitters and third, NPY potentiates the action of NA and other pressor agents on SMC. In addition to its potent vasoconstrictor capacity, NPY seems to have a dual direct effect on blood vessels: the Y1 receptors on SMC mediate vasoconstriction and the Y2 receptors on endothelial cells mediate vasodilatation [33].

Although monogenic forms of blood pressure dysregulation exist, hypertension is a complex quantitative trait that is affected by varying combinations of genetic and environmental factors. NPY is listed among the candidate genes in humans for blood-pressure homeostasis and hypertension [34, 35]. There are many studies on the effects of L7P substitution on blood pressure [10–12, 18, 21, 22, 36]. Only in one study, an independent relationship between the P7 substitution and slightly increased blood pressure has been observed [17]. The L7P substitution has been reported to be an independent predictor for myocardial infarction and stroke in a Swedish hypertensive population [36]. It seems that altered NPY regulation by L7P substitution is not a major component in the pathogenesis of hypertension in general. Instead, it may contribute to aberrant regulation of blood pressure in major stressful situations [37], during extreme conditions [38–40], or with a concomitant disease like hyperlipidemia [41]. This could lead to NPY hypersensitivity and *circus vicious*, where L7P substitution may also increase the risk of more severe complications related to elevated blood pressure [36, 42].

NPY released during stimulation of cardiac sympathetic nerves reduces the activity of cardiac parasympathetic neurons via NPY Y2 receptors. This reduces acetylcholine release and attenuates the parasympathetic negative inotrophic and chronotrophic effects in the heart [43, 44]. On the other hand, central administration of NPY increases the mean arterial pressure and heart rate probably via NPY Y1 receptors in non-adrenergic, non-cholinergic nerves [45, 46]. Although the regulation of autonomic nervous system control of the cardiovascular system seems to be very complex and may show some conflicting results, as a very general rule in normal healthy subjects' heart rate, NPY and NA are all increased proportionally during a sympathetic stimulation. This correlation may be lost with altered NPY balance, like in the presence of a functional L7P substitution. During exercise, healthy heterozygous [p.L7]+[p.P7] subjects have significantly higher NPY concentrations and significantly increased heart rates compared with homozygous [p.L7]+[p.L7] subjects, but no difference in the plasma NA levels or blood pressure [11]. In contrast, during rest, healthy heterozygous [p.L7]+[p.P7] subjects have lower plasma NPY and NA concentrations, but significantly higher heart rates during the daytime and no change in blood pressure [47, 48]. Again, we can see that the regulation of sympathetic balance and heart rate are highly complex systems probably including a central and peripheral component and depends clearly on the intensity of the sympathetic activation.

The different ratio of NPY/NA and correlation to heart rate in heterozygous [p.L7]+[p.P7] subjects suggests that these subjects may have altered cardiovascular autonomic regulation [47, 48]. The power spectral analysis of heart

rate variability (HRV) is a noninvasive method to assess cardiac autonomic modulation, and gives quantitative and qualitative data about sympathetic and parasympathetic control as well as sympathovagal interactions of cardiovascular functions. The arterial baroreflex is an important negative, mainly vagal, feedback system which rapidly adjusts the blood pressure to its physiological set point. Baroreflex sensitivity (BRS) has been suggested to provide independent prognostic values in predicting cardiovascular mortality [49]. During rest, healthy subjects have increased cholinergic and decreased adrenergic cardiovascular reactivity assessed by HRV measurements, which reflects that parasympathetic vagal control of heart function is dominant. The total HRV and sympathetic as well as parasympathetic HRV were significantly increased in the healthy subjects with heterozygous [p.L7]+[p.P7] genotype compared to matched homozygous [p.L7]+[p.L7] subjects [48]. Also in type 2 diabetic patients, L7P substitution is an independent determinant of altered sympathovagal balance [18]. Additionally, the BRS was significantly higher in the healthy subjects with heterozygous [p.L7]+[p.P7] genotype than in the matched subjects with the homozygous [p.L7]+[p.L7] genotype [48]. This data suggests that subjects with the heterozygous [p.L7]+[p.P7] genotype have increased autonomic cardiovascular regulation.

In the subjects with the [p.L7]+[p.P7] genotype, there is a significant negative correlation of NA concentrations with sympathetic HRV and with the baroreflex sensitivity, which is not the case with [p.L7]+[p.L7] genotype subjects. It seems that the cardiovascular autonomic regulation is much more sensitive to changes in plasma NA concentrations in the subjects with [p.L7]+[p.P7] genotype than in the control subjects. It also seems that the subjects with this genotype are likely to be more prone to the undesired effects of NPY when it is released during sympathetic activation and thus predisposes the individual to cardiovascular disease if they develop hypertension or type 2 diabetes later in life [18, 36].

Myocardial infarction

NPY is the most abundant peptide in the mammalian heart [50]. Since NPY is a potent vasoconstrictor, the direct cardiac effects of the peptide are difficult to distinguish from the vascular effects. *In vitro* studies with isolated myocytes suggest that NPY has inotropic and chronotropic effects and stimulates cardiac myocyte hypertrophy [51, 52], although there are some contradictions in these experimental data. As a potent vasoconstrictor, NPY causes contraction of coronary arteries, which can induce myocardial ischemia [53]. There are some clinical studies which suggest that high plasma NPY levels could correlate with myocardial ischemia and infarction [42, 54] and that the NPY levels after myocardial infarction could be used as a prognostic factor for increased mortality [55]. This evidence justifies the listing of *NPY* gene as a candidate gene for the risk of myocardial infarction [56]. Indeed, the L7P variant is an inde-

pendent predictor for myocardial infarction together with systolic blood pressure, TG and LDL for myocardial ischemia and stroke in hypertensive patients [36]. Since the severity of carotid IMT is currently recognized as an independent predictor of transient cerebral ischemia, stroke, and coronary events such as myocardial infarction, the association of L7P substitution with enhanced increase in carotid IMT future emphasizes the connection between myocardial infarction and altered NPY balance [17, 18]. It may be that the unbalanced autonomic nervous system activity leads to adverse cardiovascular events in special circumstances, like in mental stress or in pathophysiological stress involved in a disease. In such cases, the enhanced release of NPY, caused by the L7P substitution, and possibly enhanced responsiveness to NPY could increase the risk of fatal cardial events.

Vascular function and vascular growth

Endothelial cells play a crucial role in the development of atherosclerosis and its thrombotic consequences. Endothelial dysfunction is a feature of most cardiovascular risk factors and disease conditions that are associated with atherosclerosis, like dyslipidemia, type 2 diabetes, hypertension, cerebrovascular diseases, and heart diseases. In response to various mechanical (shear stress) or chemical (hypoxia, humoral factors) stimuli, endothelial cells produce nitric oxide, which dilates the vessel, inhibits monocyte and leukocyte adhesion to the vessel wall, inhibits the activation and aggregation of platelets, and inhibits SMC proliferation. In endothelial dysfunction, all these responses are disabled, which destroys the vascular homeostasis and predisposes to vascular complications. NPY is a known vasoconstrictor, endothelial growth factor, and enhancer of vascular permeability. NPY acts as a SMC mitogen and stimulates attachment, migration, DNA synthesis, and the formation of capillary tubes by human endothelial cells [57]. NPY increases nitric oxide release [58] and NPY-induced vasodilatation is endothelium- and nitric oxide-dependent [59]. In response, nitric oxide inhibits the NPY-induced vasoconstriction [60] and decreases Y1 receptor binding and expression [61], which is one of the receptors mediating the potent vascular growth-promoting activity of NPY, leading to neointima formation [62].

Ultrasound techniques are noninvasive methods to study endothelial function by measuring endothelium-dependent vasodilator responses to various stimuli. The most frequently used method is flow-mediated, endothelial-dependent vasodilatation (FMD) of the brachial artery, where increases in fluid shear stress stimulate endothelial cell release of nitric oxide, causing a dilation of the blood vessel. Our studies indicate that L7P substitution is associated with enhanced endothelial-dependent vasodilatation, in two independent study cohorts of middle-aged men and in healthy 9–11 year old children [21]. Study subjects with P7 variant had approx. 50% higher FMD than subjects with [p.L7]+[p.L7] genotype, which indicates enhanced systemic endothelial func-

tion. The finding of enhanced endothelial function in the carriers of P7 substitution is opposite to what was expected, as previous studies have linked the P7 substitution with increased risk of atherosclerosis and following cardiovascular complications, indicating endothelial dysfunction. However, this may not be surprising, if we consider the multifaceted role of NPY: a systemic neurotransmitter and a local neuromodulator, the potent vasoconstrictor and a vasodilator [33, 63]. It is possible that the cuff occlusion test produces a local shear stress, which increases the local endothelial release of NPY in the carriers of the P7 substitution. This may stimulate Y2 receptors on the endothelial cells and dilate the SMC by releasing nitric oxide.

Restenosis is a complication of 30–40% of patients undergoing coronary angioplasty [64]. It is derived by vascular responses, such as neointimal hyperplasia due to vascular SMC migration and proliferation, neointimal inflammation, and increased neoangiogenesis at the site of the injury. NPY is potentially involved in the process of restenosis: it is a mitogen for human SMCs, highly angiogenic, and promotes inflammatory responses [57]. Consequently, subjects with L7P substitution and increased secretion of NPY during sympathetic activation could release more NPY after endothelial cell damage, thus exposing the SMCs to the mitogenic, angiogenic, and pro-inflammatory effects of NPY. We tested the association of L7P substitution with restenosis after coronary stenting in a large German cohort and find no association [65]. The study was primarily designed to detect a putative association between the NPY polymorphism and acute restenosis (<6 mo); therefore, it does not elucidate the long-lasting vasoconstrictive and remodeling effect of NPY after angioplasty. Elevated levels of NPY may cause chronic coronary artery spasm and increase in peripheral vascular resistance, which may maintain ischemia during the recovery phase, as suggested by others [55].

Retinopathy and nephropathy

Diabetic retinopathy (DR) is a retinal neovascularization process which can lead to blindness. DR is associated with progressive retinal ischemia, with resulting microangiopathy that affects retinal pre-capillary arterioles, capillaries, and venules [66–68]. Ischemia, in turn, activates a number of angiogenic and mitogenic growth factors leading to increased vasopermeability, tissue edema, endothelial cell proliferation, and retinal neovascularization. Since NPY stimulates endothelial cell adhesion, migration, proliferation, capillary tube formation, and aortic sprouting [63, 69], it is justified to propose a role for NPY in the pathophysiology of retinopathy. Additionally, experimental data in $Y2^{-/-}$ -mice lacking the functional Y2-receptor gene and in rats treated with the Y2-receptor mRNA targeted antisense oligonucleotide, show that inhibition of the Y2-receptor functions, significantly limits retinal neovascularization [70]. These findings are in agreement with the data showing that angiogenic effects of NPY are mediated primarily via the Y2-receptor subtype

and are particularly linked to angiogenesis during ischemia [69, 71, 72]. The functional L7P substitution is associated with progression and development of DR in type 2 diabetes patients [70, 73]. The L7P substitution is not linked with type 2 diabetes itself, since the carrier frequency is identical between the type 2 diabetes patients not having DR and the population control, or with DR type 1 diabetes patients. The P7 variant seems to be a significant risk factor for development of DR, especially in type 2 diabetes patients. This data suggests pathophysiological mechanisms underlying type 2 diabetes together with retinal ischemia and enhanced stress-induced NPY release in patients with P7 variant activate the noxious angiogenic cascades in retinal endothelial cells. This may be mediated by local NPY overproduction and signaling via the Y2-receptor, since it is known that during ischemia, expression of NPY, Y2-receptors, and DDP IV, which converts NPY_{1-36} to Y2/Y5-receptor selective peptide $NPY_{3-36,}$ are markedly increased in the endothelium [69, 74]. Furthermore, the Leu7Pro polymorphism may promote retinopathy indirectly via alterations in the regulation of stress-related GH [12], as well, since GH is an additional etiological factor in ischemia-induced retinal neovascularization [75]. NPY and the Y2-receptor could be considered as important molecular mechanisms that are involved in the pathophysiology of retinal neovascularization diseases and as possible drug discovery targets.

The nephropathy is a serious long-term complication of diabetes and the development of persistent proteinuria predicts renal failure and early death. There seems to be a link between albuminuria (nephropathy) and atherosclerosis in coronary arteries, but albuminuria is also a predictor of microangiopathy in other organs than the kidneys, like in the retina. Evidence suggests that a genetic predisposition is involved in the pathogenesis of diabetic nephropathy, both in type 1 and 2 diabetes. Plasma NPY and urinary excretion of NPY are higher in patients with advanced nephropathy than in the control subjects [76]. The L7P substitution is independently associated with diabetic nephropathy in a cohort of Finnish type 1 diabetic patients [77]. The P7 variant was more common in patients with nephropathy in comparison to the patients with normoalbuminuria. The molecular mechanisms behind this association may be similar as speculated for L7P substitution and retinopathy (see above).

BMI and obesity

NPY plays an important role in the central regulation of food intake. NPY has been since its discovery the most potent and probably most studied neuropeptide which stimulates appetite, inhibits thermogenesis and lipolysis, all aiming to an enhanced body weight gain and obesity. Although the role of NPY in regulation of human feeding and energy balance is not totally clear, there are several human linkage studies which connect NPY to human obesity and suggest the *NPY* gene is a candidate gene for obesity related phenotypes [35, 78]. The most widely studied genetic variant of *NPY* gene, which has been used in asso-

ciation studies, is the L7P substitution. Although there are several negative studies, there are also many positive associations. The largest study, which found a positive association, was done in a Swedish population. Subjects with the P7 substitution had higher mean BMI values both in men and women. However, this effect was only observed in non-obese subjects, which suggests that other genetic or environmental factors might be involved and might override the effect in obese subjects [10]. There are also some subpopulations where a positive association has been found. L7P substitution of the *NPY* gene is independently associated with BMI in type 1 diabetics [77] and a higher birth weight in boys [16]. There is a conflicting result where the L7P substitution is associated with lower BMI in premenopausal women, but this may be due to a very low number of subjects with P7 variant (n = 7) in the study [19].

There are also studies testing the association between obesity-related phenotypes and other variations of *NPY* gene. A two base pair TG insertion/deletion (I/D) variant at the position –968 (c.–968_-969delTG) in the promoter region of *NPY* gene was found to be associated with BMI and body fat patterning in non-obese subjects [79]. In this study, they did not observe any L7P substitution, which may have depended on the population studied (Mexican–Americans). In a recent study with a Swedish population, no association of BMI and c.-968_-969delTG variations was observed [10].

Endocrine and hormonal changes

NPY modulates centrally, the feeding and energy balance. One of the peripheral energy store signals integrated in hypothalamus by NPY-containing nerves is insulin. In experimental animals, central NPY administration leads to hyperinsulinemia and peripheral insulin resistance [80], but a tonic central effect of NPY is needed with these metabolic abnormalities [81]. Insulin and NPY may also have direct peripheral interactions, since liver contains NPY-ergic neurons, which are involved in the regulation of hepatic function and hemodynamics. NPY administration reduces splanchnic glucose production, i.e., NPY inhibits adrenaline-induced glycogenolysis by inhibiting stimulated renin release and increasing insulin [82]. The L7P polymorphism is associated with decreased insulin, increased glucose concentrations, and a decreased insulin/glucose ratio after meals and oral glucose-tolerance test [11, 47, 48]. There are also opposing results: in a large, heterogenous group of middle-aged subjects, standard intravenous or oral glucose tolerance tests did not reveal any differences in insulin secretion or in insulin sensitivity between the L7P genotypes [83]. If there is a true difference in the insulin levels, it could due to a central component regulating autonomic balance (see above) or peripherally increased local NPY levels in the pancreas, where NPY has been shown to inhibit insulin secretion [84]. There is also a clear central interplay between NPY and insulin: downregulation of hypothalamic NPY by insulin is a prerequisite for its acute inhibitory effect [85]. Also, in the periphery, NPY and

insulin interact: insulin downregulates the secretion of NPY by neurons co-cultured with adipocytes, but not alone [27]. The primary mechanism leading to decreased insulin levels in subjects having the L7P substitution is not known and this difference definitely needs further research.

Ghrelin is a gut-originating growth hormone secretagogues, which stimulates eating and release of GH from the pituitary. Eating and glucose intake is followed by suppression of ghrelin concentrations in the blood. It seems that insulin is a key regulator of the postprandial ghrelin-suppression response. Ghrelin has a dual role in maintaining GH release and in relaying the peripheral signal of energy homeostasis to the CNS. There is a delayed ghrelin response in the subjects with L7P substitution during oral glucose-tolerance test, without any difference in the mean plasma ghrelin concentrations [48]. This is not due to a different leptin concentration, since there are no differences in diurnal leptin levels between L7P genotypes [47]; instead, it could be due to a different feedback signal from altered GH secretion between the two L7P substitution genotypes [12].

Hypothalamic Y2-receptors are shown to regulate bone formation and Y2 knockout mice have increased trabecular bone volume and thickness [86]. NPY receptors are involved in the local regulation of human bone metabolism in human osteoblasts and human osteogenic sarcoma cells [87]. There is an association between the L7P substitution and higher femoral neck bone mineral density, measured by dual X-ray absorptiometry in postmenopausal women [88]. This could be due to the increased growth hormone release [12], which is a known stimulus for bone formation, or it could be due to an altered balance of the autonomic nervous system, which could centrally regulate the bone metabolism. This is an interesting finding and needs further studies to find out the relevance of NPY in human osteoporosis.

Genetically determined changes in NPY levels lead to widespread consequences in the control of sympathoadrenal, metabolic, and hormonal balance in healthy subjects.

Diabetes

There are no studies associating *NPY* gene variants to prevalence or incidence of diabetes *per se*. There are, however, studies linking lower plasma NPY in diabetics to sympathetic nerve failure [89], which could be considered as an independent predictor of cardiovascular mortality in patients with type 2 diabetes. There is a study where L7P substitution associates with an earlier onset of type 2 diabetes [100] or with the worse glycemic balance in type 1 diabetic patients, as measured with HbA_{1c} [77]. However, most of the association studies found previously positive in healthy study subjects, have resulted in negative associations when studied in diabetic patients. There is no association with serum lipid levels [18, 70] or with plasma NPY levels [77] between the [p.L7]+[p.L7] genotype and [p.L7]+[p.P7] genotype in diabetic patients.

Instead, many studies have shown that diabetic patients with P7 variant are more prone to serious complications of the disease (see above). It seems that diabetes alters the metabolic status of the body in such a way, that the 'normal' interactions of NPY are disturbed and the altered NPY balance further compromises the metabolic balance of the body. This may again happen at the central autonomic level or at the peripheral, local level or at both levels.

Conclusions

Endothelial dysfunction and impaired cardiovascular autonomic nervous system regulation are both markers for increased cardiovascular disease risk. Sympathetic nerves and vascular endothelial cells balance to maintain appropriate blood vessel tone, and alterations in sympathetic activity and endothelial cell function are both observed early in the development of cardiovascular disease. This may result from an unbalance of these two systems and further contribute to disease development [90]. NPY has long been recognized as a major transmitter of sympathetic nervous system, and its neuro-modulatory cardiovascular effects are well characterized. Recent data, however, emphasize the effects of NPY as a local transmitter and trophic factor on endothelial cells. Since NPY seems to be involved in regulation of sympathetic tone and vascular function, it is an interesting candidate for studies on cardiovascular and metabolic homeostasis. There are many association studies between the *NPY* gene variants and cardiovascular and metabolic disease. Most are done by using p.L7P substitution as a marker, and these studies suggest that the L7P substitution may be a strong independent risk factor for various cardiovascular diseases. Although there is now clear evidence that the L7P substitution alters the NPY secretion in humans, the exact cellular and molecular mechanism and the functionality of this variation is not yet known. The problem with the cellular studies is that the substitution changes an amino acid in the hydrophobic signal sequence part of the preproNPY. We and others have attempted but failed several times to raise antisera to the NPY signal peptide, for possible use in localization and release studies. But this is very difficult because of high hydrophobicity (Pesonen et al., unpublished; [10]).

Another problem with the association studies with L7P substitution is that there is a considerable variation in allele frequencies in different populations. The P7 allele is found mostly in populations of Caucasian descent, and there seems to be a geographical north-east to south-west gradient from 7–14% in Europe (Pesonen et al., unpublished; [8]). The African–American, Mexican–American, and Oriental populations have a very low frequency of the P7 variant. Therefore, the effects of the L7P substitution on cardiovascular regulation and diseases are not likely to be relevant in populations other than those of European descent. This does not, however, inhibit drawing conclusions of physiological and/or pathological roles of NPY in cardiovascular control and development of diseases, since the study of DNA sequence variants that contribute

to common disease risk offers one of the best opportunities for understanding the complex cause of disease in humans.

Sequence variations within genomes are responsible for individual phenotypic characteristics, including a predisposition to complex disorders such as cardiovascular disease. Most frequently used of these variations are SNPs or small-scale multi-base deletions or insertions (also called deletion insertion polymorphisms or DIPs). The usual approach for using SNPs for the investigation of the genetics of a disorder is to examine a single diagnostic syndrome or variables relating to a cluster of symptoms and do an association study. Difficulties can arise from the diagnosis and phenotype classification, sample size and heterogeneity, which are all likely to account for variability between studies. Also, the environmental factors can modify biological processes and responses in diseases. The ultimate goal in clinical correlation studies is to identify causative SNPs – those that produce alterations in gene expression or in the expression or function of the gene product – because such SNPs will be most predictive of a possible clinical phenotype or of pharmacogenetic responses. Although each SNP can be analyzed independently of other SNPs, it is much more informative to analyze SNPs in a region of interest, simultaneously. Alleles of SNPs that are closer together (e.g., SNPs within the same gene) tend to be inherited together, a phenomenon called linkage disequilibrium (LD) or allelic association. When a new mutation occurs and is spread to the next generation, the mutated allele will be co-inherited with the alleles at the neighboring loci and all these loci will be in LD with each other. SNP can also serve as surrogates for unrecognized, neighboring, functional SNPs that may be identified by disease-marker linkage disequilibrium studies. The block of co-inherited alleles is called a 'haplotype', and haplotype analysis has more power than traditional single SNP association study design. The *NPY* gene has very few common variants (Tab. 1), which could be used in haplotype analysis in all populations. However, haplotype analysis would greatly enhance the association studies on NPY variants and cardiovascular and metabolic studies.

Although single SNP association studies have many pitfalls, they can guide functional studies. A well-designed association study requires a significant number of cases to be adequately powered to study disease genetics. It has been shown that carefully matched, moderate-sized case-control samples in Caucasian populations are unlikely to contain levels of structure that would result in significantly inflated numbers of false-positive associations. There also might be a replication problem, which is mostly due to extreme differences in power among studies, since there is variation in sample size and risk-allele frequency between the study cohorts [91].

In conclusion, SNP association studies, or preferably haplotype analysis, on *NPY* gene can be used to guide functional studies and elucidate the pathophysiological NPY related mechanisms behind cardiovascular and metabolic disorders. At the moment it seems that L7P substitution of preproNPY protein causes altered NPY secretion, which leads to haemodynamic disturbances caused by sympathetic hyperactivity, and to vascular dysfunction and trophic effects,

caused by altered local signaling by NPY. The clinical relevance of L7P genetic variant in cardiovascular regulation and clinical prediction, and the treatment of cardiovascular and hypertensive diseases, remains to be determined.

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Summary

NPY Family of Peptides in Neurobiology, Cardiovascular and Metabolic Disorders:
from Genes to Therapeutics
Edited by Zofia Zukowska and Giora Z. Feuerstein

Future directions and perspectives for therapies based on the NPY family of peptides

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The science presented and analyzed in this book provides powerful evidence of the importance of the NPY family of peptides in a variety of human diseases. Why then have 20 years of research in this area not produced any therapeutics? Part of the reason may be the redundancy of the system: one peptide may compensate for the loss of another, and multiple receptors may serve similar functions. The field, however, remains replete with promise. Novel leads for therapeutic applications are provided by the discovery of new activities of these peptides and their specific receptors, such as neuroregeneration and bone remodeling. Other well-known actions of NPY/PYY/PP such as regulation of appetite and anxiety still remain attractive targets. At the same time other activities, such as Y1 receptor-mediated vasoconstriction, appear to have lost their place as potential therapeutic targets, although new evidence of the role of Y1 receptors in vascular remodeling may require reevaluation of their utility in cardiovascular diseases: hypertension and ischemic vascular diseases. The same receptor but in the central nervous system appears to be involved in neuroregeneration, anxiety and stress disorders – suggesting therapeutic potential of drugs such as Y1 agonists or NPY-elevating drugs such as Y2 antagonists, which presynaptically increase peptide release. Y2 antagonist also emerged as new targets for osteoporosis therapy, and agonists for Y2 (and Y4) are being tested in treatment of obesity as peripherally acting appetite suppressants. Most recently, successful manipulation of the NPY system was reported via gene transfer of the leptin gene, which shuts down the brain NPY hypothalamic activity – opening possibilities of gene therapy for obesity.

This overview of NPY peptide-dependent diseases and potential therapeutics should provide a foundation for better understanding of the accomplishments and challenges that this vast field presents. Such periodic integrative analysis is necessary to critically evaluate the therapeutic potentials and clinical implications of the continually growing scientific data. The editors hope that this book will inspire cross-fertilization between distant and not so distant fields of NPY research, and serve as a reference for further scientific pursuit and drug discovery.

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