Identification of High-Potency Neuropeptide Y Analogues through Systematic Lactamization†

Dean A. Kirby, Karen T. Britton,‡ Michel L. Aubert,§ and Jean E. Rivier*

Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, Department of Psychiatry, San Diego Veterans Administration Medical Center, La Jolla, California 92037, and Division of Biology of Growth and Reproduction, University of Geneva Medical School, Switzerland

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In the pursuit of potent analogues of neuropeptide Y (NPY) that are selective for the Y_1 receptor subtype, two lactam bridge scans of a centrally truncated parent compound were synthesized. A single lactam bridge (γ-carboxyl of Glu to ϵ-amino of Lys) extending from residues i to $i + 3$ or i to $i + 4$ of the proposed α-helical region (residues 25−31 of NPY) was introduced in des-AA$^{7-24}$[Gly^6]NPY. Cyclogues (contraction of cyclic analogues), which were approximately one-half the size of native NPY, were initially screened for binding affinity at two discrete NPY receptor types using human neuroblastoma cell membranes, SK-N-MC and SK-N-BE2. Exploitation of the subtle differences present on each receptor type allowed for the identification of cyclogues which bound specifically to Y_1 receptors with increased affinity when compared to the corresponding linear parent analogue, while one short Y_1 specific cyclogue, des-AA$^{2,3,5,7-24}$cyclo(26/29)[Gly^6,Glu^{26},Lys^{29},Pro^{34}]NPY, bound with $K_i = 16$ nM. Other cyclogues showed distinct preference for Y_2 receptors and bound in the low-nanomolar range. Functionally, the compounds inhibited the norepinephrine-stimulated accumulation of cAMP indicating that all acted as agonists with varying potencies.

Introduction

Neuropeptide Y is a 36-residue member of the pancreatic polypeptide (PP) family of structurally related peptides and is one of the most abundant peptides distributed in the mammalian brain.[1] Centrally, exogenous NPY has been demonstrated to elicit numerous pharmacological effects including mediation of analgesia, anxiolysis, feeding behavior, and hormone secretion.[2−5] Peripherally, NPY is co-stored with norepinephrine in sympathetic terminals and may play a role in vasoconstriction.[6,7] Early spectral studies as well as molecular dynamics simulation suggested that the tertiary structure of native NPY may be characterized as having an N-terminal polyproline helix, a β-turn through positions 9−14, an amphipathic α-helical segment extending from residues 15 to 32, and a C-terminal turn structure,[8] though more recent examination by NMR techniques suggests a slight variation of this model.[9,10]

The distribution of NPY and its receptors is widespread throughout the periphery as well as most brain regions, particularly in the hypothalamus, hippocampus, and amygdala.[11] The earliest and best characterized forms of NPY receptors have been designated Y_1 and Y_2 receptors and are held responsible for the bulk of the pharmacological responses of NPY.[12−15] A Y_3 subtype has been proposed and characterized,[16,17] while still others have been cloned including Y_4,[18] a recently cloned Y_5 or "feeding" receptor,[19] and several PP receptors.[20−22] Both Y_1 and Y_2 receptors are known to be G-protein-coupled receptors with the downstream response resulting in the inhibition of adenylate cyclase, Ca^{2+} mobilization, and other responses.[14,23] Many cyclic and/or centrally truncated peptide analogues of NPY have been developed that show high affinity and selectivity for either Y_1 or Y_2 receptors.[24−28] Additionally, several small nonpeptide mimetics have recently been reported to display selectivity for and activation of specific receptors.[29−31]

Early in our investigations, we and others demonstrated that centrally truncated analogues of NPY (i.e., des-AA$^{7-24}$) could be optimized in such a way as to produce high affinity to the Y_2 receptor type only.[24,25,28] From these structure−activity relationship (SAR) data, we suspected that des-AA$^{7-24}$NPY contained all the significant residues needed to be recognized by the Y_1 receptor. We therefore hypothesized that constraining this molecule in a correct orientation could yield an analogue that would be short yet bind with high affinity to Y_1 receptors. Whereas peptides incorporating long range intramolecular constraint between the N- and C-termini were shown to bind to Y_2 receptors only,[24,25,27,28] we sought in the present study to constrain the conformation of the truncated peptide in the central region of the NPY fragment. The amphiphilic helix located in this region of the truncated NPY molecule may contribute to binding by providing a relatively constrained scaffold to the pharmacophore, though most likely not interacting with the receptor binding site directly. Side chain-to-side chain cyclization from residues i to $i + 3$ and i to $i + 4$ has been successfully employed in peptides which contain large α-helical segments such as growth hormone-releasing factor (GRF),[32] corticotropin-releasing factor (CRF),[33] and dynorphin A.[34] By applying this systematic scan approach, an NPY cyclogue with high

† Abbreviations: The abbreviations are in accord with the recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). The symbols represent the L-isomer except where indicated otherwise. In addition: CZE, capillary zone electrophoresis; HPLC, high-performance liquid chromatograpy; NMP, *N*-methyl-2-pyrrolidone; NPY, neuropeptide Y; TEAP, triethylammonium phosphate; TBTU, 2-(1*H*-benzotriazol-1-yl)-tetramethyluronium tetrafluoroborate.

‡ San Diego Veterans Administration Medical Center.

§ University of Geneva Medical School.

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Table 1. Physical and Biological Properties of NPY Analogues

compd no.	NPY analogue	K_i (nM) Y_1[a]	K_i (nM) Y_2[b]	selectivity[c]	purity[d] @ rt (%)	mass[e] (*m*/*z*, calcd/obsd)
1	NPY	0.8	0.3	2.6	99 @ 60	4254.7/4255.0
2	des-AA$^{7-24}$[Gly^6]	70	2.6	30	98 @ 41	2261.2/2261.6
3	des-AA$^{7-24}$cyclo(25/29)[Gly^6,Glu^{25},Lys^{29}]	1240	134	9.3	87 @ 50	2230.2/2230.2
4	des-AA$^{7-24}$cyclo(26/30)[Gly^6,Glu^{26},Lys^{30}]	1010	0.7	1440	95 @ 44	2250.2/2250.1
5	des-AA$^{7-24}$cyclo(27/31)[Gly^6,Glu^{27},Lys^{31}]	210	2	105	95 @ 36	2224.2/2224.2
6	des-AA$^{7-24}$cyclo(28/32)[Gly^6,Glu$_{28}$,Lys^{32}]	250	14	18	96 @ 40	2286.2/2286.0
7	des-AA$^{7-24}$cyclo(25/28)[Gly^6,Glu^{25},Lys^{28}]	8860	5.6	1580	95 @ 47	2231.2/2231.2
8	des-AA$^{7-24}$cyclo(26/29)[Gly^6,Glu^{26},Lys^{29}]	85	104	0.8	99 @ 49	2249.3/2249.1
9	des-AA$^{7-24}$cyclo(27/30)[Gly^6,Glu^{27},Lys^{30}]	4870	6	810	95 @ 37	2224.2/2223.9
10	des-AA$^{7-24}$cyclo(28/31)[Gly^6,Glu^{28},Lys^{31}]	6490	24	270	94 @ 35	2274.2/2274.2
11	des-AA$^{7-24}$cyclo(26/29)[Gly^6,Glu^{26},ᴅTyr^{27},Lys^{29}]	1050	700	1.5	98 @ 49	2249.3/2249.3
12	des-AA$^{7-24}$cyclo(26/29)[Gly^6, Glu^{26},ᴅIle^{28},Lys^{29}]	2320	2050	1.1	97 @ 46	2249.3/2249.3
13	des-AA$^{7-24}$cyclo(26/29)[Gly^6,Glu^{26},Lys^{29},Pro^{34}]	44	14400	0.003	96 @ 49	2218.3/2218.3

[a] Binding affinities given as K_i for Y_1 and Y_2 receptors using the human neuroblastoma cell membranes SK-N-MC[a] and SK-N-BE2,[b] respectively. [c] Selectivity represents the ratio of binding affinities to Y_1 vs Y_2 receptors, where low numbers reflect greater selectivity to Y_1 receptors. [d] Purity and elution determined by analytical HPLC using linear gradient conditions (1% B increase/min) in solvent system comprised of A, 0.1% TFA; B, 60% CH_3CN in 0.1% TFA. Column was Vydac C_{18} (5 mm particle size, 46 × 250 mm); detection was 0.1 AUFS at 210 nm. Flow rate was 1.0 mL/min. [e] Monoisotopic mass measured with LSI-MS; glycerol and 3-nitrobenzyl alcohol (1:1) matrix; Cs ion source.

affinity (K_i = 44 nM) and selectivity at the Y_1 receptor was identified (**13**), and further modifications led to yet smaller cyclogues with improved affinity.

Experimental Section

Peptide Synthesis. All peptides were synthesized manually using standard solid phase peptide synthesis (SPPS) techniques following Boc-strategy on MBHA resins prepared in our laboratory by methods previously described.[24] Side chain protection of α-Boc amino acids was as follows: Arg(Tos), Glu(OFm), His(Tos), Lys(γ-2ClZ or Fmoc), Ser(Bzl), Thr(Bzl), and Tyr(2BrZ). Following complete synthesis of peptide sequence, lactam bridges were constructed on the resin by first treating the orthogonally protected peptide with 20% piperidine to remove the Fmoc and OFm protecting groups. Cyclization resulted from the addition of 2 equiv of TBTU in NMP while solution was kept basic by the addition of *N*,*N*-diisopropylethylamine (DIPEA). The cyclized and fully protected peptide was finally cleaved from the resin in anhydrous HF.

Purification and Characterization. Crude peptides were purified by preparative reverse phase HPLC initially using a linear TEAP/60% MeCN (pH 2.25) buffer system gradient. Acceptable fractions were pooled, reloaded onto the preparative cartridge, and desalted in 0.1% TFA. Purified peptides were subjected to HPLC, CZE, and liquid secondary ion-MS analysis measured with a Jeol JMS-HX110 double-focusing mass spectrometer. Purity was found to be >95% for most analogues, with complete details shown in Tables 1 and 2.

Radioligand Binding Assays (Tables 1 and 2). Receptor binding and cAMP assays were performed at Alanex Laboratories, San Diego, CA. The activity of the compounds at NPY receptors was assessed by determining their ability to inhibit the binding of ^{125}I-labeled PYY to Y_1 or Y_2 receptors in membranes derived from clonal cell lines. PYY was radioiodinated using chloramine T, and the product was purified by reverse phase HPLC. The source of Y_1 receptors was SK-N-MC cells, while the membrane source for the Y_2 receptor binding assay was the SK-N-BE2 cell line. Briefly, SK-N-MC or SK-N-BE2 cells were harvested with an EDTA-containing saline solution, resuspended in a hypotonic buffer, and homogenized with a Polytron tissue disrupter. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 48000*g* for 20 min at 4 °C. The membranes were washed by centrifugation and resuspension, and the final pellet was stored at −70 °C until use. The binding of [^{125}I]PYY (30−50 pM) ± test compounds to thawed membranes was performed in a buffer consisting of 25 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM KCl, 135 mM NaCl, 0.1% bovine serum albumin (BSA), and 100 μg/mL bacitracin. The assays were incubated for 90 min at 37 °C and terminated by rapid filtration over glass fiber filters that had been presoaked with 0.1% polyethylenimine. [^{125}I]PYY binding was quantitated with either a Packard TopCount scintillation or Packard Cobra gamma counter. Dose−response data were analyzed by nonlinear regression using the computer program Prism (GraphPad Software, San Diego, CA). IC_{50} values representing the concentration of drug that produced a 50% displacement of binding of [^{125}I]PYY are given.

NOTE: The binding to membranes of certain peptides reported here proved to depend on the presence or absence of physiological concentrations of calcium (data not shown). Although not consistent, as much as a 40-fold decrease in binding affinity was noted in the presence of calcium.

cAMP Generation (Table 3). The inhibition of cAMP accumulation was measured in SK-N-MC cells. Cells were first washed and resuspended in DME. NPY or analogues were added at varying concentrations to the appropriate wells together with NE. Following incubation at 37 °C for 30 min, cold 0.1 N HCl was added to each well to terminate the cAMP production. Each well was sonicated for 5 s and then lyophilized. Samples were reconstituted in a sodium acetate

Table 2. Physical and Biological Properties of Deletion Analogues Based on Des-AA$^{7-24}$cyclo(26/29)[Gly^6,Glu^{26},Lys^{29},Pro^{34}]NPY

compd no.	modification to compound **13**	Y_1 K_i[a] (nM)	purity[b] @ rt (%)	mass[a] (*m*/*z*, calcd/obsd
14	des-Tyr^1	39	98 @ 47	2055.2/2055.2
15	des-Pro^2	39	97 @ 49	2121.2/2021.3
16	des-Ser^3	31	98 @ 49	2131.2/2131.1
17	des-Lys^4	36	96 @ 53	2090.2/2090.1
18	des-Pro^5	13	94 @ 49	2121.2/2121.4
19	des-Gly^6	38	95 @ 50	2161.2/2161.4
20	des-Arg^{25}	1350	98 @ 53	2062.2/2062.3
21	des-AA$^{1-6,25}$	3720	99 @ 49	1432.8/1432.9
22	[*N*-acetyl]des-AA$^{1-6,25}$	1670	98 @ 55	1474.9/1474.9
23	des-AA$^{2-6,25}$	700	97 @ 53	1595.9/1595.9
24	des-AA$^{2-6}$	100	98 @ 50	1752.0/1752.1
25	des-AA$^{2-5}$	84	96 @ 48	1809.0/1809.0
26	des-AA$^{2-4}$	18	96 @ 52	1906.1/1906.0
27	des-AA^{2,5}	13	99 @ 47	2024.2/2024.4
28	des-AA^{2,3,5}	16	97 @ 47	1937.1/1937.3

[a] Binding affinities given as K_i for Y_1 receptors using the human neuroblastoma cell membranes SK-N-MC. [b] Purity and elution determined by analytical HPLC using linear gradient conditions (1% B increase/min) in solvent system comprised of A, 0.1% TFA; B, 60% CH_3CN in 0.1% TFA. Column was Vydac C_{18} (5 mm particle size, 46 × 250 mm); detection was 0.1 AUFS at 210 nm. Flow rate was 1.0 mL/min. [c] Monoisotopic mass measured with LSI-MS; glycerol and 3-nitrobenzyl alcohol (1:1) matrix; Cs ion source.

Table 3. Activities of NPY Cyclogues on the Inhibition of Norepinephrine-Stimulated cAMP Accumulation in SK-N-MC Cells[a]

compd no.	NPY analogue	EC_{50} (nM)	SEM
5	des-AA$^{7-24}$cyclo(27/31)[Gly^{6},Glu^{27},Lys^{31}]	235	65
8	des-AA$^{7-24}$cyclo(26/29)[Gly^{6},Glu^{26},Lys^{29}]	62	20
12	des-AA$^{7-24}$cyclo(26/29)[Gly^{6},Glu^{26},D-Ile^{28},Lys^{29}]	7357	2820
13	des-AA$^{7-24}$cyclo(26/29)[Gly^{6},Glu^{26},Lys^{29},Pro^{34}]	23	7
14	des-AA$^{1,7-24}$cyclo(26/29)[Gly^{6},Glu^{26},Lys^{29},Pro^{34}]	55	13
27	des-AA$^{2,5,7-24}$cyclo(26/29)[Gly^{6},Glu^{26},Lys^{29},Pro^{34}]	24	10
28	des-AA$^{2,3,5,7-24}$cyclo(26/29)[Gly^{6},Glu^{26},Lys^{29},Pro^{34}]	12	4

[a] Values represent the mean ± SEM of independent experiments (n = 3) done in triplicate.

solution. Into prechilled tubes on ice was added buffer, standard, or sample followed by [^{3}H]cAMP trace and protein kinase. Samples were vortexed and incubated at 4 °C for 90 min. At the end of the incubation period, a charcoal slurry was added to each tube, incubated at 4 °C for 15 min, and decanted into scintillation vials containing cytoscint, and radioactivity was measured using a scintillation counter.

Results and Discussion

Early attempts at reducing the length of 36-residue neuropeptide Y produced analogues with significant loss of affinity and activity at Y_1 receptors, those receptors thought to be most responsible for NPY's role in vasoconstriction, analgesia, anxiolysis, feeding behavior, and hormone secretion.[28,35,36] High-potency truncated peptides selective for the Y_2 receptor were identified indicating that Y_1 and Y_2 receptors recognized distinct pharmacophores and that Y_1 receptors were possibly more discriminating. An alternate approach to identifying potent Y_1 selective leads has emerged from the development of nonpeptide mimetics derived from both rational design based on the C-terminal peptide pharmacophore as well as exhaustive screening of chemical libraries. Several high-affinity antagonists have been developed and characterized by various pharmaceutical corporations through these approaches, with BIBP3226, SR120819A, and various benextramine derivatives being among the most promising pharmaceutical tools and hopeful therapeutic leads.[29−31] More conservatively, we and others have continued to design peptide analogues that might yield insight into the nature of the binding and activation of the Y_1 receptor by its native ligand. This approach, though laborious and perhaps a longer route to producing potential drug candidates, is still quite necessary since it has been shown that peptide agonists and nonpeptide antagonists bind at different binding domains of the G-protein-associated receptor.[37−39] By making small incremental changes in a lead peptide analogue, it is possible to introduce specific modifications that will produce receptor specificity and trigger biological activation while avoiding deleterious effects such as nonselective binding to unrelated receptors and chemical toxicity. When optimizing a natural polypeptide as large as NPY, there are four fundamental challenges to address before a ligand can be used to study its pharmacology, mechanism of action, and assess its therapeutic potential: (1) length or size must be reduced to identify the smallest bioactive fragment, (2) flexibility must be limited to facilitate structural investigations, (3) specificity to a particular receptor must be enhanced to understand mechanism of action, and (4) highly potent agonists or antagonists must be obtained for therapeutic uses.

In meeting these challenges, our first step was to identify a lead compound in which the size was reduced. Truncation of C-terminal residues in all previous cases

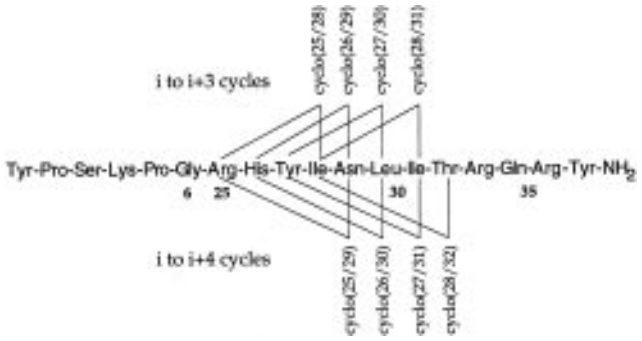

Figure 1. Sequence of des-AA$^{7-24}$NPY cyclogues showing the possible side chain bridge cycles.

produced analogues with complete loss of binding affinity for both Y_1 and Y_2 receptors, whereas the deletion of N-terminal residues produced analogues capable of binding to Y_2 receptors exclusively.[40,41] A centrally truncated NPY analogue (des-AA$^{10-17}$cyclo(7/21)-[Cys^{7,21},Pro^{34}]NPY) was shown to produce high selectivity to Y_1 receptors and was used to identify Y_1 receptor heterogeneity in rat brain.[27] Though even shorter peptides (i.e., deletion of residues 7−24) showed significant loss of affinity at Y_1 receptors, binding to Y_2 receptors was very high,[24,25] suggesting that a sequence of des-AA$^{7-24}$NPY may contain all the significant residues needed to make contact with the Y_1 receptor also.

One major problem with long linear polypeptides is that they may exist in multiple conformations, each one with the ability to present distinct pharmacophores and thereby potentially activate various receptors. By imposing local and global constraint on the flexible molecule, or in this case a truncated analogue, we can limit its ability to resonate between the various conformations. In the present study, we sought to investigate the SAR generated from a systematic scan of NPY cyclogues, all based on our smallest NPY analogue which previously displayed affinity for both Y_1 and Y_2 receptors (**2**, see Table 1 and Figure 1). By cross-linking the side chains of residues within a region expected to exist as an α-helix, we hypothesized that the added constraint might lock the putative helical conformation into a favorable fit with the receptors, a conformation that may not be readily accessible in the absence of the added constraint. Therefore, only the central segment of the molecule (positions 25−32) would be available for modification. Since one turn of an α-helix is composed of 3.6 residues, it has been demonstrated that an i to $i + 3$ or an i to $i + 4$ side chain bridge arrangement using Glu to Lys would best constrain the helix, as was the case in similar cyclic peptide scans.[32−34] From this approach it was discovered that des-AA$^{7-24}$cyclo(26/29)-[Gly^{6},Glu^{26},Lys^{29}]NPY (**8**) displayed unusually high affinity for the Y_1 receptor with K_i = 85 nM, while no other cycle in this scan produced analogues with sig-

nificant Y_1 affinity. With the exception of **5** which bound with $K_i = 210$ nM, the longer *i* to *i* + 4 cyclization produced analogues that were ineffective binders at Y_1 receptors, although Y_2 affinity was especially high for both **4** and **5**. Since our primary intention in this study was to develop analogues of NPY with high affinity for Y_1 receptors, we chose analogue **8** as our new lead compound.

Results from this scan illustrate how subtle manipulations can produce analogues with exquisite selectivity for either of the two receptor types examined. For example, **3** and **4** both bound to Y_1 receptors with very low affinity; however, **4** showed high affinity to Y_2 receptors, whereas **3** was more than 130 times less effective. Of all compounds tested in the initial scan, **8** displayed the highest affinity toward Y_1 receptors yet was a relatively poor binder to Y_2 receptors. Only full length NPY (**1**) bound to both receptors without discrimination. When examined as a whole, the present set of structurally restricted compounds again demonstrates that little similarity exists between the Y_1 and Y_2 receptor binding sites. In fact, a comparison of the sequences of cloned Y_1 and Y_2 receptors shows only 31% amino acid homology.[23] We may therefore expect that binding profiles of the present set of ligands for the two receptor types would significantly differ from one another.

Using des-AA$^{7-24}$cyclo(26/29)[Gly^6,Glu^{26},Lys^{29}]NPY (**8**) as our new lead, we proceeded to make modifications in the structure so as to improve affinity and specificity for the Y_1 receptor. Inverting the chirality of a single residue between the bridged residues (**11** and **12**) was expected to distort the overall conformation of the molecule, especially the orientation of the two termini relative to each other. Affinity to both receptors was diminished by these chiral substitutions. The substitution of glutamine by proline in residue 34 has previously been demonstrated to act as a Y_2 receptor blocker in both full length and truncated NPY analogues.[27,42-45] In the present study **13** produced the expected loss of affinity to the Y_2 receptor, while this modification, at the same time, improved the affinity to the Y_1 receptor by a factor of 2.

A second-generation scan of deletion cyclogues based on des-AA$^{7-24}$cyclo(26/29)[Gly^6,Glu^{26},Lys^{29},Pro^{34}]NPY was synthesized and is shown in Table 2. Several single-point deletion analogues (**14**−**19**) displayed yet higher affinity to the Y_1 receptor compared to parent **13**. Of greatest significance, removal of Arg^{25} (**20**−**23**) produced the greatest loss of affinity (K_i > 700 nM), highlighting the importance of this basic residue for binding contact. Removal of N-terminal Pro^{2,5} and/or Ser^3 or the AA$^{2-4}$ segment (**26**) was well tolerated by the receptor, indicating that the proposed N-terminal polyproline helix, at least in truncated NPY analogues, is not necessary for high affinity. Cyclogues having the entire N-terminus (seven residues preceding the bridgehead) removed (**21**) showed the greatest loss of binding affinity. Acetylation of the N-terminus of the same C-terminal fragment as in **22** improved Y_1 affinity by 2-fold, whereas the addition of a single tyrosine to this fragment (**23**) improved affinity by more than 5-fold.

Having identified several high-affinity compounds from the present series that are short, are constrained, and bind preferentially to one of two receptor types, we finally directed our attention at determining the intrinsic activity of these analogues. NPY receptors belong to the superfamily of G-protein-coupled receptors and are associated with the inhibition of adenylate cyclase and/or cAMP accumulation as well as other second-messenger responses.[46] When screened for cAMP response in norepinephrine-stimulated SK-N-MC cells (Table 3), all compounds demonstrated agonistic responses, with EC_{50}s closely paralleling their respective binding affinities.

The present compounds all share the C-terminal segment Thr-Arg-Pro-Arg-Tyr-NH_2 widely accepted as the pharmacophore recognized by Y_1 receptors. This fragment alone does not bind to the receptor, possibly requiring additional stabilizing forces contributed by distant residues to correctly orient functional groups to the receptor binding domain. Sautel and co-workers recently located a hydrophobic pocket in the transmembrane section of the cloned Y_1 receptor that is needed for ligand binding.[37] It is proposed that in this pocket Tyr^{36} of NPY forms hydrogen bonds with Tyr^{100}, Phe^{286}, and His^{298} of the receptor. In an earlier investigation, the same group reported on the interactions of charged residues Arg^{33}, Arg^{35}, and Arg^{19} of NPY with acidic residues in extracellular loops of the Y_1 receptor.[47] It is therefore only through the correct orientation of the C-terminal residues that docking of the peptide (or other ligand) may occur. It should be noted that the binding site for nonpeptide ligands at G-protein-coupled receptors does not share all contact points with peptide ligands at the atomic level (for a review, see Strader et al.[39]). Binding of the nonpeptide NPY mimetic BIBP3226, for example, is apparently of a distinct nature since mutants of the Y_1 receptor produced different binding profiles for NPY versus the mimetic.[38]

Several small peptides based on the C-terminal fragment of NPY and dimers of these fragments have been shown to bind to Y_1 receptors and display only antagonistic properties,[48-50] supporting the claim that the N-terminus contains residues that are critical to engender agonistic functional activation. Specifically, the N-terminal Tyr seems to be required for potent triggering of agonism since no molecule has been developed to date that activates Y_1 receptors without the presence of this moiety. More recently, the two-state model for the activation of G-protein-coupled receptors has gained acceptance and offers an alternative mechanism to explain the pharmacology of high-affinity ligands.[51] In this theory, analogues bind to the active form of the receptor (producing agonism), the inactive form (producing inverse agonism), or both forms of the receptor with equal affinity, and thus "silent activity" or competitive antagonism is produced.

Finally, we tested one of our best leads (**13**) for other known functional and behavioral actions of NPY, though data is not shown. Des-AA$^{7-24}$cyclo(26/29)[Gly^6,Glu^{26},Lys^{29},Pro^{34}]NPY failed to produce significant activity *in vivo* in both food intake and growth hormone secretion assays, performed by methods previously described.[5] Likewise, using conflict test methods described earlier,[52] this analogue was weakly active in producing anxiolysis, while some sedation was produced at high doses. All three assays measure the response to centrally administered NPY, the effects of which have been well characterized and reviewed.[11] In previous studies we

and others have suggested that a subpopulation of Y_1 receptors exists in brain that may be responsible for these actions yet has different binding and/or activation requirements as compared to peripheral Y_1 receptors.[27,49,53] Since this analogue is specific to Y_1 receptors, as shown by binding and cAMP studies, the lack of potent central activity of this analogue may imply once again that the receptor responsible for these activities may be other than the "classical" Y_1 receptor expressed on SK-N-MC cells. Very recently, in fact, a "Y_5" receptor found in certain brain tissues has been described and was shown to be responsible for the orexigenic activity of NPY.[19] In summary, awaiting further clarification by additional methods and receptor types, we have presented here a systematic study leading to the development of high-affinity Y_1 receptor specific NPY agonists.

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