MINIREVIEW

Recent Developments in Our Understanding of the Physiological Role of PP-Fold Peptide Receptor Subtypes[1]

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The three peptides pancreatic polypeptide (PP), peptide YY (PYY), and neuropeptide Y (NPY) share a similar structure known as the PP-fold. There are four known human G-protein coupled receptors for the PP-fold peptides, namely Y1, Y2, Y4, and Y5, each of them being able to bind at least two of the three endogenous ligands. All three peptides are found in the circulation acting as hormones. Although NPY is only released from neurons, PYY and PP are primarily found in endocrine cells in the gut, where they exert such effects as inhibition of gall bladder secretion, gut motility, and pancreatic secretion. However, when PYY is administered in an experimental setting to animals, cloned receptors, or tissue preparations, it can mimic the effects of NPY in essentially all studies, making it difficult to study the effects of PP-fold peptides and to delineate what receptor and peptide accounts for a particular effect. Initial studies with transgenic animals confirmed the well-established action of NPY on metabolism, food-intake, vascular systems, memory, mood, neuronal excitability, and reproduction. More recently, using transgenic techniques and novel antagonists for the Y1, Y2, and Y5 receptors, NPY has been found to be a key player in the regulation of ethanol consumption and neuronal development. Exp Biol Med 228:217–244, 2003

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The PP-fold family of peptides consists of neuropeptide Y (NPY) and the two gut hormones, peptide YY (PYY) and pancreatic polypeptide (PP). The neuronal and hormonal functions of the PP-fold peptides are intermixed because all three members are found in the bloodstream and can act at several G-protein-coupled receptors (GPCRs). Furthermore, all known PP-fold peptide receptors are found both in nerve cells of the central and peripheral nervous systems as well as in non-neuronal tissues and can bind at least two of the three PP-fold peptides. Thus, it is possible for the peptides to modulate or potentially assume the function of one another. In addition, although NPY is strictly localized in neurons, PYY and PP are mainly found in the gut. However, several studies have found PYY in neurons. Therefore, PYY may exhibit neurotransmitter functions as well. This potential redundancy is important to bear in mind, especially when discussing results from gene-targeting experiments (i.e., knockout and transgenic animals).

This article is an update of previous reviews on PP-fold peptides and receptors in this journal (1, 2). Therefore, we will focus on developments that have occurred since 1998. Since the last review, no new PP-fold peptides or PP-fold receptor subtypes have been cloned from mammals. In fact, knowledge about the full sequence of the human genome will end the complete discovery of new mammalian genes. Nevertheless, new receptors have been cloned from lower vertebrates and invertebrates. However, these will only be discussed briefly in this review when binding data and sequence comparisons can be extrapolated to the mammalian receptors. The cloning of PP-fold receptors during the first 7 years of the 1990s has given us the tools to explore the function of the PP-fold peptides and their receptors both *in vivo* and *in vitro*. Therefore, we will discuss advances in our understanding of the role of PP-fold peptides using genetic

techniques such as knockout or overexpressing rodents. By comparison, at the time of the latest review, there were only a few Y1-selective nonpeptide antagonists available, and peptides previously thought to be selective were found to be nonselective. Since then, several subtype-selective ligands, both agonists and antagonists, have been discovered that provide better tools to dissect what receptor is responsible for a particular effect.

Structure(s) of PP-fold Peptides. NPY, PYY, and PP, together with the fish peptide Y (PY), share a common hairpin-like three-dimensional structure called the PP-fold (3). All four peptides are 36 amino acids long (except chicken PYY, which is 37) with an amidated carboxy-terminus. The general structure of the PP-fold peptides has been established using x-ray crystallography of avian PP (4) and confirmed in several studies using nuclear magnetic resonance, most recently for PYY (5) and the synthetic analog [Leu^{31},Pro^{34}]NPY (6). Amino acid residues 1–8 form a type II proline helix followed by a loop. Residues 15–32 form an α-helix, and the four most carboxy-terminal residues are in a flexible loop conformation. NPY is one of the most evolutionary conserved peptides known. Only two of the 36 amino acids of NPY are variable between mammals. PYY has evolved at a higher rate than NPY and has eight variable amino acids between different orders of mammals, whereas the third member of the PP-fold family of peptides, PP, has evolved very rapidly and is one of the least-conserved peptides known (see Ref. 7 for review). However, despite the low degree of conservation in amino acid sequence between PP from different species as well as between PP and PYY and NPY, the general three-dimensional structure seems to be conserved in all PP-fold peptides (4–6).

There are several reports on endogenous circulating amino terminally truncated fragments of NPY (and PYY), such as NPY2-36 and NPY3-36 (8) and PYY3-36 (9). These fragments are intermediate degradation products from peptidergic breakdown of NPY and PYY or result from specific cleavage by aminopeptidases (8, 10) and may have a physiological role resulting from the affinity and selectivity of amino terminally truncated NPY fragments for Y2 and Y5 receptors.

PP Function. PP was the first of the PP-fold peptides to be identified and sequenced. It was found as a contaminant in extracts of chicken insulin (11, 12). Fish lack PP, indicating that PP probably arose as a local gene duplication of the PYY gene in early tetrapod evolution (7). The genes for PP and PYY are located only 10 kb apart from one another on chromosome segment 17q21.1 in humans (13). PP is almost exclusively expressed in endocrine pancreas and is released in response to meals (14). The effects of PP, including inhibition of pancreatic secretion, gall bladder activity, and intestinal motility, are mainly located in the gastrointestinal (GI) tract (1). Recently, PP has been found to inhibit ileum contractions (15) and to stimulate colon contractions (16). In addition, PP affects metabolic functions, including glycogenolysis, and decreases fatty acid levels (2). However, binding sites for PP have been found in several rat brain regions including the interpeduncular nucleus, hypothalamus, and brainstem (17–19), suggesting that PP may also have direct effects of brain function. This may explain why intracerebroventricular injection of PP has been found to stimulate feeding in several different species of experimental animals (20–22).

PYY Function. PYY was first isolated from porcine intestine using a method that captured peptides with an amidated carboxy terminus (23, 24). The peptide had tyrosines in both ends and was therefore named peptide YY after the single letter abbreviation for tyrosine (Y). PYY is released in the GI tract in response to meals. Many of the GI effects described for PP, including inhibition of gall bladder secretion, gut motility, and pancreatic secretion, can also be provoked by PYY (1). Other peripheral effects produced by PYY are the inhibition of fluid and electrolyte secretion in the intestinal tract (25) and vasoconstriction (26). PYY has also been found in neurons indicating that PYY may also have a neuronal function (see Ref. 27 for review).

The chromosome segment that harbors the genes for PYY and PP (17q21.1 in humans; Ref. 13) has been duplicated one more time in primates to generate two new genes, PYY2 and PP2 (28). Specific mutations have changed the processing of the protein generating a product with very low structural similarity to the PP-fold peptides. The function of these gene products is presently unknown.

NPY Function. NPY was first isolated from pig brain using the same method as for PYY (29, 30). It is widely distributed throughout the mammalian brain but it is also found in many peripheral areas. NPY is one of the most potent orexigenic peptides known. The feeding effect of NPY appears to be very well conserved because NPY has been found to induce food intake with a preference for carbohydrate-rich food when injected intracerebroventricularly in several species, including snake (31), goldfish (32, 33), chicken (34), rat (20, 35), mouse (36, 37), rabbit (38), guinea pig (39), sheep (40), dog (41), pig (42), and rhesus monkey (43). When administered centrally, antisense oligonucleotides against NPY reduce feeding (44, 45). Interestingly, a synthetic fatty acid synthesis inhibitor, C75, dramatically decreases hypothalamic mRNA levels for NPY and causes an almost total inhibition of feeding in both lean and obese mice (46, 47). Centrally administered NPY also regulates metabolism by decreasing energy expenditure (48). Other centrally mediated effects of NPY are decreased thermogenesis (49), anticonvulsant activity (50, 51), inhibition of sedation (52, 53), mood, and memory (see Refs. 2, 54, and 55 for reviews). Recently, it was found that NPY-deficient mice have an impaired development of neurons in the olfactory epithelium (56, 57), which suggests a role in neuronal development. NPY is involved in regulation of reproduction because it stimulates luteinizing hormone-releasing hormone (LHRH) release (58), an effect that can be inhibited by NPY antisense oligonucleotides (59). Fur-

thermore, when NPY is injected into the suprachiasmatic nucleus, it inhibits cell firing (60) and induces a forward shift in circadian rhythms (60–62), possibly through reduction of Per1 and Per2 mRNA levels. NPY is colocalized with noradrenaline in sympathetic nerves and acts to enhance noradrenaline-mediated vasoconstriction, especially upon strong stimulation (see Ref. 63 for review). Contrary to this, centrally administered NPY reduces arterial blood pressure and heart tone shown in both rats and dogs (64). Another vascular effect is stimulation of smooth muscle proliferation (65). In addition, NPY can act as an antinociceptive peptide (66), probably through inhibition of substance P release in the dorsal horn of the spinal cord. NPY and the Y2 receptor are also dramatically upregulated after axotomy of the sciatic nerve in the dorsal root ganglion. This provides further evidence for the involvement of NPY in pain modulation (67–69).

Several disorders and pathological conditions are associated with altered NPY function. Based on the strong orexigenic effect of NPY, the most obvious are various eating and metabolic disorders. NPY levels are changed in all conditions involving a disturbed energy balance, such as anorexia, bulimia neurosa, and diabetes (2). Moreover, several cardiovascular dysfunctions as well as some tumor diseases (70) are associated with increased plasma levels of NPY.

One of the most important developments in NPY research is the recent finding that NPY regulates ethanol consumption. In a genetic study comparing the ethanol preferring and nonpreferring rats, the locus that harbors the gene for NPY was identified (71), suggesting that dysfunction in the gene locus contributes to ethanol preference in these rats. The role of NPY as a potential regulator of alcohol consumption has also been investigated in transgenic mice (72) where an inverse correlation between NPY levels and drinking has been shown (see also transgenic section). At this point, it is unclear how these findings relate to the well-established anxiolytic actions of NPY (73). Furthermore, a point mutation (leucine^7 to proline) in the pre-pro NPY gene has been associated with higher alcohol consumption in humans (74). This mutation has also been linked to higher cholesterol, serum lipids, and progression of carotid atherosclerosis (75, 76). NPY appears to be involved in regulation of neuronal excitability as mice lacking NPY are more susceptible to seizures (50), and people with temporal lobe epilepsy have increased NPY expression in CA3 regions as well as prominent rearrangements in receptor distribution (77).

NPY is regulated by several other neuropeptides and hormones. Two hormonal signals that act on NPY neurons are ghrelin and leptin (78). Ghrelin is a 28 amino acid peptide that is released from the gut and binds to a specific GPCR to signal release of growth hormone from the pituitary (79, 80). Both intravenous and intracerebroventricular injections of ghrelin result in a dose-dependent increase in growth hormone levels in plasma (see Ref. 80 for review). Interestingly, this peptide is present in neurons of the hypothalamic arcuate nucleus where ghrelin acts to increase the hypothalamic levels of NPY and agouti-related protein mRNA levels (81) and feeding and body weight in rats (81, 82). In contrast, the adipose hormone leptin acts as a satiety factor possibly by inhibiting NPY release in the hypothalamus (83). Another peptide system, the melanocortins, has been found to interact with NPY (84–86). When co-injected intracerebroventricularly, the endogenous agonist α-melanocyte-stimulating hormone dose-dependently inhibits NPY-induced feeding (86). It is likely there are additional neuropeptides and endocrine hormones that regulate or are regulated by NPY. Research over the next 5 years should help unravel these undoubtedly complicated interrelationships.

PY. Fish lack PP but have a fourth PP-fold family peptide, PY, which can be found in the pancreas of several bony fishes (87). This peptide is equally identical to mammalian NPY as to mammalian PYY but displays higher identity to fish PYY than to fish NPY. Thus, PY is most likely the result of an independent evolutionary event in the acantomorph fish lineage (87). Recently, the genes for NPY, PYY, and PY were cloned from sea bass providing strong evidence that the PY gene is the result of a unique gene duplication in fish, most likely of the PYY gene (87). Therefore, it is unlikely that a mammalian ortholog of PY exists.

Receptors for the NPY Family of Peptides

Structural and General Features of PP-fold Receptors. NPY, PYY, and PP bind to a large and very heterogeneous family of GPCRs belonging to the rhodopsin like superfamily (class 1) of receptors. Five PP-fold family receptors have been cloned from mammals (Y1, Y2, Y4, Y5, and y6; see Table 1 for cloning references and ligand binding preferences) and several additional receptors have been postulated from pharmacological profiles using various tissue preparations (55). However, no additional mammalian receptor subtypes have been identified since 1996. The y6 receptor has been given the lower case designation (IUPAR nomenclature; Ref. 54) because it encodes for a truncated receptor in most mammals, including humans (54). Cloned Y1, Y2, Y4, Y5, and y6 receptors have all been shown to couple to inhibitory G-proteins (G_i) and thus mediate inhibition of cAMP synthesis (88–92). The Y1 receptor has also been shown to activate mitogen-activated protein kinase in gut epithelial cells (IEC-6; Ref. 93). Very recently, it was shown that activation of PP-fold receptors involves pertussis toxin-sensitive phosphorylation of extracellular signal-regulated protein kinase 1 and 2 in Chinese hamster ovary cells, confirming that these receptors couple to Gi/Go (94). Furthermore, whereas protein kinase C seems to be necessary for Y5 receptor signaling, a protein kinase C-independent pathway may also be involved in Y1, Y2, and Y4 signaling (94). Y1, Y2, Y4, and Y5 receptors can also couple to phospholipase C to provoke release of Ca^{2+} from intracellular stores (88, 89, 91, 95).

The PP-fold receptors display many of the structural features of rhodopsin-like receptors, including two extracel-

Table I. Cloned NPY Receptors with Ligand-Binding Profiles

Receptor	Ligand-binding profile	Cloned from (Ref.)
Y1	NPY ≈ PYY ≈ [Leu^{31}, Pro^{34}]NPY > NPY2-36 > NPY3-36 ≥ PP > NPY13-36	Human (88, 89) Rat (97, 297) Mouse (298) Guinea pig (299) Dog (105) Pig (105, 139) Rhesus monkey (300) *Xenopus laevis* (190) Chicken (189)
Y2	NPY ≥ NPY2-36 ≈ NPY3-36 ≈ NPY13-36 >> [Leu^{31}, Pro^{34}]NPY	Human (134–136, 205) Rat (204, 301) Mouse (302) Guinea pig (138) Pig (105, 139) Cow Accession no: AAB40600 Rhesus monkey (300) Chicken (140)
Y4	PP > PYY ≥ NPY > NPY2-36	Human (90, 95, 154) Rat (152, 154) Mouse (303) Guinea pig (155) Pig (139) Chicken (304)
Y5	NPY ≈ PYY ≈ NPY2-36 > hPP > [D-Trp^{32}]NPY > NPY13-36 > rPP	Human (91, 164) Rat (91, 164) Mouse (165, 166) Guinea pig (168) Pig (139) Dog (166) Rhesus monkey (300) Chicken (189)
y6*	1) NPY ≈ PYY ≈ [Leu^{31}, Pro^{34}]NPY >> PP 2) PP > [Leu^{31}, Pro^{34}] NPY > NPY ≈ PYY	Human (183, 184, 186) Mouse (184, 185) Rabbit (183) Guinea pig (188) Pig (139)

* Two different labs cloned the mouse y6 receptor but reported very different binding profiles: 1) according to (185) and 2) according to (184). No pharmacology is available for the truncated human, guinea pig, and pig y6 receptors.

lular cysteines that are believed to form a disulfide bond between extracellular loop (EL) I and EL II as is known from the x-ray crystallography structure of bovine rhodopsin (96). Y1, Y4, and y6 also have two additional cysteines, one in the amino-terminal tail and one in EL III that potentially can form a second disulfide bound. All PP-fold receptors have one or several consensus sites for N-linked glycosylation (Asn-X-Ser/Thr). The Y1 and Y4 receptors have three such glycosylation sites located on the extracellular side in the amino terminus and one in the second extracellular loop whereas the Y2 and Y5 receptors have only one and two glycosylation sites in the amino terminus, respectively. Furthermore, all PP-fold receptors have at least one cysteine in the cytoplasmic tail that probably anchors the tail to the inside of the membrane by palmitoylation similar fashion as bovine rhodopsin (96). The amino acids immediately after transmembrane region (TM) 7 of bovine rhodopsin were found to form an α-helix located on the inside of the membrane (96). These amino acids are well conserved among the PP-fold receptors compared with rhodopsin, suggesting that this structure is present in these receptors too (Fig. 4).

The Y1 Receptor. The Y1 receptor was the first PP-fold peptide binding receptor to be cloned. It was originally published as a rat orphan receptor (97) and later identified as the Y1 receptor based on its distribution (88, 89; see Table I for additional cloning references).

The tissue expression of the Y1 receptor can be regulated differently because of the use of different promoters regulated by alternative splicing (98). The gene for Y1 is located in a cluster together with Y2 and Y5 on human chromosome 4q31. Contrary to the other PP-fold receptors, the coding region of the Y1 gene harbors an intron of about 100 bp in all species explored. This intron has been shown to enhance the expression of the Y1 and Y5 receptors *in vitro* (99). Interestingly, two *in vivo*-expressed splice variants of the mouse Y1 receptor have been found (100). The short form (307 amino acids) of the Y1 receptor ends a few

amino acids after the third extracellular loop, yielding a receptor with an incomplete TM7. NPY binds to this short form of the Y1 receptor with similar affinity as to the complete 384 amino acid protein. However, the signaling of the short form of the receptor was impaired, indicating that the TM7 and the carboxy-terminal tail are not essential for ligand interaction but rather for G-protein activation. Like many GPCRs, the Y1 receptor can be internalized together with the ligand upon agonist stimulation shown by radioligand binding (101), confocal microscopy with fluorescent ligands (102), as well as by tagging the receptor with green fluorescent protein (103). Upon stimulation with PYY, the Y1 receptor was rapidly internalized into endosomes and recycled to the surface within 60 min (103).

Most of the vascular (65, 104–106) and antinociceptive effects (107, 108) of NPY are transduced via the Y1 receptor. For example, the Y1-selective antagonists SR120819A (109), BIBP3226 (110; Fig. 1), BIBO3304 (111), and H394/84 inhibit NPY-induced vasoconstriction in a variety of species (106, 109, 112, 113). Interestingly, the centrally induced vascular effects of NPY (reduced blood pressure and heart rate) are also signaled mainly through Y1 (64), as are many of the psychological functions of NPY, such as decreased anxiety and depression (114–118). Y1 is involved in the feeding response of NPY (43, 111, 119–123). Hypothalamic Y1 mRNA levels decrease during fasting (124). Although intracerebroventricular injection of Y1-selective agonists increase feeding (125), Y1 antagonists can inhibit NPY-induced feeding (37, 43, 126). Surprisingly, Y1-antisense oligonucleotides have been found to increase food intake in energy-deprived rats (127). The Y1 receptor is also involved in the cross-talk between NPY and another orexigenic peptide in the hypothalamus as the feeding response of melanin concentrating hormone is attenuated by administration of Y1 receptor antagonists (128).

Recent reports described an emerging role for NPY in the regulation of ethanol consumption. Injections of NPY into the paraventricular nucleus increase ethanol intake in rats (129). The importance of Y1 in the regulation of ethanol consumption has been confirmed using both Y1 specific

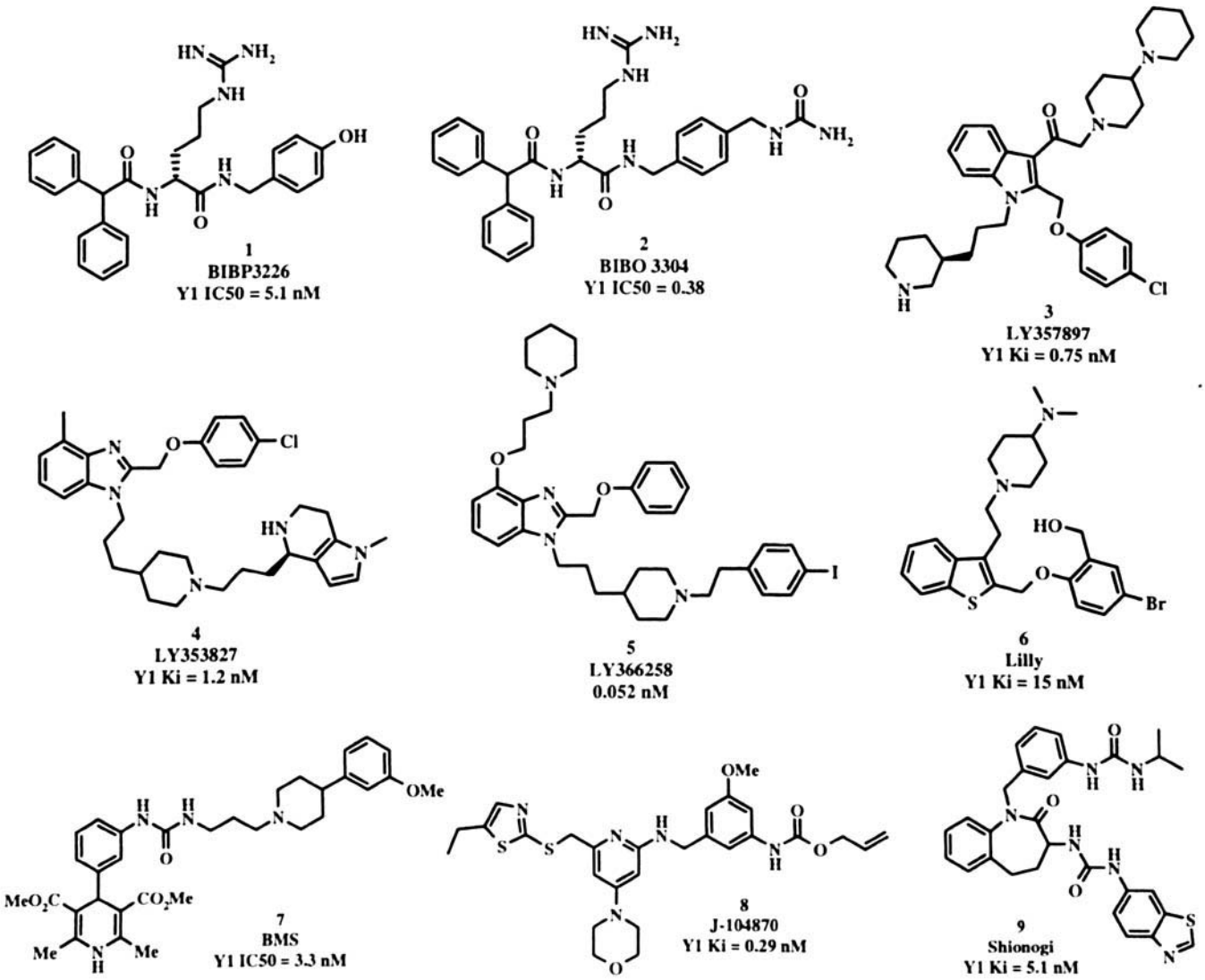

Figure 1. Novel nonpeptide analogs with selectivity for the Y1 receptor.

antagonists (129) and knockout animals (130). In addition, the ability of NPY to regulate arousal is mediated by Y1, but Y2 receptors are also involved in an opposing fashion (52, 53).

The Y2 Receptor. Originally, the Y2 receptor was identified using vascular preparations and defined by the activity of amino terminally truncated fragments of NPY and PYY, such as NPY3-36 and NPY13-36, that are full agonists with similar potency as the native peptides (Table I; Ref. 131). When positions 31 and 34 of NPY or PYY (Ile and Gln) are replaced by the corresponding amino acids in PP, Leu, and Pro, respectively, the resulting peptides do not bind to Y2 (132), although this peptide remains a potent full agonist at the other PP-fold receptors. It was later shown that only the Pro^{34} substitution was essential for preventing Y2 receptor binding (133). When the Y1 receptor was cloned, it was assumed that the Y2 receptor would display a high degree of sequence identity to Y1. When homology screening failed for the Y2 receptor, several research groups finally turned to various expression cloning approaches and found cDNA clones coding for proteins with PYY-binding abilities (134–136). The Y2 receptor gene codes for a 381 amino acid protein and is located close to the Y1 gene on chromosome 4 (137). Like Y1, the Y2 receptor is highly conserved between species with more than 90% identity between orders of mammals (105, 134–136, 138, 139) and about 80% identity when comparing mammalian and chicken Y2 (140). Surprisingly, the Y2 receptor was only about 30% identical to the Y1 and Y4 receptors explaining the failure of homology screening approaches. Unlike the Y1 receptor, Y2 does not appear to internalize after prolonged agonist stimulation (101, 103) or does so very slowly.

The Y2 receptor is mainly located presynaptically where it acts as an autoreceptor, inhibiting further release of neurotransmitter (131, 141, 142). This may explain why agonists specific for the Y1 receptor are anxiolytic (114, 115) whereas Y2 agonists like NPY13-36 and C2-NPY appear to be anxiogenic (114, 143). The same opposing relationship between Y1 and Y2 is evident for the central effects of NPY on blood pressure as Y2 specific agonists increase blood pressure whereas activation of central Y1 receptors decreases it (64). The Y2 receptor is also directly involved in some of the vascular effects of NPY (144). For instance, in pig spleen, a Y2-specific agonist, evoked potent vasoconstriction (105) that could be inhibited by a Y2-selective antagonist, BIIE0246, (145; Fig. 2). In addition, Y2 is involved in NPY-induced angiogenesis (65) and NPY-induced effects on circadian rhythms (60, 146, 147). In addition, centrally administered Y2 agonists delay gastric emptying (148, 149). Knockout studies of the Y2 receptor have shown that also this receptor may be involved in the feeding response to NPY (150) as well as in bone formation (151).

The Y4 Receptor. The third PP-fold receptor to be identified by cloning was a receptor with high affinity for PP. The most interesting feature of the Y4 receptor is perhaps the low degree of sequence identity between species. When comparing the rat and human sequences, only 75% of the amino acids are identical, making Y4 one of the most rapidly evolving GPCRs known (90, 152). In addition, the human Y4 receptor gene is highly polymorphic. No less than seven variable positions have been found within the coding part of the gene (Genbank XM_011916). However, all seven polymorphisms found in the hY4 receptor are silent and do not affect the resulting receptor protein. Thus, the PP-fold systems harbor three of the most (Y1, Y2, and Y5) and one of the least (Y4) conserved receptors known as well as a very conserved peptide (NPY) and one very rapidly evolving peptide (PP). The Y4 receptor has been reported to be internalized after agonist stimulation (101). However, another group has reported that Y4 does not internalize (153).

The pharmacological profiles of the rat and human Y4 receptors differ in that the affinity for the rat receptor increases when position 34 of NPY or PYY is replaced by proline (152, 154, 155) whereas the human receptor is unaffected by this change (90, 95). In addition, the difference in affinity between the preferred ligand, PP, compared with NPY and PYY is much greater in rat Y4 than in the human Y4 receptor (90, 152, 155–157). Determination of the pharmacological profile of the Y4 receptor is complicated by the fact that ^{125}I-PYY appears to recognize only a fraction of the receptor population recognized by ^{125}I-PP (157, 158), which may explain some of the differences in pharmacological profiles of cloned Y4 receptors.

As PP is the preferred ligand at the Y4 receptor, it is likely that this receptor mediates many of the GI effects produced by PP like rabbit ileum contractions (15). Centrally located Y4 receptors may be involved in the regulation of reproduction as the Y1 antagonist/Y4 agonist 1229U91 (159, 160) induced release of luteinizing hormone when injected intracerebroventricularly (161).

The Y5 Receptor. NPY and NPY2-36 are equally potent in producing a large increase in feeding after intracerebroventricular administration (162), suggesting the receptor mediating the feeding response to NPY differs from Y1, Y2, and Y4. In addition, NPY with position 32 replaced with D-tryptophan ([D-Trp^{32}]NPY) selectively inhibited NPY-induced feeding (163) though it had relatively low affinity for Y1 and Y2. Thus, a feeding receptor was proposed with the profile NPY = PYY = NPY2-36>NPY3-36≥[D-Trp^{32}]NPY (Table I). Expression cloning from a hypothalamic rat cDNA library resulted in a gene coding for a 446 amino acid protein (91, 164). The Y5 receptor protein is much larger than the other NPY receptors because of the extended third cytoplasmic loop with about 100 amino acids more than the other PP-fold receptors. However, the carboxy-terminal tail of the Y5 receptor is much shorter than in Y1, Y2, and Y4. Interestingly, in the mouse Y5 receptor gene, the 63 nucleotides encoding amino acids 15–35 have been duplicated in tandem, yielding a receptor that is 21 amino acids longer than the otherwise highly identical rat receptor (165, 166). However, this addition does not appear

to affect the pharmacology or signaling properties of the receptor (166). The gene for Y5 is located on human chromosome 4 (4q31) and overlaps with Y1 but is transcribed in the opposite direction. In fact, one of the alternative promoters and 5′ exons of the Y1 gene is located within the coding sequence of the Y5 gene, suggesting at least partially coordinated transcriptional regulation (167).

[D-Trp^{32}]NPY was found to be a modestly selective agonist at Y5 expressed in HEK293 cells acting to inhibit cAMP synthesis but with a lower potency than NPY, PYY and NPY2-36 (91). Although the Y5 receptor is very well conserved (88–90% overall amino acid identity and 95–98% when the third intracellular loop is not accounted for; Refs. 166, 168), between orders of mammals there may be species differences in the endogenous ligand for the Y5 receptor. One interesting feature about the Y5 receptor is that the rat PP binds with very low affinity to the Y5 receptor from various species whereas PP from human and other species has much higher affinity (166). Thus, it is possible that PP is involved in Y5 signaling in humans but most likely not in rats. This may indicate a difference of potential importance when extrapolating effects produced by PP-fold peptides in rodents to physiological and behavioral effects in humans.

The role of the Y5 receptor in NPY-induced feeding has been confirmed by studies involving antisense knockdown (169–172), knockout animals (173), and Y5-selective agonists (48, 91, 174–176). The selective Y5 antagonist CGP71683A (177; Fig. 3) was reported to antagonize NPY induced feeding; however, recent findings suggest that it inhibits feeding by a non-Y5 mechanism (178). Activation of the Y5 receptor also results in a decrease in energy expenditure (48). Other effects of NPY that are mediated by the Y5 receptor are reproduction through inhibition of luteinizing hormone release (179) and regulation of brain excitability and seizures (180, 181). Furthermore, it has been shown that the Y5 selective agonist [D-Trp^{32}]NPY inhibited neuronal activity in the suprachiasmatic nucleus without generating a phase-shift (60) indicating that Y5 may also be indirectly involved in regulation of circadian rhythms (60, 182).

The y6 Receptor. The y6 receptor was first cloned in rabbit (183) and mouse (184, 185). Later, when the human ortholog was cloned, it was found to be a nonfunctional pseudogene because of a frameshift mutation (single base deletion) in the third intracellular loop causing an in-frame stop codon after the sixth TM region (183, 184, 186). All y6 sequences from primate species studied (i.e., chimpanzee, gorilla, marmoset, and human) contain this stop indicating that the mutation causing the stop codon occurred early in the primate evolution before the split into new- and old-world monkeys (183). Yet, mRNA for the truncated form of y6 is expressed in several areas of the primate brain including hypothalamus (183). Although a human y6 receptor with an extra "T" to counteract the frameshift has been expressed *in vitro*, the resulting receptor did not bind any radiolabeled PP-fold peptides (183, 184). The gene for human y6 is localized on chromosome 5 in the 5q31 region (184). Although the gene for y6 is present in most mammals, including mouse, neither mRNA nor the gene encoding y6 were detected in rats (187). A full-length open reading frame for the receptor has also been found in the peccary (188) whereas the gene is frameshifted in the peccarys closest relative, the domestic pig (139). Therefore, the y6 receptor gene must have been shut off or resurrected independently in several evolutionary lineages (188). Thus, it is likely that a subtle mutation occurred in early mammalian evolution and the subsequent loss of the y6 receptor did not have profound physiological consequences allowing survival in its absence.

Messenger RNA for the y6 receptor is expressed in the hypothalamus and in the kidney of the mouse (185). However, because of the lack of pharmacological tools to distinguish the y6 receptor from Y1 and Y5, it has not been conclusively proven that the y6 receptor protein is indeed expressed in the animals where it is not frameshifted. The fact that mice have a functional y6 receptor is also a concern for the interpretation of results from receptor knockout studies.

Receptors from Nonmammalian Species. Y1, Y2, and Y5 have been cloned in chicken (140, 189) and the Y1 receptor has also been cloned from the frog *Xenopus laevis* (190). In addition, there are currently five cloned and functionally expressed Y1-/Y4-/y6-like PP-fold receptors from fish, zebrafish (z) Ya (191), Yb (192), Yc (193), and cod Yb (194), as well as a receptor from the river lamprey (195). The fish Yb/c receptors display Y1-like pharmacology with a gradual loss of affinity by progressive amino-terminal truncation of the NPY molecule as well as recognition of Pro^{34}-substituted analogs. In contrast, amino terminally truncated peptides like NPY3-36 and NPY13-36 as well as Pro^{34}-substituted analogs bind with the same affinity as NPY to the zYa receptor. However, neither the two Y1-selective antagonists BIBP3226 (110) and SR120819A (109) nor the Y2-selective BIIE0246 (196) bind to any of the fish receptors (193–195, 197). Several proposed PP-fold like receptors have been cloned from various invertebrate species (198, 199), but most of them display very low sequence identity to the cloned vertebrate PP-fold receptors. In fact, most of the invertebrate receptors display as low amino acid identity to PP-fold receptors (20–30%) as to other peptide receptors. However, the amino acid sequence of the Y1, Y2, and Y5 receptors are also only 25–30% identical to each other. Also, a few invertebrate peptides with PP-fold receptor like sequence have been reported (198, 200). A mutation in a PP-fold like receptor in *Caenorhabditis elegans* was found to change the feeding behavior of this organism (199), suggesting that the role of the PP-fold peptides and receptors in the regulation of feeding is not limited to vertebrates.

Other Proposed Receptors

The Y3 Receptor. The existence of an NPY-preferring receptor, often referred to as the Y3 receptor, has

been inferred from pharmacological studies of a variety of tissues (2, 201). The typical rank order of peptide potency of the receptor is NPY > NPY13-36 > PYY. Despite numerous attempts, no cloned receptor has been identified with a Y3-like pharmacology. Recently, it was shown that bovine chromaffin cells, which display typical Y3-like pharmacology, express high levels of the Y1 receptor (202). One explanation for the lack of cloning success with the Y3 receptor is that Y1 receptors display a different pharmacological profile in these areas or that the Y3 binding profile is in fact the result of a mix of several of the cloned receptors.

The PYY-Preferring Receptor. The presence of a PYY-selective receptor has been reported using tissue preparations from the GI tract (see Ref. 2 for review) and also electrophysiology of the rat dorsal vagal complex (203). Very recently, the rat intestinal PYY-preferring receptor was found to be identical to the rat Y2 receptor expressed in peripheral organs (204).

Peripheral Y2 Receptor. When the human Y2 receptor was cloned, it was striking how little of the mRNA that could be detected in peripheral organs (134, 136, 205). However, high levels of Y2-like binding have been detected in several organs, for instance, kidney (105, 206). This discrepancy might be explained by the existence of another not yet cloned receptor with a Y2-like binding profile in the periphery. However, such a receptor has not been identified to date. Considering the presynaptic localization of the Y2 receptor (see Y2 section), one possible explanation could be that the peripheral Y2-like binding may be caused by the already cloned Y2 receptor presynaptically located on peripheral nerve terminals.

Recent Findings on Distribution of PP-fold Peptide Receptors

General. NPY as well as all mammalian PP-fold receptors are expressed both in the brain and in the periphery, and there is an overlap in the distribution of the different receptors in the brain. Furthermore, determination of the receptor distribution is complicated because there are extensive species differences in the distribution of the receptors between orders of mammals as well as within the rodent and primate families for all PP-fold receptor subtypes (2, 207, 208). Thus, it appears that although the functions of the PP-fold peptides are very well conserved throughout evolution, the receptor system involved in a particular effect can vary dramatically between closely related species.

There are also discrepancies between the distribution of mRNA and protein (209). For example, although the highest mRNA concentrations in the human hypothalamus were found to be Y5, the binding profile of human hypothalamic homogenate identified Y2 as being the predominant receptor in this brain region (210). This has also been observed using autoradiography (211). A possible explanation for these discrepancies could be that Y2-expressing neurons have their nuclei outside the hypothalamus and Y2 binding in this tissue is to presynaptic sites. Another explanation for the discrepancies between mRNA and protein distribution are differences in mRNA stability and protein breakdown in post-mortem tissues (212). We therefore chose to discuss expression of the gene and protein separately. For more details, especially on the distribution of the peptides, see the previous reviews in this journal as well as others (1, 2, 27, 54) for reviews.

Receptors: mRNA. The distribution of the PP-fold receptor mRNAs in the brain differs dramatically between species. There are even reports about differences between different primate species (211) as well as differences between rat and mouse (213). By reverse-transcription polymerase chain reaction (RT-PCR), the relative levels of mRNA for Y1, Y2, and Y5 in the human hypothalamus were determined (210). The Y5 mRNA levels were 400 and 200 times higher than for the Y1 and Y2 receptors, respectively. Interestingly, Y5 receptor mRNA appears to be consistently colocalized with the Y1 mRNA in the rat (214) and mouse (213) brain whereas Y1 has a much broader distribution and is expressed in many areas without the presence of Y5.

Messenger RNA for Y1 has been found in brain areas important for the regulation of feeding, especially the arcuate nucleus of the hypothalamus where it is sometimes but not exclusively localized on pro-opiomelanocortin-expressing neurons (215). Y1 mRNA is also highly concentrated throughout the cerebral cortex and cerebellum in human brain with moderate levels in pyramidal cells of CA1-3 (212).

In human post mortem brain, the highest levels of Y2 mRNA was found in dentate gyrus (216). High levels were also found throughout the cerebral cortex as well as in lateral geniculate nucleus, amygdala, substantia nigra, hypothalamus, cerebellum, and choroid plexus (216). Furthermore, in the cerebral cortex, hippocampus, striatum, and amygdala Y2 (but not Y1) mRNA was found on NPY-positive cells suggesting that Y2 is an autoreceptor in these areas (217). In the arcuate nucleus of the rat hypothalamus, Y2 mRNA is mainly found in NPY expressing neurons in agreement with a presynaptic function (215). In the rat, a similar distribution is observed with the highest levels of Y2 mRNA in hippocampal areas (218, 219) and in the arcuate nucleus of the hypothalamus (218). In the intestinal tract of the rat, Y1 mRNA is exclusively found in non-epithelial colon while Y2 is found in all crypt cells, villus, colon epithelium, and jejunal epithelium (220). RT-PCR detected Y1 as the predominant receptor subtype in human adipocytes although Y4 and Y5 transcripts were also detected (221).

The Y4 receptor is highly variable across species both with regards to pharmacology and distribution, which may explain why its exact role may differ between species (90, 152). In humans, Y4 mRNA is found in prostate, colon, pancreas, and small intestine (90) as well as skeletal muscle (154). Lower levels of human Y4 mRNA was also found in brain by RT-PCR (95, 220) and Northern blot (154). A high level of Y4 mRNA in the rat was only found in the testis

(152). In a more detailed study using RT-PCR, rat Y4 mRNA was found in all intestinal tissues examined with the highest levels localized to colon epithelium (220). Y4 mRNA has also been found in rat hypothalamus by RT-PCR (220) and *in situ* hybridization (214). Furthermore, *in situ* hybridization has also detected high levels of Y4 mRNA in neurons of the rat dorsal vagal complex, area postrema, and nucleus of the solitary tract (222). Very recently, the presence of the Y4 receptor in several colon adenocarcinoma cell lines was reported (223).

By *in situ* hybridization, Y5 mRNA is found in many brain regions of the rat. High levels can be found in many areas, including the paraventricular and arcuate nuclei of the hypothalamus, lateral hypothalamus, medial thalamus, suprachiasmatic nucleus, and hippocampus (91, 219, 224, 225). A similar distribution was found in the human brain (225). Low levels of Y5 mRNA are present throughout the cerebral cortex of the rat (224). However, compared to rat and human, the overall levels of Y5 mRNA appear to be very low in mouse brain (213). The Y5 receptor is also found in several organs in the periphery. The highest level of Y5 expression in the periphery has been found in the testis (91). By RT-PCR, rat Y5 mRNA has been detected in colon crypt cells, non-epithelial colon (220), spleen, and pancreas (210).

During mouse embryo development, Y1, Y2, and Y5 are turned on at about embryonic day 12-15 and the expression remains rather stable with relative levels Y1>Y2>Y5. In cerebellum, however, Y1 and Y2 expression is reduced after post-natal day 4 (213).

Receptors: Protein. Some initial successes have been reported using antibodies directed against PP-fold receptors. In general these studies have used synthetic peptides corresponding to unique regions of the receptors to obtain antisera from rabbits. Antibodies directed against the rat Y1 detected the highest levels in the subiculum of the hippocampal formation with lower levels found in the dentate gyrus and the CA2 region (226). Strong Y1-ir was also detected in striatum, claustrum, piriform cortex, arcuate nucleus of the hypothalamus, interpeduncular nucleus, paratrigeminal nucleus, and lamina II of the spinal trigeminal nucleus as well as in the entire spinal cord of the rat (227). In the hypothalamus of the mouse, Y1-ir was found to be colocalized with thyrotropin-releasing hormone and CART in parvocellular neurons (228). In the periphery, Y1-ir has been detected on NPY- and vasoactive intestinal peptide (VIP)-positive neurons throughout the intestine of the rat. Y1-ir can also be found in endothelium and on some endocrine cells (229), as well as in arterial smooth muscle in testis (230). The use of subtype selective antagonists to inhibit PYY-evoked vasoconstriction revealed the existence of Y1 and Y2 receptors in pig spleen (26). To our knowledge, there are no reports yet on successful use of antibodies raised against the Y2 receptor protein.

Fewer studies are available using antisera directed against the other PP-fold receptors. Using immunohistochemistry, high levels of Y5 were detected in the rat hypothalamus, where the highest levels were found in the magnocellular neurons of the paraventricular hypothalamus, the supraoptic nucleus, and in the arcuate nucleus (172). Scattered immunopositive neurons were observed in the thalamus, including paraventricular nucleus of the thalamus, habenula, mediodorsal thalamic nucleus, and zona incerta. Furthermore, high levels of Y5-ir were found throughout the hippocampus and cortex of the rat (172). Using double-label immunofluorescence, Y5-ir was colocalized with corticotropin-releasing hormone (CRH) and gamma-aminobutyric acid (GABA) in brainstem (231). In the preoptic area, the Y5 receptor is present on about 55% of the gonadotropin-releasing hormone (GnRH) neurons (172). Y5-ir was also found on CRH-, neurophysin-, and GABA-positive neurons in the hypothalamus (172, 231).

Until recently, autoradiographic studies of the PP-fold receptors have been hampered by the lack of specific tools to delineate receptor subtypes. All peptide agonists used (^{125}I-PYY, ^{125}I-PYY3-36, ^{125}I-[Leu^{31},Pro^{34}]PYY/NPY, ^{125}I-hPP, and ^{125}I-1229U91) bind to several receptor subtypes with high affinity. The recent development of highly specific antagonists will undoubtedly help future studies of receptor distribution using this method in that it will be possible to selectively mask unwanted binding. However, consistent with previous studies using less selective radioligands, the Y1 and Y2 receptors appear to be the predominant receptor subtypes in rat brain when using autoradiography.

When ^{125}I-[Leu^{31},Pro^{34}]PYY binding was compared with the distribution of ^{125}I-PYY3-36 binding (Y2-like) in human postmortem brain (211), high levels of Y2 binding was found throughout the cortex and in the hypothalamus whereas ^{125}I-[Leu^{31},Pro^{34}]PYY only detected moderate levels of receptors in the dentate gyrus of the hippocampal formation and in the caudate nucleus confirming the low abundance of Y1 and Y5 receptors in the human brain. However, ^{125}I-[Leu^{31},Pro^{34}]PYY and ^{125}I-[Leu^{31},Pro^{34}]NPY bind to all PP-fold receptors except Y2. Dumont and coworkers identified low densities of BIIE0246 (Y2-selective antagonist, see Fig. 2 and Ref. 196) -insensitive ^{125}I-PYY3-36 binding sites, most likely Y5, in olfactory tubercle of the rat and several hippocampal areas of the rat, monkey (marmoset), and human (232). A similar approach used

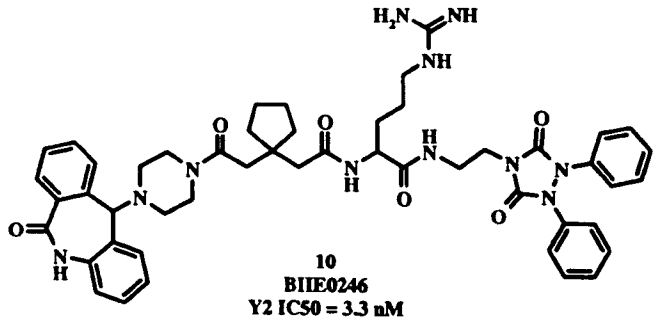

Figure 2. Novel nonpeptide analogs with selectivity for the Y2 receptor.

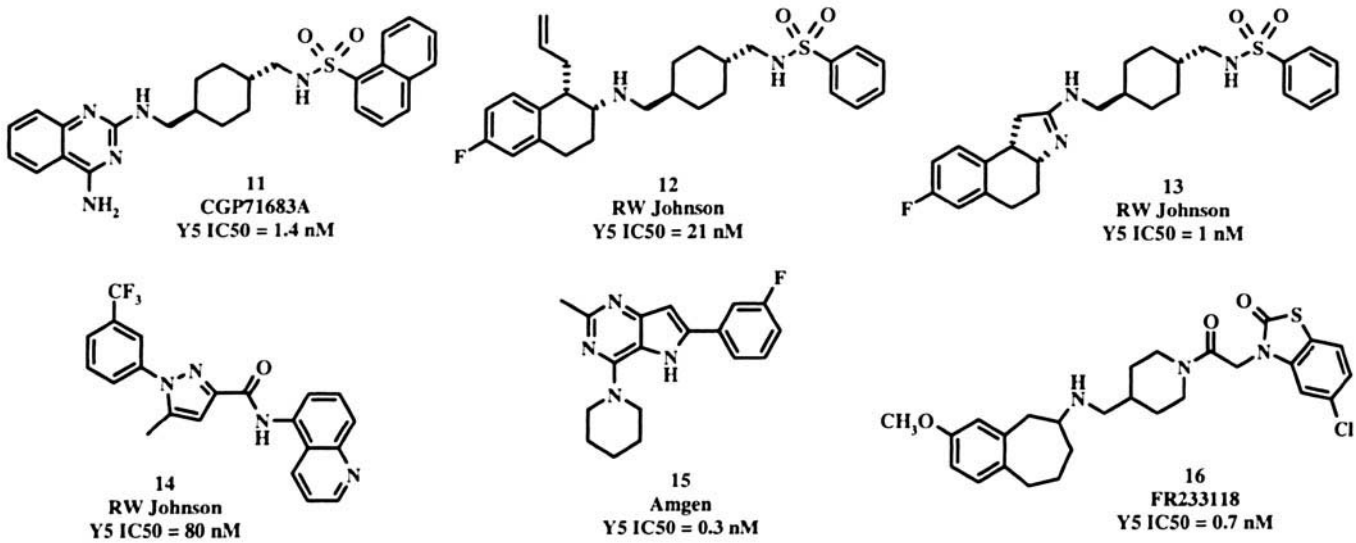

Figure 3. Novel nonpeptide analogs with selectivity for the Y5 receptor.

BIBO3304 (Y1-selective antagonist; Ref. 111; Fig. 1) and CGP71683A (proposed Y5 antagonist; Ref. 177; Fig. 3) to compete with ^{125}I-[Leu^{31},Pro^{34}]PYY binding to rat brain homogenate. In these studies, it was found that the Y1-like binding outnumbered Y5 by 3:1 (233). Y5-like binding (BIBO3304 insensitive) was found mainly in the olfactory bulb and hippocampal areas with very little in the hypothalamus (233).

When comparing Y1-like and Y2-like binding in human and rat, it has been found that Y2 is the predominant receptor in human brain whereas Y1 dominates in the rat (2, 211) and mouse (213) brain. However, in another study, homogenate binding of mouse forebrain detected similar amounts of binding sites for ^{125}I-[Leu^{31},Pro^{34}]PYY (Y1, Y4, and Y5) as for ^{125}I-PYY3-36 (Y2 and possibly Y5) (234). Using autoradiography, the highest concentrations of ^{125}I-[Leu^{31},Pro^{34}]PYY sites were found in the islands of Calleja and dentate gyrus of the mouse whereas ^{125}I-PYY3-36 binding displayed a much broader distribution (234). Dumont and colleagues only detected low levels of BIBP3226-insensitive binding sites for ^{125}I-[Leu^{31},Pro^{34}]PYY (i.e., Y5 or possibly Y4) in the rat hypothalamus (235). Instead, the highest Y5-like binding was detected in CA3 area of the hippocampus, lateral septum, olfactory bulb, the nucleus tractus solitarius, and the area postrema (235).

The radioligand ^{125}I-1229U91 binds with very high affinity to both Y1 and Y4 receptors (159, 160). Using ^{125}I-1229U91, binding was detected throughout the rat brain, including the lamina I-III of the cerebral cortex, olfactory tubercle, many thalamic areas, and the dorsal hypothalamus (236, 237). Interestingly, ^{125}I-rPP binds to the interpeduncular nucleus and the paraventricular nucleus (PVN) of the rat (208, 236), whereas these areas were not labeled by ^{125}I-1229U91, suggesting the presence of an atypical PP binding site in these areas (236). In another study of the rat brain, high levels of BIBO3304-insensitive but PP-sensitive ^{125}I-1229U91 binding (Y4-like) sites were detected in area postrema with lower levels in the olfactory bulb and hippocampus (237).

In the periphery, autoradiography studies using rat and rabbit kidney revealed that Y1 is the predominant receptor subtype in the rat kidney, whereas Y2 is the predominant PP-fold receptor in rabbit kidney (238). Human adipose tissue contains a BIBP3226-sensitive receptor that bound ^{125}I-[Leu^{31},Pro^{34}]PYY and ^{125}I-PYY and thus confirmed the presence of the Y1 receptor and absence of Y2 in adipocytes (221).

Studies of Genetically Engineered Rodents

Overview. Numerous genetic manipulations have been made to create mice that are deficient in NPY and individual NPY receptor subtypes (Table II). In addition, a line of rats, which exhibit a 3- to 4-fold increase in NPY concentrations in certain tissues, has been created. In general, these animals exhibit very little overt phenotype when raised under standard conditions (for review, see Ref. 239). For instance, the NPY knockout mice eat normally, grow normally, and refeed normally after fasting conditions. Furthermore, the endocrine responses to fasting under normal conditions are within normal parameters. The NPY overexpressing rats exhibit a similar normal phenotype with little change in body weight and resting blood pressure. In general, the receptor knockouts also exhibit a normal phenotype but with a mild late onset obesity in most cases. Given these modest changes in resting phenotype, it has been difficult to discern the role of NPY in normal physiology and in the maintenance of body weight, blood pressure, epilepsy, and in models of certain emotional and psychiatric indications. Now that these tools are available to understand the NPY system, numerous investigators have begun to alter the physiological parameters of these animals to understand the role of NPY in disease pathology.

NPY Knockout Mice. NPY knockout mice were generated by homologous recombination to eliminate ex-

Table II. Results from Studies of Transgenic Animals

Animal type	Major findings	References
NPY Knockout mice	Mild, late onset obesity	(241)
	When crossed with ob/ob mice, partial reduction in obese state	(241)
	Increased sensitivity to leptin	(305)
	Spontaneous seizures (<9 mos. of age)	(241)
	Increased deaths after kainic acid-induced seizures	(306)
	No change in food consumption	(241)
	No change in fasting-induced food consumption	(241)
	Decreased fasting-induced food consumption	(240)
	Increased anxiety-like behavior	(239, 240)
	Decreased nociception	(240)
	Normal spatial and contextual memory	(239)
	Increased voluntary consumption of ethanol	(72)
	Decreased sensitivity to ethanol	(72)
	Impaired olfactory neurogenesis	(56, 57)
	Attenuated hyperphagia in mice with streptozotocin-induced diabetes	(307)
Y1 Knockout mice	Mild, late onset obesity in females	(121, 122)
	Reduction in the feeding response to NPY	(121, 245)
	Hyperalgesia to acute thermal, cutaneous and visceral pain	(108)
	Increased "neuropathic" pain	(108)
	Decreased NPY-induced pressor response	(121)
	Resistance to sedative actions of barbiturates, anesthetics, and ethanol	(53, 130)
	Obesity and increased insulin levels and glucose turnover without hyperphagia	(308)
	Increased voluntary alcohol consumption	(130)
Y2 Knockout mice	Mild, late onset obesity	(150)
	Increased food intake	(150)
	Normal response to leptin and starvation	(150)
	Normal blood pressure response but increased heart rate	(150)
	Reduced locomotor activity	(150)
	Reduction in the acute effects of leptin	(150)
	NPY has no effect on parasympathetic activity to the heart	(144)
	Hypothalamic regulation of bone formation	(151)
Y4 Knockout mice	Rescues fertility when crossed with ob/ob mice	(247)
	Reduced body weight, white adipose tissue, and increased plasma level of PP	(247)
	Increased aggressive behavior in males	(247)
Y5 Knockout mice	Mild, late onset obesity	(173)
	Partial reduction in feeding response to NPY	(173)
	No change in the feeding response to NPY but partial reduction to Y5 agonists	(245)
	When crossed with ob/ob mice, no change in the obese state	(173)
	Increased sensitivity to kainic acid-induced seizures	(180)
NPY-overexpressing rats	Normal arterial pressure and heart rate	(250)
	Increased total vascular resistant	(250)
	Reduced stress related behaviors	(251)
	Impaired spatial memory acquisition	(251)
NPY-overexpressing mice	Decreased voluntary ethanol consumption	(72)
	Increased sensitivity to sedative/hypnotic effects of ethanol	(72)
	No difference in anxiety-like behavior	(72)
PP-over-expressing mice	Decreased food intake and body weight	(252)

pression of NPY. Under standard conditions, these mice exhibit very normal behavior, grow normally, and refeed normally after a fast (50). In addition, all of the endocrine responses to fasting are normal. However, another group (240) used a different line of NPY knockout mice and observed a reduced food intake in response to a 24-hr fast. These mice were subjected to diet-induced obesity and chemically induced obesity (monosodiumglutamate and gold thioglucose) with no substantial difference in food consumption (239). Finally, NPY was proposed to be an important mediator of the actions of leptin on feeding (83), these animals exhibited an increased sensitivity to peripherally and centrally administered leptin (50). Crossing the NPY knockout animals with the ob/ob mice produced a line of animals that were deficient in both NPY and leptin. These animals exhibited a partial amelioration of the obese phenotype and also were more sensitive to leptin (239, 241). Therefore, NPY may normally have a tonic inhibitory effect on leptin-mediated satiety signals. NPY-deficient mice also exhibited a normal response to central administration of NPY when food consumption was measured. In addition, these mice appeared to have normal metabolism, general

activity, and also normal spatial and contextual learning ability. Interestingly, the NPY –/– mice have been reported to have occasional spontaneous seizure-like events (242). Also, these animals are less able to terminate seizures induced by chemicals, such as GABA receptor antagonists or a glutamate agonist. These data emphasize the previously shown importance of NPY in dampening excitotoxicity in the brain (243).

A more recent study conducted with NPY –/– mice examined the behavioral phenotype created by this mutation (240). Based on data from behavioral models, the NPY –/– mice exhibit increased anxiogenic-like behavior and also appear to be hypoalgesic on the hot plate test of pain. Knockout studies have also suggested NPY to be a neuroproliferator. Mice lacking NPY displayed an impaired development of olfactory neurons (56, 57). Very recently, NPY –/– mice were crossed with mice lacking agouti-related peptide (AgRP; Ref. 244). AgRP acts as an antagonist at melanocortin receptors (see NPY section) and is thus, like NPY, an orexigenic peptide. Interestingly, AgRP is expressed by the same hypothalamic neurons as NPY. Like the NPY –/– mice, these double knockout animals exhibited a normal feeding behavior and weight gain under normal conditions (244).

Y1 Receptor Knockout Mice. Y1 receptor knockout mice have been generated by numerous investigators (108, 121, 122, 245). Again, under standard vivarium conditions, little overt phenotype is discernible between the knockout and control wild-type animals. Peripherally, the Y1 knockouts exhibit a substantially reduced blood pressure increase after NPY administered intravenously (246), confirming the role of this receptor in the pressor effects of NPY. In initial studies, female Y1 knockout mice display a late onset increase in body weight compared to their wild-type litter mates (121, 122); however, male animals exhibited no difference in body weight when compared with wild-type male litter mates. When NPY was injected intracerebroventricularly, there was a significant reduction in feeding observed in the Y1 knockout mice when compared to the wild-type litter mate controls (121, 245). Therefore, NPY may produce its effects on food consumption at least partially by activation of the Y1 receptor. However, a significant increase in NPY-induced food consumption was still observed in the Y1 knockout animals. This may be the result of the cooperative interaction with other receptor subtypes to produce the full effect of NPY on feeding (see Y5 knockout data below). In a recent report, Naveilhan and colleagues (108) have demonstrated that the Y1 knockout mice develop hypoalgesia to acute thermal, cutaneous, and visceral pain. In addition, these animals exhibit increased mechanical hypersensitivity in pain models. In models of neuropathic pain, there is also an increased response and a complete absence of the pharmacological effects of NPY analgesia. Therefore, it is likely that the Y1 receptor participates in the analgesic effects of NPY centrally and, perhaps, peripherally as well.

In another study (52), NPY was found to potentiate pentobarbital-induced sedation in wild-type animals but not in Y1 knockout animals. This suggests the Y1 receptor plays an important role in mediating the sedation seen with GABAergic compounds. Similar results were obtained for Avertin but not Kealar (NMDA antagonist)-induced sedation, thus reinforcing the proposed GABAergic/NPY hypothesis.

Y2 Receptor Knockout Mice. In initial studies, the Y2 receptor knockout mice exhibit a small increase in body weight, slightly increased food intake and increased fat deposition (150). The mutant mice also showed a blunted response to leptin but a normal response to NPY-induced food intake and intact regulation of refeeding and body weight after fasting. Therefore, the Y2 receptor may be involved in the regulation of hypothalamic NPY release in a tonic fashion. An absence of the Y2 receptor subtype also appeared to affect the basal control of heart rate in mice but did not affect normal blood pressure (150). The Y2 receptor knockouts exhibit very interesting phenotypes in behavioral studies. In a study that evaluated the role of both the Y1 and Y2 receptors in sedation (53), an increased sensitivity to pentobarbital-induced sedation was observed in the Y2 –/– mice. Therefore the Y2 receptor appears to modulate GABAergic-induced sedation physiologically and by agents such as pentobarbital. Furthermore, the Y2 receptor appears to produce an opposing function when compared to the role of the Y1 receptor (see above). Because reports about Y2 knockout animals have just recently entered the scientific literature, it will be interesting in future studies to evaluate the role of Y2 in analgesia, seizure formation and propagation, anxiolytic action, and endocrine function. Recently, another group reported a separate strain of Y2-knockout mice (142). In initial reports, it appears that the Y2 receptor may also be involved in the effects of NPY on parasympathetic innervation to the heart (142) and, via the hypothalamus, in the regulation of bone formation (151).

Y4 Receptor Knockout Mice. The first knockout of the Y4 receptor was published during the spring 2002 (247). These animals have an increased plasma level of PP (2- to 3-fold) and put on weight more slowly than control animals despite a normal food intake. The most exciting finding with the Y4 knockout mice came when these animals were cross-bread with the leptin deficient ob/ob mice. Although the ob/ob mice are virtually infertile, the resulting double knockout was fertile. Furthermore, male mice lacking the Y4 receptor displayed a very aggressive behavior (247).

Y5 Receptor Knockout Mice. Mice with an inactivated Y5 receptor gene were first reported in 1998 (173). The interest in the Y5 receptor was spurred on by the pharmacological identification of this receptor as one that may mediate the feeding response to NPY (91). Interestingly, the Y5 receptor null mice feed and grow normally. However, much like the Y1 receptor and Y2 receptor knockouts, they develop a mild late onset obesity characterized by increased body weight and food intake and subsequent storage of calories in adipose tissue (173). When the Y5 receptor

knockout animals were fasted, normal refeeding behavior was observed in the younger knockout mice. In addition, these animals exhibited a normal sensitivity to leptin. As seen with the Y1 receptor knockouts, the Y5 receptor knockouts exhibited a reduced feeding response after intracerebroventricularly administered NPY. The Y5 receptor knockouts have also been crossed with animals deficit in leptin (ob/ob). However, no changes in body weight or food intake were observed in the Y5 null ob/ob mice (173). Thus, it appears that the Y5 receptor does participate in the effects of centrally injected NPY on food intake. However, its presence does not appear to be critical for the normal physiological regulation of feeding in mice (248). In other studies of the Y5 receptor knockout animals, an important role for this receptor has been proposed for its suppression of seizure-like activity induced either by chemical or electrical convulsants (180). In a study by Kanatani *et al.* (245), the relative contributions of Y1 and Y5 receptors in the feeding response were evaluated in Y1 and Y5 receptor-deficient mice. This study concluded that both the Y1 and Y5 receptors and, possibly, a yet-unknown Y1-like receptor may be involved in the regulation of the feeding response to NPY. In a study by Thiele and co-workers (249) the effects of NPY null-mutations and Y5 receptor null mutations were evaluated in both male and female mice. The NPY receptor knockouts exhibited an increased locomotor activity when compared with the wild-type controls. This was observed in the female animals but not in the males. This observation appears to confirm the suggested role for NPY in sedation. In the Y5 receptor knockouts, no significant difference was observed in locomotor activity. In addition, the NPY receptor knockout mice exhibited an increased consumption of ethanol during the 2-day trial whereas the Y5 receptor knockouts exhibited similar consumption when compared with control. However, NPY knockouts exhibited a normal sensitivity to ethanol whereas the Y5 receptor knockouts exhibited increased ethanol induced sedation as well as increased plasma levels of ethanol. It should be noted that the increased ethanol intake observed in the Y5 receptor knockouts was only seen with a 20% volume-to-volume solution of ethanol and not at lower concentrations (249).

Based on the data from studies using knockout animals, there are some initial conclusions that can be reached regarding the roles of NPY receptor subtypes and the effects produced by NPY. It is clear from the initial knockout data that the Y1 receptor mediates the vasoconstrictor responses observed when NPY is administered intravenously. However, in mice, NPY does not appear to play an important role in the maintenance of normal pressure. When NPY is given intracerebroventricularly to induce a feeding response, it appears that both Y1 and Y5 receptors contribute to the response seen in feeding paradigms. In addition, there are other metabolic and endocrine actions of NPY, which are not lost in the Y1 or Y5 nonmutation mice. Further studies will need to characterize the effect of double deletion of both Y1 and Y5 receptors. Future studies should focus on a better understanding of the effects of NPY on endocrine function and metabolism as seen in obese animals. In mouse pain studies, the Y1 receptor mediates the majority of the actions of NPY on the antinociception seen both in acute and chronic pain paradigms. In anxiety studies, the Y1 receptor appears to contribute an important function in production of the anxiolytic-like effects of the centrally administered NPY. Likewise, the sedative actions of NPY appear to be mediated by the Y1 receptor with the Y2 receptor influencing the tonic release of NPY to alter the effects. Finally, in seizure paradigms only the Y5 receptor has been studied extensively to date. This receptor appears to contribute importantly to the anticonvulsant effects of NPY. In the future it will be interesting to do additional studies with Y2 and Y1 receptor knockouts to fully understand their role in seizure propagation in mice.

NPY-Overexpressing Mice. A line of NPY-overexpressing mice has been reported in the literature (72). These animals exhibit elevated staining for NPY peptide expression in brain regions including the cerebral cortex, and amygdala. The staining for NPY-IR in the arcuate nucleus of the hypothalamus was similar to control. These mice have been studied in a limited number of paradigms. In tests of ethanol consumption, the NPY-overexpressing mice showed a decrease in voluntary consumption and a greater sensitivity to the sedative/hypnotic effects of ethanol (72). However, these animals did not exhibit differences in tests of anxiety-like behavior. These data, along with those from NPY –/– mice, suggest that NPY is a key modulator of ethanols actions in the brain. It is possible that this is related to the proposed anxiolytic-like actions of NPY mediated by the amygdala. Broader study of these animals should assist the further understanding of NPY in behavioral disorders as well as substance abuse.

NPY-Overexpressing Rats. A line of transgenic NPY-overexpressing rats has been generated, which allows the study in a number of paradigms where the effects of intracerebroventricular NPY have been evaluated. These animals have a modest increase in plasma NPY concentrations and normal arterial pressure and heart rate (250). Consistent with the vasoconstrictor role proposed for NPY, these animals have increased vascular resistance. Perhaps the most interesting data have been found in behavioral studies (251). In the brain, the NPY-overexpressing rats were found to have a marked increase in the hippocampal CA1 and CA2 expression of NPY mRNA and a marked reduction in Y1 receptor binding in all subregions of the hippocampal formation. In behavioral models designed to evaluate the effects of stress, responses were markedly attenuated. In addition, these animals exhibited a significant impairment in spatial memory acquisition in the Morris water maze. These studies open an important question as to whether the antistress effects of NPY are selective or reflect an impaired acquisition of behavioral memory. Further studies on these animals will undoubtedly elucidate the importance of NPY and memory.

PP-Overexpressing Mice. A transgenic line of mice with a 20-fold higher plasma concentration of PP than that of wild type mice has been reported in the literature (252). These PP-overexpressing mice are leaner and display a reduced food intake and lower fat mass than control, adding support for the role of PP in the regulation of metabolism.

Recent Developments in Selective Agonists and Antagonists for PP-fold Receptors

Peptides. Over the past 4 years there has been considerable progress in the development of novel selective analogues of NPY. A number of these analogues have been directed toward discovering tools to understand the role of the Y1 and Y5 receptors in the feeding produced by central administration of NPY. For instance, several novel analogues of NPY have been found to be highly selective for the Y1 receptor (125). Three of these analogues are included in Table III. These all produce an increase in feeding after intracerebroventricular administration. Over 4 hours, [D-Arg^{25}]-NPY produced a similar increase in feeding compared with NPY. This increase in feeding was blocked by the peptidic Y1 antagonist 1229U91. However, it was found that 1229U91, besides being a Y1 antagonist, also is a potent agonist at the Y4 receptor (159, 160). Soll and coworkers (253) have reported a very potent and Y1 selective peptide [Phe^7, Pro^{34}]-NPY. Although this peptide is a full agonist at the Y1 receptor, no work has been reported on its effects *in vivo*. In addition to novel Y1 receptor agonists, an analog of the Y1-selective peptide antagonist 1229U91 was recently reported (254). This analog consisted of an OMe replacement for the amide in 1229U91 (GR231118). This peptide retained high affinity for the Y1 receptor but had much reduced affinity for the Y4 receptor when compared to 1229U91. In functional assays, this peptide analog potently antagonized the NPY inhibition of forskolin-stimulated adenylate cyclase in Y1 receptor-containing cells while having no effect (agonist or antagonist) in Y4 receptor-containing cells. When administered intrahypothalamically, this Y1 antagonist inhibited both NPY stimulated feeding as well as feeding in schedule-fed rats.

More limited work has been performed to obtain Y2-selective peptide analogs. One of the more promising peptide analogs was recently published by Soll *et al.* (253). This peptide (Cyclo S-S [Cys^{20}, Cys^{24}]pNPY) exhibits subnanomolar affinity for the Y2 receptor with low micromolar affinity at Y1 and Y5. No additional information is available in the scientific literature to further characterize the properties of this compound. On the other hand, considerable progress has been made for developing Y5 selective agonists. However, several of these peptide analogs have considerable affinity for both the Y4 and Y5 receptors (Table III). One of these analogues, 2-36[K^4, RYYSA$^{19-23}$]-PP, produced a dose-dependent increase in food consumption after intracerebroventricular administration (175). The magnitude of this increase in food intake exceeded that produced by identical doses of NPY. More selective Y5 agonist peptides have also been reported that have little affinity for the Y4 receptor. One of these, [Ala^{31}, Aib^{32}]-NPY was found to produce a small increase in food intake when compared to control animals (176). Another analog, [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP, which exhibited an approximately 20-fold higher affinity for the Y5 receptor when compared to analogue [Ala^{31}, Aib^{32}]-NPY, produced increases in food intake that exceeded NPY at similar doses (176). Finally, the relatively low affinity analogue, p[D-Trp^{34}]-NPY, was reported to be a selective Y5 agonist (255). This peptide produced a similar inhibition of forskolin-stimulated adenylate cyclase to that seen for NPY and was found to increase food intake after intracerebroven-

Table III. Novel Peptide Analogs with Selectivity for PP-fold Peptide Receptor Subtypes

Peptides	K_i (nM) or IC_{50} (nM)				Reference
	Y1	Y2	Y4	Y5	
Y1 agonist					
[D-Arg^{25}]-NPY*	0.9	11.6	74.6	43.4	(125)
[D-His^{26}]-NPY*	2.0	29.0	20.1	34.6	(125)
Des-AA$^{11-18}$[Cys^{7,21}, D-Lys^9(Ac), D-His^{26},Pro^{34}]-NPY[a]	1.2	801	31.4	2363	(125)
[Phe^7, Pro^{34}]-pNPY[a]	0.009	32.1	ND	34	(253)
Y1 antagonist					
1229U91 (GR231118)-OMe substituted[a]	0.46	624	65.5	7890	(254)
Y2 agonist					
Cyclo S-S [Cys^{20}, Cys^{24}]pNPY[a]	1525	0.38	ND	6296	(253)
Y4/Y5 agonist					
[cPP$^{1-7}$, pNPY$^{19-23}$, His^{34}]-hPP	5.7	22.2	0.06	0.04	(309)
2-36[K^4, RYYSA$^{19-23}$]-PP[a]	0.87	1.95	0.004	0.029	(175)
Y5 agonist					
[Ala^{31}, Aib^{32}]-NPY	>700	>500	>1000	6.0	(176)
[cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	530	>500	51	0.24	(176)
p[D-Trp^{34}]-NPY[b]	>10,000	631	1905	41.7	(255)

[a] K_i values.
[b] Converted to K_i values from pK_i values.
ND = not done.

tricular administration. However, the increase was not as substantial as that observed with similar doses of NPY. The putative Y5 selective antagonist, CGP71683A (Fig. 3), antagonized the increase in food intake produced by this peptide; however, the specificity of CGP71683A has recently been called into question (178).

Small Molecule NPY Medicinal Chemistry

This portion of the review will concentrate on the primary, small molecule medicinal chemistry literature appearing over the last 5 years. Although an effort was made to catalog all ligands that appeared in journal publications, a particular emphasis has been placed on those series that have reported *in vivo* biological data. The structures of all compounds discussed below are presented in Figures 1–3.

NPY Y1 Ligands. Studies with the Karl Thomae NPY Y1 antagonist BIBP3226 (compound **1**) (110) have continued. This compound, which is a selective NPY Y1 antagonist with low nanomolar affinity, had been shown to block intracerebroventricular NPY-induced feeding in rats (119, 256). However, later it was demonstrated that the opposite and NPY Y1 inactive enantiomer was similarly active (257). This suggests that the feeding behavior effects observed with BIBP3226 were nonspecific. Furthermore, both enantiomers of this compound have been found to provoke release of intracellular Ca^{2+} in Chinese hamster ovary cells in the absence of recombinant Y1 receptors (258). BIBP3226 has also been found to bind with high affinity to the rat receptor NPFF1 ($K_i = 25$ n*M*; Ref. 259) and to block the effect of NPFF on forskolin-induced cAMP synthesis at the human receptor NPFF2 (260), providing further evidence for the nonspecific nature BIBP3226. Very recently, the same group has published data on a new more water-soluble analog, BIBO3304 (compound **2**; Ref. 111). This potent and selective antagonist was also shown to block intracerebroventricular NPY-induced feeding in a dose-dependent manner. Furthermore, the Y1-inactive S-enantiomer did not inhibit feeding in the same model, suggesting that the effects observed with BIBO3304 may be related to its effects at the NPY Y1 receptor.

Several novel series of Y1 antagonists have been published by Lilly Research Laboratories (261–266). A heterocyclic core attached to some type of basic group and additional aryl functionality characterizes many of the examples from this program. An indole-based compound (**3**) was reported to be a potent and selective Y1 antagonist and, furthermore, it was reported that this agent blocked intracerebroventricular NPY-induced feeding in satiated Long-Evans rats. A lack of oral bioavailability prevented further development of this particular chemical series.

Bristol-Myers-Squibb has also been active in the Y1 arena with at least two recent publications (267, 268). The disclosed structure–activity studies focused on a dihydropyridine type structure exemplified by compound **7**. Compound **7** was described as a Y1 receptor ligand with low nanomolar potency and was found to be active in two models of food consumption behavior (intracerebroventricular NPY-driven food consumption; 57% inhibition at 30 mg/kg ip; spontaneous dark cycle feeding, 54% inhibition at 20 mg/kg ip). Unfortunately no *in vivo* food consumption behavior data on a close structure analog without activity was used as a NPY Y1 control compound, so it is difficult to conclude the exact mechanistic origin of the observed effects.

Banyu provided an additional analog with observed *in vivo* effects (269). Compound **8** was purported to displace radiolabeled [Leu^{31},Pro^{34}]NPY from NPY Y1 receptors with a K_i of 0.29 n*M*. In *in vivo* studies, the compound was found to be active in NPY-driven feeding in normal Sprague-Dawley rats (74% reduction, 200 μg icv) and also active in blocking food consumption in Zucker Fatty Rats via both the icv (200 μg) and oral (100 mg/kg) routes.

An additional series from Shionogi was also disclosed in the primary literature (270, 271). A benzazepine compound (**9**) was described as a 5.1 nM Y1 antagonist, but unfortunately no further data was disclosed.

NPY Y2 Ligands. There has been only one report in the primary medicinal chemistry literature describing potent, selective NPY Y2 ligands (196, 232). BIIE0246 (compound **10**) was reported to displace [^{125}I]-PYY(3-36) from NPY Y2 receptors in human embryonic kidney (HEK293) cells with an IC_{50} of 3.3 n*M*. It was also shown to be a functional antagonist in both the rat vas deferens and dog saphenous vein assays blocking the effect of NPY with a pA_2 of 8.1 and 8.6, respectively. More recently, BIIE0246 has also been used to evaluate Y2 receptors in colonic tissue (272, 273) and in the hippocampus (274). Unfortunately its high molecular weight (896 Daltons) limits the *in vivo* utility of this tool, and no further *in vivo* studies have been reported in the primary literature. Clearly additional medicinal chemistry entries in the Y2 field would aid the full elucidation of the therapeutic potential of an NPY Y2 ligand.

NPY Y5 Ligands. Several groups have been very active in the pursuit of a diverse array of NPY Y5 ligands. Novartis has disclosed the pharmacology surrounding CGP71683A (compound **11**; Refs. 177, 275). This highly selective NPY Y5 antagonist (radioligand binding assay, IC_{50} = 1.4 n*M*, HEK293 cells; functional activity, IC_{50} = 5.8 n*M*, NPY-induced calcium transients, LMTK cells) blocked food consumption behavior in NPY-induced, 24-hr fasted, dark-cycle free-feeding and streptozotocin diabetic rats in a dose response manner (177). Recently, it has been reported that this compound reduced feeding in Y5 knockout mice and that its suppression of food intake may be related to other receptor activities or the release of inflammatory mediators (178).

The RW Johnson group has reported on the development of two series of NPY Y5 ligands represented by compounds **12** and **13** (276–278). Analog **12**, a tetrahydronaphthalene derivative was discovered to displace [^{125}I]-PYY from Y5 receptors in HEK293 cells with an IC_{50} of approximately 21 n*M*. Despite the modest *in vitro* potency, the group reported a decrease in food consumption behavior in

a fasted rat model with this agent (22% reduction; 30 mg/kg, ip). This group also reported that compound **5** was fairly enantiospecific (36-fold) in the *in vitro* radioligand-binding assay, but unfortunately no *in vivo* data on the less active enantiomer was disclosed.

An interesting series of pyrazoles were also reported by RW Johnson investigators (279, 280). Amongst these, compound **14** stands out as an example. This compound was described as fairly weak in radioligand displacement binding (IC_{50} = 80 n*M*); however, in *in vivo* studies this agent was found to be active (fasted rat model, 43% reduction in food consumption behavior versus a fluoxetine control; 30 mg/kg, ip). Unfortunately no oral activity was observed.

Amgen has reported on the development of two series of NPY Y5 ligands (281, 282). One series of pyrrolopyrimidines, represented by compound **15** (IC_{50} = 0.3 n*M*), was described as highly selective ligands with antagonistic properties. The related des-fluoro compound, which incidentally was reported as a direct high-throughput screening hit, was also observed to block food consumption behavior in fasted Long-Evans rats and BALB-C mice (30 mg/kg). The Amgen group has also published a pharmacophore model for this series of NPY Y5 antagonists.

Finally, the Fujisawa group also has been actively pursuing the NPY Y5 field. Compound **16**, reminiscent of the Novartis/Synaptic series, is just one example (283, 284). Unfortunately no further data have been reported.

Structure and Mutagenesis Studies of PP-fold Receptors

Mutagenesis of the Y1 Receptor. Several mutagenesis studies have been conducted to identify the key amino acids in receptor-ligand interaction at the Y1 receptor (285–291). One study (290) used the rat Y1 receptor whereas the other six published thus far used the human Y1 receptor. All in all, 68 positions of the 384 of the Y1 receptor have been mutated (Fig. 4), in most cases to alanine. Initially, a hydrophobic pocket formed by amino acids Tyr^{100} in TM4, Phe^{286} in TM6, and His^{298} at the top of TM7 (286) together with several acidic amino acids in the extra-

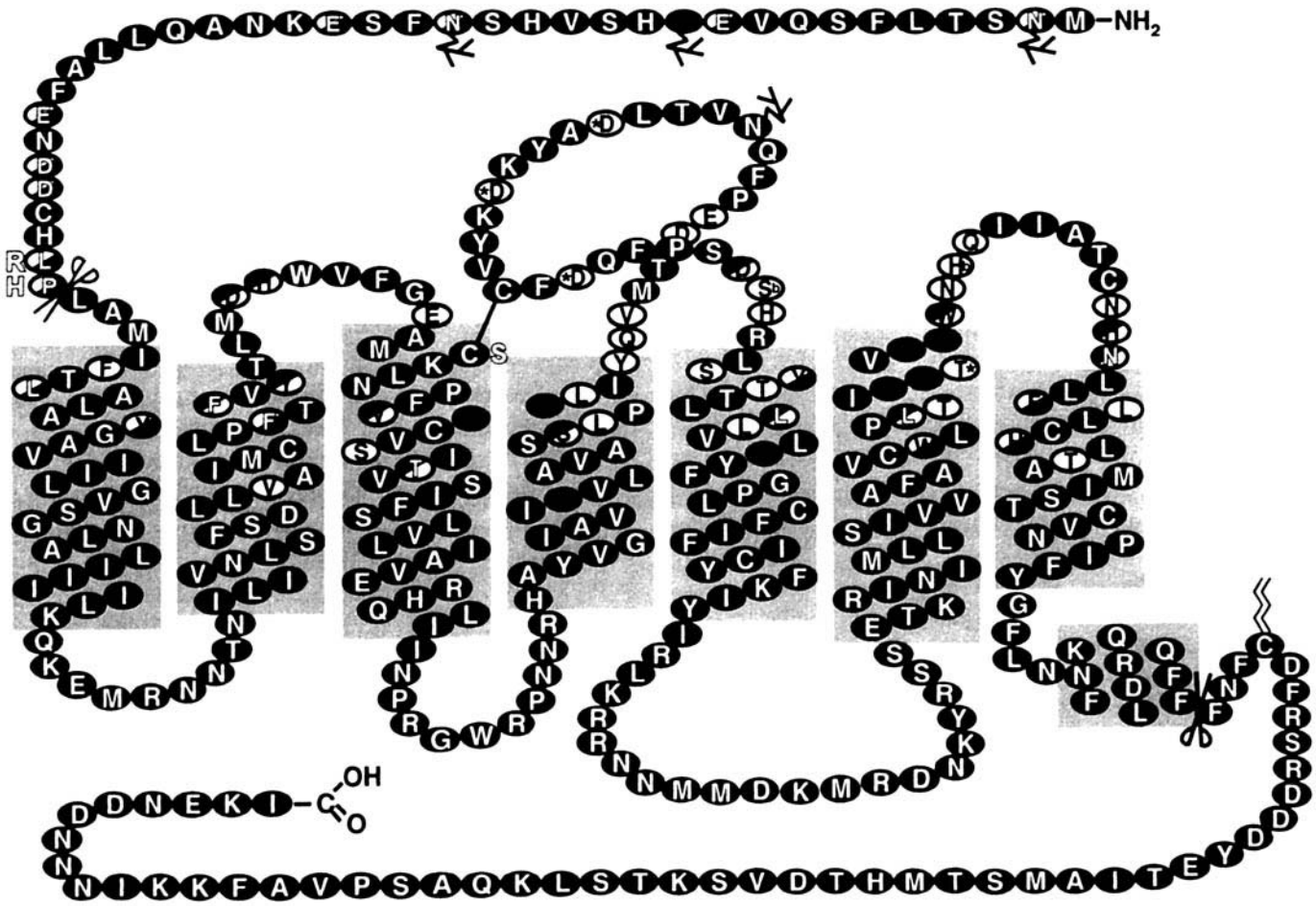

Figure 4. Summary of structural and mutagenesis studies performed on the mammalian Y1 receptor. Amino acids were mutated to alanine except in three positions. Those are indicated by a shaded amino acid next to the original sequence. Red indicates a loss in affinity >2.5-fold. Upper left: effects on NPY/PYY binding, lower right: antagonist. White: no effect. Black: not done. Special cases: (a) Mutation affects binding of BIBP3226 but not 1229U91 and J-104870, (b) an increase in affinity was detected, (c) when Gln^{120} was mutated to tyrosine, no binding of NPY was detected while mutation to glutamine abolished BIBP3226 binding, (d) triple mutant [N2Q, N11Q, N17Q] could not be expressed whereas the double mutant [N2Q, N17Q] bound ^{3}H-NPY with the same affinity as the wild-type receptor. Deletion of the carboxy-terminus did not affect binding (black scissor) whereas deletion of the amino-terminus abolished binding of NPY (red scissor). A star (*) indicates that there are differences between the data from different mutagenesis studies at that particular amino acid.

cellular parts of the Y1 receptor (Asp^{104}, Asp^{194}, Asp^{200}, and Asp^{287}; Ref. 285) were proposed to interact with NPY. Although later studies confirmed the effect of the mutation D104A in NPY binding, this mutation was found to have no affect on binding of antagonists (288, 291). Similarly, Y100A was found to bind BIBP3226 (Y1-selective antagonist; Fig. 1; Ref. 110) but not NPY (288). In contrast, in later studies, D194A bound NPY with the same affinity as the wild-type receptor (287, 291) and D204A bound both NPY and BIBP3226 with wild type affinity in two other studies (287, 289). In a third study, Robin-Jagerschmidt and colleagues reported that the D204A mutation caused a 6-fold loss in affinity of NPY (290). Although no NPY binding to F286A was detected by Munch *et al.* (287) and Sautel *et al.* (286, 288), Du and colleagues (289) found that this mutation decreased BIBP3226 binding affinity but not PYY binding. According to Sautel and colleagues, Tyr^{211} in TM5 was the only amino acid that, when mutated to alanine, affected ^{3}H-BIBP3226 binding without affecting binding of ^{125}I-NPY (288). In contrast, mutation of amino acids Tyr^{100}, Asp^{104}, Trp^{288}, and His^{298} to alanine abolished binding of ^{125}I-NPY with virtually no effect on ^{3}H-BIBP3226 binding (288). Alanine replacement of Trp^{163}, Phe^{173}, Gln^{219}, Asn^{283}, and Asp^{287} resulted in loss of binding for both ^{125}I-NPY and ^{3}H-BIBP3226 (288). The mutant F286A did not display any NPY binding whereas the affinity of BIBP3226 decreased 6-fold (288). In the other study (289), the effect on antagonist binding for the Y211A mutation was confirmed but, in contrast to Sautel *et al.* (288), Phe^{173}, Phe^{286}, and His^{298} were also found to affect binding of BIBP3226 with little or no effect on ^{125}I-PYY binding. For the mutations at His^{298}, the effect was totally opposite when comparing Sautel *et al.* (288) and Du *et al.* (289). However, in Sautel, His^{298} was mutated to alanine, whereas in Du, it was mutated to glycine (the corresponding amino acid in the Y4 receptor that does not bind BIBP3226). Therefore, there is a possibility that this difference could account for the discrepancy in the results. In addition, Tyr^{47} in TM1 and Phe^{302} in TM7 was also found to affect binding of the antagonist BIBP3226 in (289) but not J-104870 (291). Although there are many contradictions in the various mutation studies, there are some important conclusions that can be made. Overall, the binding domain for nonpeptide antagonists (BIBP3226 and J-104870 in this case) appears to be more restricted to the TM regions of the Y1 receptor (mainly TM5-7) whereas mutations affecting binding of NPY and PYY are found both in loops and TM regions. It also appears the recognition elements required for NPY and PYY are very similar.

Interactions between NPY and the amino terminus of the Y1 receptor can also be inferred. Insertion of the FLAG epitope in this part of the receptor, right after the start-codon, abolished binding of NPY (285) whereas deletion of the first 20 amino terminus of the rat Y1 receptor prevented cell surface expression (290). Curiously, addition of green fluorescent protein at the very amino terminus (i.e., the same position as the FLAG epitope was placed in Walker *et al.*, Ref. 285) did generate a receptor that was able to functionally couple to G-proteins in HEK293 cells (103) upon agonist binding. It is likely that certain parts of the amino terminus are important for proper folding, glycosylation, and trafficking of the receptor. Thus, effects of mutations in this area may reflect misfolded receptors or lack of cell surface expression rather than actual points of interaction. When all possible sites for N-linked glycosylation in the amino terminus of the rat Y1 receptor were mutated to glutamine (N2Q+N11Q+N17Q), cell surface expression of the mutated receptor was totally abolished (290). However, mutation of Asn^{2} and Asn^{17} to glutamine (N2Q+N17Q) did not affect the expression or ligand binding suggesting that Asn^{11} is essential for cell surface expression. Another explanation for this is that at least one glycosylated asparagine at the amino terminus is necessary for receptor function, though there are no compelling data that this region is required for ligand binding or G-protein coupled signal transduction. Interestingly, *Escherichia coli* has been used successfully to express the human Y1 receptor (287, 292). The receptor bound ^{125}I-NPY with similar affinity as when expressed in mammalian cell lines. As bacteria do not exhibit N-linked glycosylation of proteins it can be assumed that glycosylation is important for the trafficking of the receptor in mammalian cells rather than the actual binding of the PP-fold peptide. The Y1 receptor has also successfully been expressed in insect (Sf21) cells (293) where a protein of similar size as the endogenous human Y1 receptor in SK-N-MC cells, was detected suggesting that the Y1 receptor is glycosylated in Sf21 cells. In contrast to the amino terminus, the complete carboxy-terminus could be deleted with no effect on NPY binding to the Y1 receptor (100, 285). Also, the Y1 receptor has been successfully expressed carrying green fluorescent protein in the carboxy-terminus (99).

Some of the differences between the different mutagenesis studies can probably be explained by the various and very heterogeneous expression systems used. For instance, Walker and colleagues used a vaccinia virus vector to express the human Y1 receptor in HeLa cells (285, 286) and later found differences when these mutants were compared with the same mutants expressed in *E. coli* and mammalian cell lines (287). It is also possible that the usage of different mammalian cell lines may alter the pharmacology of the very same receptor protein depending on what other proteins are expressed in the cell.

Sequence Comparisons and Ligand Recognition. Cloning of receptors from various species can, apart from providing exciting insights into evolutionary processes, also result in interesting data on how the primary sequence influence ligand binding and function. A high degree of sequence identity between two receptors with different pharmacology can be used to identify amino acids important for a particular function or binding profile and help pinpoint where to start a mutagenesis study. For example, when the chicken Y2 receptor was cloned it was

found to exhibit a pharmacological profile very unlike the mammalian Y2 receptors in that it bound the prototypical Y1-like receptor agonist [Leu^{31},Pro^{34}]NPY but not the Y2 selective antagonist BIIE0246 (140; Fig. 2). Furthermore [Leu^{31},Pro^{34}]NPY could inhibit forskolin stimulated cAMP synthesis at the chicken but not at the human Y2 receptor (140). Sequence comparison between the human and chicken Y2 receptors as well as computer modeling of the human Y2 revealed several candidate positions that could be responsible for the pharmacological differences. Reciprocal point mutations of Gln^{135} in TM3 (His^{139} in chY2), Leu^{227} in TM5 (Gln^{231} in chY2), and Leu^{284} in TM6 (Phe^{288} in chY2) could almost totally exchange the binding properties of the human and chicken Y2 receptors with regards to BIIE0246 binding (294). The triple mutant of the chicken receptor (chY2[Q135H, L227Q, L284F]) bound BIIE0246 with similar affinity as the hY2 receptor while BIIE0246 bound to the triple mutant of the hY2 receptor with micromolar affinity. An alanine scan of these three amino acids in the hY2 receptor suggested that Leu^{227} may form a direct interaction with the antagonist whereas the roles of Gln^{135} and His^{284} are more uncertain (294). Similarly, the cloning of the Y1 receptor in chicken revealed interesting differences to the mammalian receptors as it binds the Y1 selective antagonist BIBP3226 but not SR120819A (189). This lays the groundwork for future studies on the recognition domains for these antagonists.

The fish receptors also present opportunities to understand ligand recognition in this receptor family. Fish Ya-c (191–194) display reasonably high sequence identities (≈50% between subtypes) to mammalian Y1, Y4, and y6 receptors but very different pharmacological properties. As described above, seven amino acids were found to be important for BIBP3226 binding (288). Interestingly, all these amino acids are conserved in fish Ya-c. Yet, Y1 is the only receptor that binds BIBP3226 (197). Of the additional positions described in (289), Tyr^{47}, Trp^{276}, and Phe^{302} are conserved in all PP-fold receptors known (including Y2 and Y5) while Phe^{286} varies between subtypes but is conserved in fish Yb and Yc.

Possible explanations for the lack of BIBP3226 binding to Y4, y6, and the fish receptors could be that there may be additional interaction points for BIBP3226 in the Y1 receptor besides the amino acids identified in (288) and (289), or that adjacent amino acids in the other receptors may occupy the space between TM4-6 where BIBP3226 has been proposed to bind. However it is obvious that more structural studies of the Y1 receptor are necessary to elucidate how agonists and antagonists bind. Finally, to our knowledge, there are no published reports on mutagenesis of the Y4 and Y5 receptors.

Overview

Over the past 5 years, significant advances have been made in our understanding of the physiological role for the PP-fold peptides. Numerous new pharmacological tools have become available that have improved specificity for the respective receptor subtypes. In addition, knockouts for three of the receptor subtypes in mice have become available and, in many cases, have facilitated the identification of new potential functions for these receptors. As we stated in the previous review, we have entered the "golden age" of research in the PP-fold peptides.

In the previous review the question was posed, "How many receptors? How many peptides?" During the past 5 years, no new peptide or receptor sequences have been identified; therefore, it appears less likely that additional peptides or receptors exist. We also speculated that receptor knockouts would play an increasingly important role in understanding the PP-fold peptide system. This has proven to be correct, with numerous authors publishing studies that use this technology to understand the system. Undoubtedly, numerous other researchers will tap into the potential of this technology in the upcoming years. In addition, NPY-overexpressing rats and mice have been produced and been found to be useful in understanding potential pathological role of NPY. Furthermore, a PP-overexpressing mouse strain has also been published. Unfortunately, the PYY system has received less attention than NPY and to date, no transgenic PYY animals have been reported. It will be interesting in the future to obtain animals overexpressing PYY as well as knockouts of PYY and PP, which would enable studies of double knockouts, especially of animals lacking both NPY and PYY. Finally, it was speculated that increased understanding of PP-fold peptides in human biology would help us understand the relevance of animal studies. Primarily due to the lack clinically useful small molecule antagonists, very little progress has been made in this area. It is our hope that in the next 5 years subtypes selective antagonists will enter clinical development and enlighten us as to the role of these peptides in human health.

Are There Additional Receptor Subtypes in Mammals? To date, no new human receptor sequences have been identified. However, many indications for additional receptors come from evolutionary studies. The fact that fish have receptors that evolutionarily do not fall distinctively under any of the known mammalian receptor subtypes may infer the presence of additional subtypes in mammals. However, fish are so unrelated to mammals that the additional receptors may very well reflect separate evolutionary events in fish rather than yet undiscovered receptor subtypes in mammals (195). Furthermore, the human Y4 and y6 receptors have been mapped to chromosome 10q11.2 (295) and 5q31 (184), respectively. If the Y4 and y6 receptor genes arose as the result of chromosome duplications one would expect to find Y2 and Y5 receptor like genes in close proximity to both the Y4 and y6 receptor genes, as the chromosome duplications must have taken place after the local duplications that generated the Y1-Y2-Y5 complex on chromosome 4 (see Ref. 296 for review). However, these Y2- and Y5-like genes on chromosome 5 and 10 may very well have been lost during the evolution.

Several additional receptors have also been suggested from pharmacological evaluations of different organ preparations (see receptor section). However, it is likely that some, if not all of these in fact are combinations of the known receptors or that some of the known receptors may display different pharmacological profiles dependent upon cells or organs in which they are expressed. In some cases, the discrepancies might be explained by species differences as has been shown for the Y4 receptor.

Another important area for future research will be the ligand recognition involved in the PP-fold receptors. Studies using site-directed mutagenesis should be used to understand the interaction of agonists and antagonists with the Y4 and Y5 receptors. It will also be important, if technically feasible, to obtain crystal structures of these receptors with and without bound ligands.

How Far Will Receptor Knockout Animals Further Our Understanding of the Functional Role of This System? Perhaps the most controversial aspect of NPY research has been the results obtained using transgenic knockout mice. In most cases, the receptor knockout mice have exhibited increased adiposity. This is surprising in light of the vast body of research demonstrating that NPY and receptor-selective peptide agonists increase food intake, decrease metabolism, and increase adiposity. It has also been difficult to delineate the receptor subtypes that contribute to NPY-induced feeding using knockout mice. The best information to date is suggestive of both Y1 and Y5 receptors contributing to this effect in mice. This is further confounded by the expression of a functional y6 receptor in mouse hypothalamus that may also contribute to mouse feeding. In addition, several Y1 antagonists have been demonstrated to block the increase in food intake produced by NPY. More controversially, Y5 antagonists have had mixed results in feeding studies. At this point in time, it is likely that both the Y1 and Y5 receptors contribute to the feeding observed after central administration of NPY. Future studies will need to look at the interaction of these receptors to further understand their contributions to feeding. In addition, knocking out several receptor subtypes in the same animal will be an important line of research as well. Finally, more refined feeding studies need to be conducted to better understand the role of NPY in situational feeding in knockout mice. For instance, the feeding response to hyperglycemia has not been investigated nor has macronutrient selection.

On the other hand, the use of knockout animals has opened up new avenues of research. Particularly interesting has been the evolving understanding of the NPY system and ethanol consumption. Clearly, this is an important area for future research, which will require the use of receptor specific knockouts such as the recent study by Thiele and coworkers (130). In addition, results from early studies suggest that the PP-fold peptides play important roles in endocrine and peripheral organ function. A key missing component of this line of research is the low abundance of studies of peripheral functions using receptor knockouts. Given the evolutionary divergence of the Y4 receptor with regards to sequence and distribution, this will be the most challenging of the receptors to understand in terms of its physiological function.

Because mice express a functional y6 receptor, this species may not be the most appropriate to understand the human function of the PP-fold receptor subtypes. Significant progress has been made in developing NPY overexpressing rat. It will be interesting to note developments in rat-based receptor knockout models over the next 5 years.

How Important Are the Endocrine and Peripheral Organ Functions? Although the importance of NPY in the regulation of luteinizing hormone release is well established, there is more limited data outlining the role of NPY in the release of other hormones. This will need to be important area of research. Particularly interesting are recent studies showing hypothalamic NPY Y2 receptors may be involved in regulation of bone formation. It will be exciting to link these central actions to the peripheral effects via the endocrine system. Although there have been exciting developments in our understanding the role of PP and PYY in gastrointestinal function, this is an area that would also benefit of additional research and understanding. The presence of multiple receptor subtypes along the GI tract suggests a coordinated function in regulation of the digestive process. It will be important to understand how these receptor subtypes interact and what signal transduction processes are affected.

What Will It Take to Get Clinical Development of PP-fold Peptide-Based Pharmaceuticals? Over the past 5 years, a number of novel peptide agonists and nonpeptide antagonists have been discovered. Several of these tools have been useful to define the functional role of NPY receptor subtypes *in vivo*. However, all these molecules have substantial limitations as drug development candidates. Improvements will be necessary in the oral bioavailability and central nervous system and penetration of all these compounds. Nevertheless, it is apparent that Y1 antagonists are capable of reducing NPY-driven feeding as well as naturally driven feeding responses. On the other hand, Y5 receptor antagonists do not produce consistent inhibition of feeding responses in rats. It is not clear whether this is caused by limited brain penetration and bioavailability or a flaw in the hypothesis. The very low level of protein expression for both Y1 and Y5 in the hypothalamus makes it difficult to reconcile the *in vivo* effects with the receptor distribution. For the Y2 receptor, the pharmacological antagonist tools are currently limited to a single compound (Fig. 2). Further compound discovery in particular, the discovery of centrally penetrating antagonists will be necessary to understand the opportunity this receptor presents. Certainly, this receptor appears to play an important role in brain function, endocrine function, and GI function. Much like the receptor knockout mice, better pharmacological tools will be the key not only to understanding the physiological role of PP-fold peptides but also the role in human

pathology. Along with development of novel pharmaceutical agents for PP-fold receptor subtypes, it will be important to understand the molecular interaction of the agents with the receptors. Further studies using site directed mutagenesis or, if possible, receptor crystallization will be important steps in understanding transmitter and antagonist interactions with these receptors.

Conclusion

Substantial progress has been made in our understanding of the role of PP-fold receptor subtypes in physiological function. These advances have been facilitated by receptor knockout animals, NPY-overexpressing animals, novel peptide analogues, novel nonpeptide antagonists, and creative cutting-edge science. Perhaps the greatest advances have been in our understanding of the role that individual receptor subtypes play in physiological function. However, these stories are not yet complete. Significant gaps still exist in even the most important and well-understood actions of these peptides. Particularly perplexing has been our inability to understand the importance of NPY in normal and pathological feeding or understanding of the receptor subtypes involved in this response. Looking ahead, further exploitation of the technological advances made in the last 5 years will be required to fully understand the system. Undoubtedly, more surprises await us in the future.

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