Acc. Chem. Res. **2001,** *34,* 389−397

Design in Topographical Space of Peptide and Peptidomimetic Ligands That Affect Behavior. A Chemist's Glimpse at the Mind−Body Problem

VICTOR J. HRUBY*

Department of Chemistry, University of Arizona, Tucson, Arizona 85721

Received March 30, 2000

ABSTRACT

Efforts to determine the bioactive conformations of peptide ligands for membrane-bound proteins such as G-protein-coupled receptors (GPCRs) have been particularly challenging due to the flexibility of the ligands and the lack of 3D structural information (X-ray, NMR, etc.) for integral membrane proteins. An approach to determining these conformations by conformational constraint of the backbone template (ϕ and ψ angles) and by topographical constraint (χ^1, χ^2, etc. constraint) is outlined. Special attention is given to peptide neurotransmitter ligands that affect critical behaviors (feeding, sexual, addiction, pain, etc.). It is demonstrated that small changes in structure or a single torsional angle are sufficient to dramatically modify complex behaviors.

Introduction

Recent advances in the design, synthesis, and conformation−topography/biological activity relationships, in conjunction with advances arising from the human genome project and rapid advances in methods to examine the chemical basis of brain function and behavior, have opened up a new dimension in chemistry. In particular, through the design of ligands that affect behavior (learning, addictions, pain, etc.), including those that are volitional (e.g., feeding, sexual, etc.), chemists can apply chemical principles to examine relationships of chemical structure and dynamics to various behaviors and help address the classical mind−body (mind−brain) problem. Most simply stated, the problem is to determine to what extent the mind's functions (thought, volitional behavior, etc.) can be accounted for by the chemical/physical properties of the brain. In this brief report, we outline some of the approaches we and others are taking to address this problem. This will be discussed as determining the chemical−physical basis for information transduction in biological systems (e.g., refs 1 and 2).

Our hypothesis is that the "information" possessed by hormones, neurotransmitters, growth factors, and other intercellular and intracellular chemical messengers that are involved in information transduction and subsequent behavior is directly related to their conformational, topographical, and dynamic properties. Some of these molecules are biogenic amines (e.g., epinephrine or amino acids), but the vast majority are peptides. The approach of designing ligands that are more potent and selective for their receptors (mostly seven transmembrane G-protein-coupled receptors, GPCRs) as a way of addressing this problem will be discussed. It will be shown that changing a few atoms, bonds, or torsional angles in a ligand can have a major effect on the binding and transduction properties with their receptors, and this in turn can result in dramatically different behaviors. A correlary hypothesis is that agonists and antagonists *using the same binding site* will have different structure−biological activity relationships, and that the ligand−receptor (acceptor) complexes will have different structures.

Most neuromodulators that affect behavior are peptides and proteins, and one might reasonably ask, why peptides and proteins? There are many ways to look at this question, but it is interesting to note that the 20 amino acids used to form proteins and peptides derived from genes provide enormous chemical and structural diversity (Table 1), as well as H-bond-accepting and -donating properties, hydrophobicity, etc., and can incorporate the rest of the elements of the periodic chart and other chemical moieties into their structures. In addition, there are astronomical numbers of possible structures for even a small protein (Table 1). Indeed, there is not enough matter in the known Universe to make even one molecule each of all the possible molecules for a 100 amino acid peptide/protein. Furthermore, a single peptide or protein can assume different conformations (ϕ/ψ space) and many topographies (different χ angles, χ space) (Table 1). Thus, nature has chosen proteins and peptides to serve as enzymes, catalysts, structural elements, mechanical elements, receptors, information carriers, etc. The critical insight of Ramachandran[3,4] was that the structures Nature chooses (α-helix, β-sheet, β-turns, etc.) are the low-energy secondary structures found in proteins. This and related insights have served as the basis for peptide and protein design and provide the opportunity to examine in detail the structural and dynamic components of peptide molecules that affect information transduction and behavior. We now outline some of the critical chemical consider-

Victor J. Hruby was born in North Dakota and received his B.S. and M.S. Degrees at the University of North Dakota. He received his Ph.D. from Cornell University (A. T. Blomquist) working on synthetic and theoretical chemistry of benzocyclobutadiene dianion, and then went to Cornell Medical College as an Instructor, where he did a Postdoctoral with Vincent du Vigneaud in peptide science. In 1968, he joined the Department of Chemistry at the University of Arizona, where currently he is Regents Professor of Chemistry, Professor of Biochemistry, Professor of Neuroscience, and Professor in the Arizona Research Laboratory. He has served on three NIH Study Sections and several other national panels, and has been President of the American Peptide Society and a Councilor of the American Chemical Society. He has been recipient of a Guggenheim Fellowship, a Senior Humboldt Fellowship, the Pierce Award in Peptide Sciences, a Fulbright-Hays Fellowship, a Javits Award, a MERIT Award, and several other awards. Professor Hruby's research interests include de novo design of bioactive peptides and peptide mimetics; asymmetric synthesis; computational chemistry; conformational analysis; combinatorial chemistry; bioorganic chemistry; conformation−biological activity relationships; design of conformationally and topographically constrained peptide and peptide mimetic hormone and neurotransmitter agonists and antagonists for exploring the chemical−physical basis for information transduction and behavior in biological systems; and the structure−function of GPCRs.

* For reprint or other requests, contact the author at the University of Arizona. E-mail: hruby@u.arizona.edu.

10.1021/ar990063q CCC: $20.00
Published on Web 03/22/2001

Table 1. Diversity of Peptides and Proteins

I.	chemical diversity	–	chemical constituents including acidic, basic, nucleophilic, electrophilic, aliphatic, aromatic, heterocyclic, alcohol, thiol, sulfide functional groups plus; can incorporate the rest of the elements and other molecules into their structures
II.	structural diversity	–	decapeptide, 20^{10}– $\sim 1 \times 10^{13}$ structures 100-residue peptide, 20^{100}– $>10^{130}$ structures
III.	conformational diversity	1.	ϕ/ψ space; each amino acid residue and various segments can occupy α-helical, or β-sheet, or β-turn or extended structures in conformational space
		2.	χ space; for each amino acid (except gly, which can be a D-amino acid in ϕ/ψ space), each χ angle generally has three low-energy conformations: *g*(−), *g*(+), and *trans*
IV.	dynamic diversity	–	peptides and proteins can readily change conformations and topographies at ambient temperatures

Table 2. Favorable Properties of Conformationally Constrained Bioactive Peptides

property	example reference
1. are more potent in binding affinity than native peptide and often smaller	18
2. are more receptor (acceptor) selective	19
3. are stable against proteolytic enzymes	20
4. can penetrate membrane barriers better	20
5. are more efficacious in biological systems	21
6. X-ray crystal structure & NMR solution structure obtained more readily	22
7. "biologically active" conformation can be proposed	15, 16, 22,23

ations and associated biological observations that allow us to demonstrate that small structural, conformational, and dynamic changes in peptides can change behaviors.

Peptide and Protein Design

During the past 25 years, there has been enormous progress in the de novo design of peptides and proteins, and several excellent reviews have appeared. This has been critical for peptide ligand design, because most bioactive peptide are conformationally flexible. A working hypothesis that we and others have investigated is that conformationally constrained peptides and peptidomimetics, with greatly restricted conformations (e.g., see refs 5−16), can provide critical insights into the preferred "biologically active" conformations[17] and provide other unique biophysical and biological properties (Table 2). Success in de novo design of peptide and protein secondary structure templates with desired biological activities is considerable and has led in turn to some success in peptide mimetic de novo design of structural moieties related to α-helices and β-turns (e.g., refs 7−10, and 14).

χ Space—Topographical Design

Much less has been done to design bioactive peptides and proteins so that side chain groups of key amino acid residues have particular χ torsional angles (χ^1, χ^2, etc.). Side chain groups for amino acid residues in peptides prefer torsional angles about the $C_\alpha-C_\beta$ bond in an amino acid residue (the χ^1 angle) of −60° [*g*(−)], ±180° [*trans*], and +60° [*g*(+)]) (Figure 1). The energy differences between the different preferred conformations are small (generally less than 1 kcal/mol), and the energy barriers between the different *gauche* conformations are small (<10 kcal/mol). Hence, the topographical features of peptide ligands or protein surfaces, in which the side chains of three or four residues are critical for binding and/or information transduction, can be quite different for a particular peptide backbone structure. (Note that each R group will be directed toward the N-terminal of the peptide backbone for *gauche*(−), toward the C-terminal for *trans*, and over the peptide bond for *gauche*(+) (Figure 1). Thus, for three residues, there are 27 different topographical arrangements of the R groups in χ space on a single backbone template.) If these three residues are all critical pharmacophore elements for molecular recognition and transduction, only one will give the highest potency and efficacy. But which one? In the past 15+ years, we have systematically investigated approaches that would energetically distinguish the different side chain *gauche* conformations and thus allow design in χ space.[7,24]

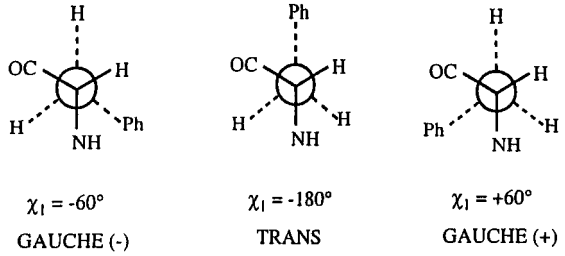

FIGURE 1. Three low-energy *gauche* conformations about χ^1 in an α-amino acid.

Success has depended on several considerations: (1) side chain groups must be constrained to the preferred side chain conformations of natural amino acids; (2) the constraint must be compatible with backbone conformations (α-helix, etc.); and (3) the constraint must be compatible with efficient binding kinetics. These are critical issues because the constrained amino acid residues must not significantly interfere with the conformation of the constrained template, with binding of the ligand to its receptor, or with the dynamic processes and conformational changes that accompany ligand−receptor interaction and information transduction. We have been able to meet these requirements and have had considerable success utilizing such topographically constrained amino acids as 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) (Figure 2), and we have designed and developed asymmetric synthesis for several others (Figure 2). By incorporating these into bioactive peptides, we have found that χ^1 and χ^2 torsional angles of key pharmacophore amino acids are as critically important as the template for potent and selective bioactivity.[24] This complete discrimination provides a new tool for examining peptide ligand−receptor/acceptor molecular recognition and in-

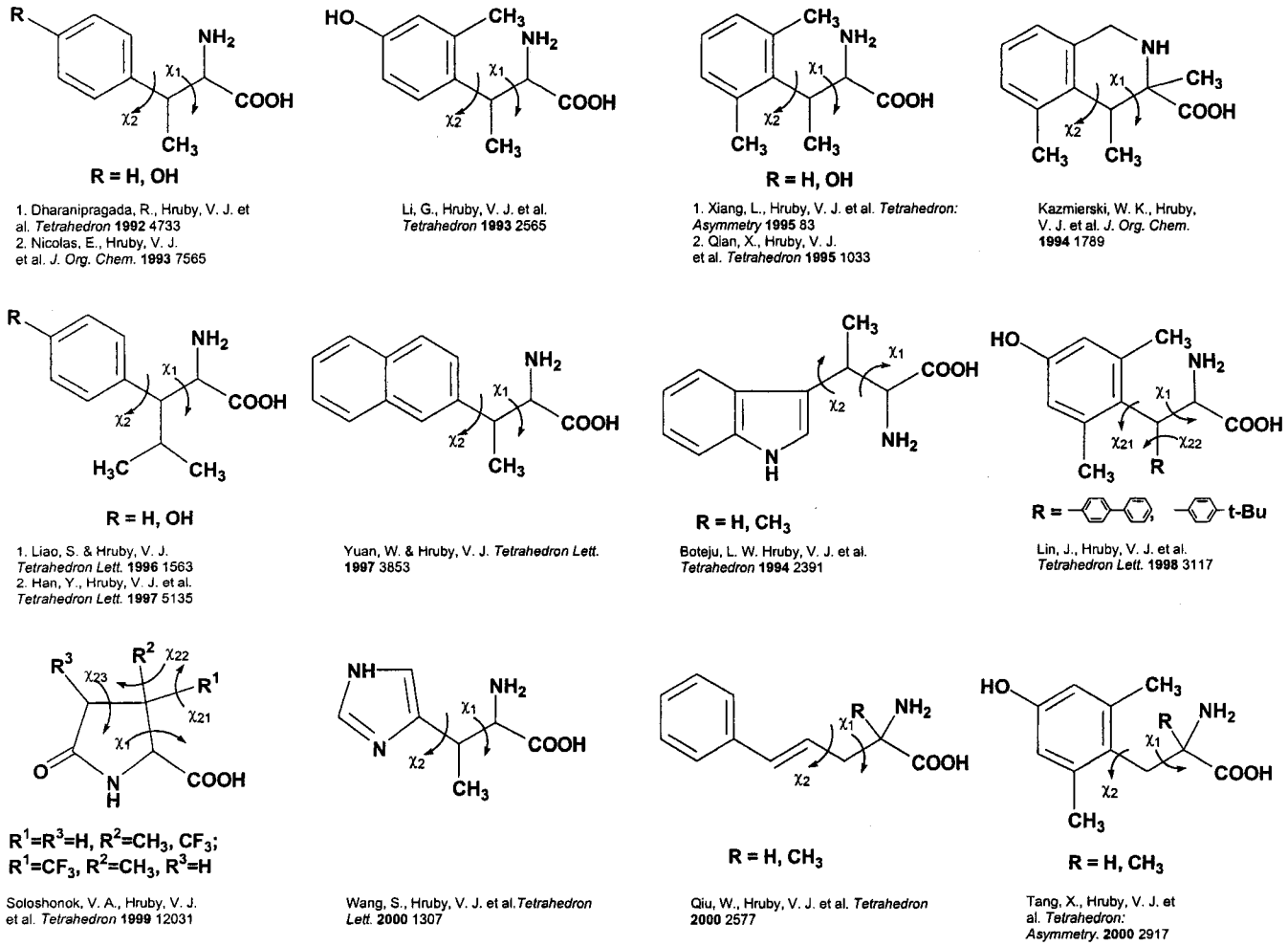

FIGURE 2. Structures of several topographically (χ) constrained amino acids prepared by asymmetric synthesis (references are to key synthetic methods).

formation transduction. Indeed, a single change in a torsional angle or in a covalent bond attachment can have a profound effect on animal behavior, thus providing a way to examine the chemical origins of behaviors.

Use of Topographical Constraint in the De Novo Design of Non-Peptide Peptide Mimetic Agonist Ligands for the δ Opioid Receptor

The modulation of pain and addiction involves opioid receptors. Thus far, three opioid receptors have been cloned: the μ, δ, and κ receptors. The putative natural ligands for these receptors are the endomorphins (μ), enkephalins (δ), and dynorphins (κ). Opioid ligands used to treat severe pain, such as morphine and codeine, interact with the μ receptor and have severe side effects, including respiratory depression, severe constipation, and high addiction potential. A promising alternative would be a potent δ opioid ligand.

Using the cyclic conformational constraint strategy discussed above, we designed the cyclic enkephalin analogue H-Tyr-c[DPen-Gly-Phe-DPen]-OH (c[DPen^2,-DPen^5]enkephalin, DPDPE),[19] which is highly δ receptor selective. In addition to its potent analgesia, DPDPE does not inhibit transit through the gut (no constipation), does not cause respiratory depression, has a low addiction potential, and may reverse aspects of addiction. Its solution conformation has been determined by NMR[25] and its crystal structure by X-ray crystallography.[22] It was found that its two key pharmacophore moieties, the side chain groups of Phe^4 and of Tyr^1, were flexible and could exist in all three *gauche* conformations. Thus, we examined which *gauche* conformations were required for δ opioid receptor binding affinity and selectivity. For the Phe^4 residue, we used all four isomers of β-methylphenylalanine (2*S*,3*S*; 2*R*,3*R*; 2*S*,3*R*; 2*R*,3*S*) and concluded from their biological activities and conformations that the preferred Phe^4 side chain conformation for δ receptor recognition was *gauche*(−). However, the results with β-methyltyrosine analogues were equivocal, and thus we designed the amino acid β-methyl-2′,6′-dimethyltyrosine (TMT) and developed an asymmetric synthesis for all four isomers (Figure 2) and a force field to calculate the preferred χ^1 and χ^2 conformations for TMT (Figure 3).[24] We also experimentally determined the validity of the force field by examining the barriers to rotation about the $C_\alpha-C_\beta$ bond (χ^1) using dynamic NMR.[26] Barriers to rotation of 15−20 kcal/mol were determined for various derivatives, comparable to the force field calculations of about

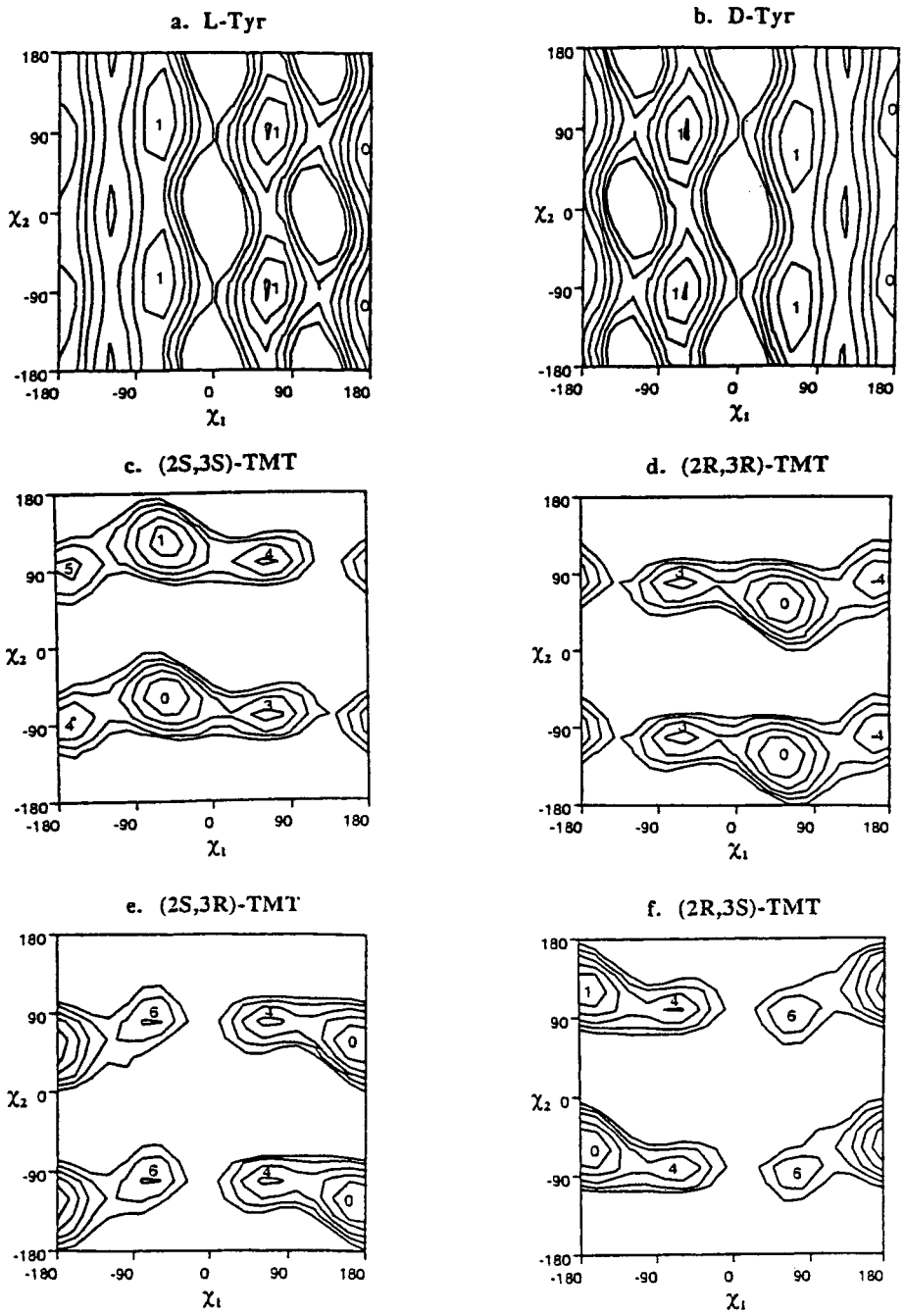

FIGURE 3. χ^1/χ^2 plots for L- and D-tyrosine and for the four isomers of β-methyl-2′,6′-dimethyltyrosine (TMT). Adapted from ref 24.

Table 3. Binding Affinities for [TMT[1]]DPDPE Analogues[a]

peptide	binding IC_{50} (nM) vs [^{3}H]CTOP (μ)	binding IC_{50} (nM) vs [^{3}H[*p*-ClPhe^4]DPDPE (δ)	selectivity (μ/δ)
DPDPE	609	1.6	380
[(2*S*,3*S*)-TMT[1]]DPDPE	722	211	3.4
[(2*S*,3*R*)]-TMT[1]]DPDPE	4270	5.0	850
[(2*R*,3*R*)-TMT[1]]DPDPE	77 100	3500	22
[(2*R*,3*S*)-TMT[1]]DPDPE	0% at 10 μM	9% at 10 μM	na

[a] Data adapted from ref 27.

17−18 kcal/mol. We incorporated all four isomers into DPDPE and studied their binding (Table 3) and biological activities[27,28] and their conformations in solution using NMR and computational chemistry.[27] Only the [(2*S*,3*R*)-TMT[1]]DPDPE analogue had high binding affinity and selectivity for the δ opioid receptor. Interestingly, the (2*S*,3*S*)-isomer (Table 3) binds moderately well to the μ opioid receptor but is not selective. Clearly, the δ and μ receptors have quite different topographical requirements for the key Tyr[1] pharmacophore. The NMR and compu-

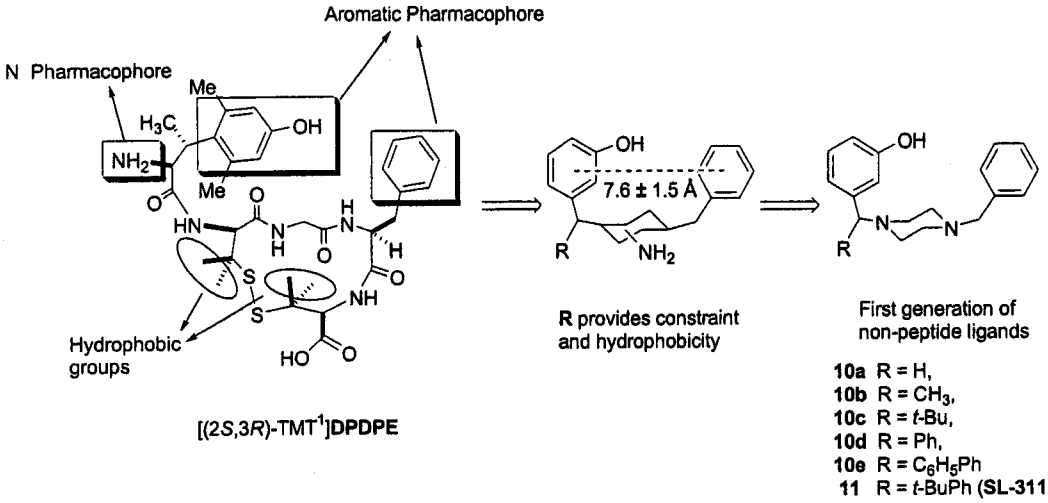

FIGURE 4. Schematic representation of the consideration in the de novo design of a non-peptide peptide mimetic from a constrained peptide.

Table 4. Binding Affinity of De Novo Designed 1,4-Piperazine-Substituted Non-Peptide Ligands to δ and μ Opioid Receptors[a]

	binding IC_{50} (nM)		
compound	[^{3}H]DAMGO (μ)	[^{3}H]*p*-ClPhe^4[DPDPE] (δ)	selectivity (μ/δ)
[(2*S*,3*R*)-TMT^1]DPDPE	4300	5.0	860
analogue **1** (R = H)	8100	6400	1.3
analogue **2** (R = ^{i}Bu)	2100	420	5.0
analogue **3** (R = Ph)	500	34	15
analogue **4** (R = *p*-*t*-BuPh)	17 000	8.4	2000
analogue **5** (OH → OMe)	>8000	1800	>4
[*p*-OMe Tyr^1]DPDPE	11 000	230	48
DPDPE	610	1.6	380

[a] Analogues are substituted-1,4-piperazines whose structures are shown in Figure 4. Adapted from ref 30.

tational studies clearly showed that the four DPDPE analogues have the same conformation in the 13-membered ring but differ in the topography of the Tyr^1 moiety, with the (2*S*,3*R*)-TMT^1 analogue having a *trans* conformation.[27] We concluded that for molecular recognition for an agonist, the Tyr residue had to have a *trans* χ^1 angle. Especially intriguing was the observation that [(2*S*,3*S*)-TMT^1]DPDPE is a μ receptor antagonist.[27,28] Thus, a simple change in χ^1 for Tyr^1 from *trans* in the (2S,3R)-Tyr^1-containing analogue, to *g*(−) in the (2S,3S)-Tyr^1-containing analogue, can convert an agonist to an antagonist at the μ opioid receptor, changing an animal behavior from not feeling pain to feeling it.[28]

De Novo Design of a Non-Peptide δ Receptor Agonist from a Peptide Lead

We next sought to design a non-peptide ligand for the δ opioid receptor on the basis of the above conformationally *and* topographically constrained peptide lead. Extensive computation studies were carried out on [(2*S*,3*R*)-TMT^1]-DPDPE to determine the three-dimensional disposition of the key pharmacophore units.[29] In addition, we recognized the need for a hydrophobic patch with steric bulk for δ vs μ opioid receptor selectivity, as demonstrated by the need for the four methyl groups on the two DPen residues in DPDPE[19] (Figure 4). We explored a variety of "simple" non-peptide scaffolds on which we could place the key pharmacophore units. Several scaffolds were found, and we chose to examine the 1,4-piperazine scaffold. Figure 4 gives a summary of the design considerations in going from peptide pharmacophore to the non-peptide pharmacophore using a 1,4-piperazine.[30] The R groups examined ranged from hydrogen to *p*-*tert*-butylphenyl, and a straightforward convergent synthetic methodology was designed. Table 4 gives the binding affinities of selected analogues. Clearly, analogue **4** is highly potent and δ selective, validating our design approach. Using site-specific mutagenesis, it was shown that peptide and non-peptide agonist ligands bind differently to the δ opioid receptor. Would our designed ligand **4** (Table 4) behave as a peptide or a non-peptide? Binding was examined on wild-type and mutagenized δ opioid receptors,[30] and **4** was found to bind in a manner similar to the binding of peptides. This could be further tested since it had been shown that O-methylation of the phenol OH pharmacophore in a non-peptide led to a potent, much more δ receptor selective ligand,[31] while O-methylation of the Tyr^1 in DPDPE, and the −OH group on **4**, led to a 150-fold loss in binding affinity for DPDPE at the δ opioid receptor, and to a 200-fold drop in binding affinity for the δ opioid receptor for **5** (Table 4).[30] Clearly, compound **5** is behaving like the peptide on which its design was based and not like a non-peptide. Nonetheless, it must be emphasized that we cannot state with certainty that the non-peptide mimetic binds to the receptor precisely as the peptide does since we have no structural

(X-ray or NMR) proof. We can only state that they have the same structure−activity relationships, which implies similar structural interactions.

Use of Topographical in the Design of Peptide Ligands that Modulate Feeding, Sexual, and Other Behaviors

The human genome project presents many opportunities for design of specific ligands that interact with receptors to modulate behaviors. Treatment of diseases related to these behaviors will involve multidisciplinary interactions and social inputs from law, religion, and cultural institutions. In this section we briefly discuss how topographical and other chemical considerations can lead to the design of peptide ligands that can greatly affect/modify such behaviors.

The proopiomelanocortin gene produces several peptides with diverse biological activities including α-melanocyte stimulating hormone (α-MSH, pigment cells, pigmentation), adrenocorticotropic hormone (ACTH, adrenal gland, fear−flight, stress response), γ-MSH, β-endorphin (pain modulation),[32] etc. Only recently have their receptors been cloned and their structures determined (for a review see[33]). Five receptors have been found: melanocortin 1 receptor (MC1R, pigmentation), MC2R (adrenal gland), MC3R (brain and other tissues), MC4R (primarily brain), and MC5R (found throughout the body and brain). The functions of the first two receptors are well established for pigment and adrenal cells, but the functions of the others were not known. This provides an enormous opportunity for design of novel agonist and antagonist ligands with selectivity for these receptors, and for discovery of novel mechanisms of bioactivity, as discussed below.

α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) studies go back to the early 1960s. By the late 1970s, it had been determined that the structure Met-Glu-His-Phe-Arg-Trp-Gly contained the biological activity for pigmentation, and then it was found that the sequence His-Phe-Arg-Trp- could mimic all the bioactivities at the pigment cell. Efforts to obtain biostable (half-life of α-MSH in serum is minutes) and prolonged-acting analogues met their first success with the introduction of a D-Phe^7 for L-Phe^7 and Nle^4 for Met^4 of the native hormone to give Ac-Ser-Tyr-Ser-Nle-Glu-His-*D*Phe-Arg-Trp-Gly-Pro-Val-NH_2 (NDP-α-MSH),[34] which has been widely used worldwide for in vitro and in vivo biological activity studies. It was proposed[34] that the conformational origin of the superior biological properties of [Nle,^{4}DPhe^7]α-MSH was a β-turn. Pseudoisosteric cyclization (Met^4···Gly^{10} ⟹ c[Cys^4···Cys^{10}]) was developed to stabilize this β-turn. Ac-c[Cys^4,Cys^{10}]α-MSH was found to be superactive in the classical frog skin bioassay.[35] Subsequent NMR studies, molecular modeling, computational calculations, and molecular dynamics simulations led to the design of truncated cyclic lactam analogues such as Ac•Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2 (Ac•Nle^4-c[Asp^5,DPhe^7,Lys^{10}]α-MSH(4−10)-NH_2).[18] The analogue was superpotent, had further increased stability to proteolytic breakdown, and showed prolonged biological activity (hours to days in vitro and in vivo) (Table 5). The discovery of these cyclic conformationally constrained analogues of α-MSH provided a unique opportunity to examine the effect of topographical change on a variety of biological effects.

Table 5. Biological Activities of β-MeTrp^9 Analogues of MT-II in Frog Skin Bioassays

compound	frog skin bioassay IC_{50} (nM)[a]	prolonged[b] activity	preferred χ^1 [c] side chain conformations (NMR)	
α-MSH	0.10	−	nd	
MT-II[d]	0.10	+++	H-*t*	f-*t*
			R-*t*	W-*t*
[(2*S*,3*S*)β-MeTrp^9]MT-II	0.44	−	H-*t*	f-*t*
			R-*t*	W-*g*(−)
[(2*S*,3*R*)β-MeTrp^9]MT-II	28.6	+	H-*t*	f-*t*
			R-*t*	W-*g*(+)
[(2*R*,3*S*)β-MeTrp^9]MT-II	0.060	++	H-*t*	f-*g*(+)
			R-*t*	w-(*g*+)
[(2*R*,3*R*]β-MeTrp^9]MT-II	0.33	+++	H-*g*(+)	f-*t*
			R-*t*	w-*g*(+)

[a] Adapted from ref 38. [b] Prolonged activity: −, not prolonged; +++, highly prolonged (irreversible); +, modestly prolonged (minutes); ++, quite prolonged (hours). [c] H = His^6; f = DPhe^7; R = Arg^8; W = Trp^9 or β-MeTrp^9 isomer; t = *trans* (180°); *g*(−) = *gauche*(−) (−60°); *g*(+) = *gauche*(+) (+60°) for an L-amino acid. [d] MT-II = Ac−Nle^4-c[Asp^5,DPhe^7,Lys^{10}]α-MSH(4−10)-NH_2.

Topographical Basis for Prolonged Biological Activities

A particularly interesting biological characteristic of NDP-α-MSH (MT-I) and of Ac−Nle^4-c[Asp^5,DPhe^7,Lys^{10}]α-MSH-(4−10)-NH_2 (MT-II) is their prolonged activities. When the ligand is removed from the bathing fluid of the frog skin (or other tissue), the skin remains dark for hours. In vivo, a single dose leads to skin darkening for weeks.[36] The effect is Ca^{2+} dependent, but efforts to explain the effect biochemically have failed. Generally, stimulation of a c-AMP-dependent process via a GPCR leads to down-regulation by receptor internalization. We have demonstrated that when these ligands interact with the MC1R, receptor internalization occurs,[37] but nonetheless prolonged bioactivity continues.

To obtain structural insight into the mechanism responsible for this prolonged activity, we have explored the use of topographically constrained amino acids. The Trp^9 residue, a key residue for recognition of the MC1R, was chosen, and the four isomers (2*S*,3*S*; 2*S*,3*R*; 2*R*,3*R*; 2*R*,3*S*) of β-MeTrp^9 were incorporated into Ac-Nle^4-[Asp^5,DPhe^7,-Lys^{10}]α-MSH(4−10)-NH_2.[38] First, note (Table 5, Figure 5) that there is no correlation between potency and prolonged biological activity. Most interesting are the topographical requirements for prolongation. At the frog MC1R, a Trp^9 *g*(−) conformation (determined by NMR) is incompatible with prolonged biological activity, and a *g*(+) conformation for Trp^9 is preferred. Interestingly, when His^6 has a *gauche*(+) conformation, prolongation is enhanced. In the human MC1R, similar differentiations are seen, but in this case the [(2*S*,3*S*)-β-Me-Trp^9]MT-II

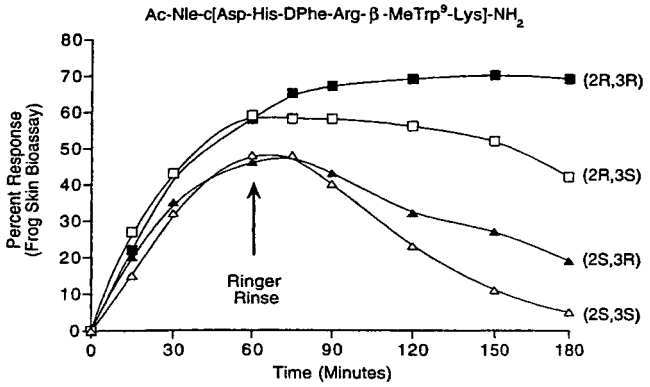

FIGURE 5. Prolonged biological activities of the four diasterioisomers of the potent α-melanotropin analogue Ac-Nle-c[Asp^5,DPhe^7,β-MeTrp^9,Lys^{10}]α-MSH-(4−10)-NH_2 in the frog skin (*R. pipiens*) bioassay. The 2*R*, 3*R*, etc. designation refers to the absolute configuration of the β-MeTrp amino acid in the cyclic peptide analogue. Adapted from ref 38.

analogue is the most prolonged acting.[39] Clearly, different MC1Rs have different topographical requirements for bioactivity. This provides a powerful tool for examining the mechanism of prolonged biological activity without down-regulation.

Development of Melanocortin Receptor Antagonists

A universal antagonist for the MC1R has not been reported, but potent antagonists for the frog skin system have been obtained.[40] In one example, substituting the *p*-hydrogen in DPhe^7 with most halogens led to potent agonists,[40] but the *p*-iodo-*D*Phe^7 analogue was a potent *antagonist* in the frog skin bioassay and at the mammalian MC4R and MC3R (with weak residual partial agonist activity) and a potent *agonist* at the MC5R and MC1R. Remarkably, the compound was a more potent antagonist at the MC4R than agouti, an endogenous MCR antagonist protein. We next investigated other bulky aromatic amino acids in the 7 position of MT-II (Table 6), including 2′-naphthylalanine and 1′-naphthylalanine (Figure 6). It was found that the D-2′-analogue Ac-Nle^4-c[Asp^5,DNal(2′)-Lys^{10}]α-MSH(4−10)-NH_2 (SHU-9119) was a potent antagonist at the MC4R and MC3R with 8−10-fold selectivity for the MC4R, but the D-1′-analogue was a potent agonist at both receptors (Table 6). Thus, very subtle changes in topographical structure (1′ to 2′ naphthyl) change a potent melanotropin agonist to a potent antagonist. Subsequently, we[41] and Kask et al.[42] used the D-Nal(2′) substitution to create more MC4R-selective antagonists, with Ac-Nle^4-c[Asp^5,(3-Me)His^6,DNal(2′),^{7}Lys^{10}]α-MSH(4−10)-

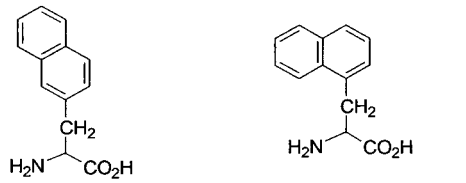

FIGURE 6. Structure of naphthylalanine analogues.

NH_2 being about 80-fold selective[41] and Ac-c[Cys^3, Nle,^{4}Arg^5,DNal(2′),^{7}Cys^{11}]α-MSH(3−11)-NH_2 being about 20-fold selective.[42]

Use of Potent and Stable Topographically Modified Melanotropin Analogues To Examine Novel Behavioral Effects

The availability of potent, biologically stable melanotropin agonists and antagonists allowed examination of the biological roles of the new melanocortin receptors. Here we will discuss behavioral effects, namely feeding behavior and sexual behavior. In collaborative efforts, a number of other critical biological activities have been discovered, including (1) differential central and peripheral effects on heart rate and blood pressure by α-MSH and γ-MSH, respectively;[43] (2) the antipyretic role of melanotropin receptors associated with endotoxin-enduced fever;[44] and (3) the role of melanotropins in exocrine gland functions,[45] and several others.

Melanotropins are primary regulators of feeding behavior. This was demonstrated by the ability of the antagonist SHU-9119 to block decreased feeding caused by MT-II[46] in normal and obese animals (and by the use of MC4R knockout animals). Long-term administration of a melanocortin 4 antagonist led to a long-term increase of eating and obesity in rats.[47] It is remarkable that a minor change in a naphthylalanine analogue, a single bond isomer, can have such different effects on behavior.

In the course of investigating the effects of Ac-Nle^4-c[Asp^5,DPhe^7,Lys^{10}]α-MSH(4−10)-NH_2 (MT-II) on pigmentation in humans, it was discovered that MT-II had important effects on sexual behavior and erectile function. A placebo-controlled double blind crossover study in men with psychogenic erectile dysfunction demonstrated[48] that MT-II, at very low doses, could induce erectile function[48] and that the melanotropin agonist also increases sexual desire.

These effects of melanotropin agonists and antagonists on volitional behavior in animals and humans suggest that neuropeptide ligands and their receptors strongly modu-

Table 6. Biological Activities of Selected Analogues of Ac-Nle^4-c[Asp^5,DPhe^7,Lys^{10}]α-MSH(4−10)-NH_2 (MT-II)[a]

	EC_{50} (nM) or pA_2			
compound	mMC1R	mMC3R	mMC4R	mMC5R
Ac-Nle^4-c[Asp^5,DPhe^7,Lys^{10}]α-MSH(4−10)NH_2 (MTII)	0.037	0.011	0.083	0.17
Ac-Nle^4-c[Asp^5,DNal(2′)^7,Lys^{10}]α-MSH(4−10)-NH_2 (SHU 9119)	0.64	pA_2 9.5	pA_2 10.4	2.31
Ac-Nle^4-c[Asp^5,DNal(1′)^7,Lys^{10}]α-MSH(4−10)-NH_2	0.037	0.65	0.65	0.25
Ac-Nle^4-c[Asp^5,Nal(2′)^7,Lys^{10}]α-MSH(4−10)-NH_2	3.7	5.4	2.0	2.1
Ac-Nle^4-c[Asp^5,DPhe(pI)^7,Lys^{10}]α-MSH(4−10)NH_2	9.0	−	pA_2 9.5	−

[a] Adapted from refs 40 and 41. [b] Human MC1R.

late critical behaviors, and they suggest possible avenues for the treatment and cure of many diseases and disorders related to human behavior.[49]

Conclusions and Discussion

The studies reported here suggest that conformational and topographical constraint of flexible bioactive peptides can considerably improve on nature in providing ligands that are more potent, receptor selective, stable, and bioavailable than the endogenous peptides. The ability of these ligands to modulate and control critical bodily functions such as heart rate, blood pressure, and immune response, and critical behaviors such as feeding, addictive behaviors, sexual behavior, pain, learning, and many others suggests their enormous potential. However, this research raises important issues about the mind−body problem. For example, to what extent do volitional behaviors such as addictive behavior have a chemical basis, and to what extent can chemical design be used to modulate these behaviors? In view of the complexities of the biological systems, how is it that minor changes in structure (a single torsional angle of a side chain group, or a simple isomeric change) can have such dramatic effects on behavior?[50]

A few rather modest conclusions can be suggested at this time.

1. Backbone conformational constraint (ϕ, ψ constraint) *and* topographical constraint (χ constraint) of side chain groups critical to molecular recognition and information transduction provide a useful approach to develop peptide and peptide mimetic ligands that have superior binding, information transduction, receptor/acceptor selectivity, stability, and bioavailability properties relative to those of the native ligand.

2. Structural chemistry can examine in great detail the stereostructural requirements for intracellular communication and information transduction in complex biological systems.

3. Small changes in torsional angles, or simple isomer differences, can have profound effects on molecular recognition, information transduction, and behavioral responses.

4. Simple chemical principles can provide tools that can be used by biologists, psychologists, and medical doctors to probe the underlying mechanisms of diseases related to behavioral disorders.

5. Though one must be cautious in interpreting the considerable behavior changes that can result from modest stereostructure changes in peptide ligands, it seems clear that chemical design provides a very important method for obtaining ligands that can affect many behaviors. There is a need for chemists to make society fully aware of the novel chemical methods for affecting behavior that are being developed.

In summary, peptide and peptide mimetic ligands can provide very useful chemical and biological insights for understanding the functions of genes that are discovered in the human genome projects, for examining the underlying chemical mechanisms for biological processes, and for the development of new medicines and diagnostics. It is an exciting time for chemists to make critical contributions to the understanding of life processes.

The author thanks the Alexander Humboldt Foundation for a Senior Humboldt Fellowship and Professor Horst Kessler for his hospitality during a sabbatical, and the U.S. Public Health Service for research support. Special thanks to my students and colleagues whose names appear in the references for their enormous talents, hard work, and critical comments that make this report possible.

References

(1) Hruby, V. J.; Hadley, M. E. Binding and Information Transfer in Conformationally Restricted Peptides. In *Design and Synthesis of Organic Molecules Based on Molecular Recognition. Proc. XVIII Solvay Conf. on Chemistry*; Van Binst, G., Ed.; Springer-Verlag: Heidleberg, 1986; pp 269−289.

(2) Hruby, V. J.; Yamamura, H. I.; Porreca, F. Molecular Organization of Receptors: Efficacy, Agonists and Antagonists. *Ann. N. Y. Acad. Sci.* **1995**, *757*, 7−22.

(3) For a review see Ramachandran, G. N.; Sasisekharan, V. Conformations of Polypeptides and Proteins. *Adv. Protein Chem.* **1968**, *23*, 283−438 and references therein.

(4) For an examination of the ϕ/ψ space accessible to normal amino acids, see: Roterman, I. K.; Lambert, M. H.; Gibson, K. D.; Scheraga, H. A. A Comparison of the CHARMM, AMBER and ECEPP Potentials for Peptides. II. $\phi-\psi$ Maps for N-Acetylalanine-N′-Methylamide: Comparisons, Contrasts and Simple Experimental Tests. *J. Biomol. Struct. Dyn.* **1989**, *7*, 421−453.

(5) Kessler, H. Conformation and Biological Activity of Cyclic Peptides. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512−523.

(6) Hruby, V. J. Conformational Restrictions of Biologically Active Peptides Via Amino Acid Side Chain Groups. *Life Sci.* **1982**, *31*, 189−199.

(7) Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. M. Emerging Approaches in the Molecular Design of Receptor Selective Peptide Ligands: Conformational, Topographical and Dynamic Considerations. *Biochem. J.* **1990**, *268*, 249−262.

(8) Veber, D. F. Design and Discovery in the Development of Peptide Analogues. In *Peptides: Chemistry and Biology*; Smith, J. A., Rivier, J. E., Eds.; ESCOM Sci. Publ.: Leiden, 1992; pp 1−14.

(9) Rizo, J.; Gierasch, L. M. Constrained Peptides: Models of Bioactive Peptides and Protein Substructures. *Annu. Rev. Biochem.* **1992**, *61*, 387−418.

(10) Goodman, M.; Ro, S. Peptidomimetics for Drug Design. In *Burger's Medicinal Chemistry and Drug Design: Principles of Drug Discovery*, 5th ed.; Wolff, M. E., Ed.; John Wiley & Sons: New York, 1995; Vol. 1, pp 803−861.

(11) De Grado, W. F. Designs of Peptides and Proteins. *Adv. Protein Chem.* **1988**, *39*, 51−124.

(12) Smith, C. K.; Regan, L. Construction and Design of β-Sheets. *Acc. Chem. Res.* **1997**, *30*, 153−161.

(13) Imperiali, B.; Ottesen, J. J. Uniquely Folded Mini-Protein Motifs. *J. Pept. Res.* **1999**, *54*, 177−184.

(14) Toniolo, C. Conformationally Restricted Peptides Through Short-Range Cyclizations. *Int. J. Pept. Protein Res.* **1990**, *35*, 287−300.

(15) Veber, D. F.; Holly, F. W.; Palaveda, W. J.; Nutt, R. F.; Bergstrand, S. J.; Torchiana, M. L.; Glitzer, M. S.; Saperstein, R.; Hirschmann, R. F. Highly Active Cyclic and Bicyclic Somatostatin Analogues of Reduce Ring Size. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2636−2640.

(16) Meraldi, J.-P.; Hruby, V. J.; Brewster, A. I. R. Relative Conformational Rigidity in Oxytocin and [1-Penicillamine]-oxytocin: A Proposal for the Relationship of Conformational Flexibility to Peptide Hormone Agonism and Antagonism. *Proc. Nat. Acad. Sci. U.S.A.* **1977**, *74*, 1373−1377.

(17) The term "bioactive conformation" is generally used only when conformational constraints provide conformationally stable peptide templates (as determined by NMR and X-ray crystallography) from which one can design predictably potent ligands. In this regard, the constraints generally used include cyclization using disulfide, lactam, lactone, and other side chain-to-side chain, side chain-to-backbone, or backbone-to-backbone groups to give macrocyclic ring structures. For examples of the many approaches that have been taken, refs 6−25 would be a good starting point.

(18) Al-Obeidi, F.; Hadley, M. E.; Pettitt, B. M.; Hruby, V. J. Design of a New Class of Superpotent Cyclic α-Melanotropins Based on Quenched Dynamic Simulations. *J. Am. Chem. Soc.* **1989**, *111*, 3413−3416.

(19) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. Bis-Penicillamine Enkephalins Possess Highly Improved Specificity Toward Delta Opioid Receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871−5874.
(20) Weber, S. J.; Greene, D. L.; Hruby, V. J.; Yamamura, H. I.; Porreca, F.; Davis, T. P. Whole Body and Brain Distribution of [^{3}H]Cyclic-[D-Pen^2, D.-Pen^5]Enkephalin After Intraperitoneal, Intravenous, Oral and Subcutaneous Administration. *J. Pharmacol. Exp. Ther.* **1992**, *263*, 1308−1316.
(21) Kramer, T. H.; Davis, P.; Hruby, V. J.; Burks, T. F.; Porreca, F. In Vitro Potency, Affinity and Agonist Efficacy of Highly Selective Delta Opioid Receptor Ligands. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 577−584.
(22) Flippen-Anderson, J. L.; Hruby, V. J.; Collins, N.; George, C.; Cudney, B. X-Ray Structure of [D-Pen^2, D.-Pen^5]Enkephalin, A Highly Potent, Delta Opioid Receptor Selective Compound: Comparisons With Proposed Solution Conformations. *J. Am. Chem. Soc.* **1994**, *116*, 7523−7531, and subsequent publications.
(23) Kazmierski, W. M.; Yamamura, H. I.; Hruby, V. J. Topographic Design of Peptide Neurotransmitters and Hormones on Stable Backbone Templates: Relation of Conformation and Dynamics to Bioactivity. *J. Am. Chem. Soc.* **1991**, *113*, 2275−2283.
(24) For a review and overview, see: Hruby, V. J.; Li, G.; Haskell-Luevano, C.; Shenderovich, M. D. Design of Peptides, Proteins, and Peptidomimetics in Chi Space, *Biopolymers* (*Pept. Sci.*) **1997**, *43*, 219−266.
(25) Hruby, V. J.; Kao, L.-F.; Pettitt, B. M.; Karplus, M. The Conformational Properties of the Delta Opioid Peptide [D-Pen^2, D.-Pen^5]-enkephalin in Aqueous Solution Determined by NMR and Energy Minimization Calculations. *J. Am. Chem. Soc.* **1988**, *110*, 3351−3359.
(26) Jiao, D.; Russell, K. C.; Hruby, V. J. Locally Constrained Tyrosine Analogues with Restricted Side Chain Dynamics. *Tetrahedron* **1993**, *49*, 3511−3520.
(27) Qian, X.; Shenderovich, M. D.; Kövér, K. E.; Davis, P.; Horváth, R.; Zalewska, T.; Yamamura, H. I.; Porreca, F.; Hruby, V. J. Probing the Stereochemical Requirements for Receptor Recognition of δ Opioid Agonists Through Topographic Modifications in Position 1. *J. Am. Chem. Soc.* **1996**, *118*, 7280−7290.
(28) Bilsky, E. J.; Qian, X.; Hruby, V. J.; Porreca, F. Antinociceptive Activities of [β-Methyl-2′,6′-dimethyltyrosine^1]-Substituted Cyclic-[DPen^2,DPen^5]Enkephalin and [D-Ala^2,Asp^4]Deltorphin Analogues. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 151−158.
(29) Shenderovich, M. D.; Liao, S.; Qian, X.; Hruby, V. J. A Three-Dimensional Model of the δ Opioid Pharmacophore: Comparative Molecular Modeling of Peptide and Non-Peptide Ligands. *Biopolymers* **2000**, *53*, 565−580.
(30) Liao, S.; Alfaro-Lopez, J.; Shenderovich, M. D.; Hosohata, K.; Lin, J.; Li, X.; Stropova, D.; Davis, P.; Jernigan, K. A.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. De Novo Design, Synthesis, and Biological Activities of High-Affinity and Selective Non-Peptide Agonists of the δ-Opioid Receptor. *J. Med. Chem.* **1998**, *41*, 4767−4776 and references therein.
(31) Knapp, R. J.; Santoro, G.; De Leon, I. A.; Lee, K. B.; Edsall, S. A.; Waite, S.; Malatynska, E.; Varga, E.; Calderon, S. N.; Rice, K. N.; Rothman, R. B.; Porreca, F.; Roeske, W. R.; Yamamura, H. Y. Structure−Activity Relationships for SNC80 and Related Compounds at Cloned Delta and Mu Opioid Receptors. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 1284−1291.
(32) Hadley, M. E., Ed. *The Melanotropic Peptides*; CRC Press: Boca Raton, FL, 1988; Vols. I, II, and III.
(33) For a review, see: Cone, R. D.; Mountjoy, K. S.; Robins, L. S.; Nadeau, J. H.; Johnson, K. R.; Roselli-Rehfuss, L.; Mortrud, M. T. Cloning and Functional Characterization of a family of receptors for the Melanotropic Peptides. *Ann. N. Y. Acad. Sci* **1993**, *680*, 342−343.
(34) Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. [Nle^4,D-Phe^7]-α-Melanocyte Stimulating Hormone: A Highly Potent α-Melanotropin with Ultralong Biological Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754−5758.
(35) Sawyer, T. K.; Hruby, V. J.; Darman, P. S.; Hadley, M. E. [4-Half-Cystine, 10-Half-Cystine]-α-Melanocyte Stimulating Hormone: A Cyclic α-Melanotropin Exhibiting Superagonist Biological Activity. *Proc. Nat. Acad. Sci. U.S.A.* **1982**, *79*, 1751−1755.
(36) Hadley, M. E.; Anderson, B.; Heward, C. B.; Sawyer, T. K.;. Hruby, V. J. Calcium-Dependent Prolonged Effects on Melanophores of [4-Norleucine, 7-D-Phenylalanine]-α-Melanotropin. *Science* **1981**, *213*, 1025−1027.
(37) Sharma, S. D.; Jiang, J.; Hadley, M. E.; Bentley, D. L.; Hruby, V. J. Melanotropic Peptide-Conjugated Beads for Microscopic Visualization and Characterization of Melanoma Melanotropin Receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13715−13720.
(38) Haskell-Luevano, C.; Toth, K.; Boteju, L.; Job, C.; Castrucci, A. M. de L.; Hadley, M. E.; Hruby, V. J. β-Methylation of the Phe^7 and Trp^9 Melanotropin Side Chain Pharmacophores Affects Ligand−Receptor Interactions and Prolonged Biological Activity. *J. Med. Chem.* **1997**, *40*, 2740−2749.
(39) Haskell-Luevano, C.; Miwa, H.; Dickinson, C.; Hadley, M. E.; Hruby, V. J.; Yamada, T.; Gantz, I. Characterization of the Unusual Dissociation Properties of Melanotropin Peptides From the Melanotropin Receptor, hMC1R. *J. Med. Chem.* **1996**, *39*, 432−435.
(40) Hruby, V. J.; Lu, D.; Sharma, S. D.; Castrucci, Ana de L.; Kesterson, R. A.; Al-Obeidi, F. A.; Hadley, M. E.; Cone, R. D. Cyclic Lactam α-Melanotropin Analogues of Ac-Nle^4-c[Asp^5,D.-Phe^7,Lys^{10}]α-MSH(4−10)-NH$_2$ with Bulky Aromatic Amino Acids at Position 7 Show High Antagonist Potency and Selectivity at Specific Melanocortin Receptors. *J. Med. Chem.* **1995**, *38*, 3454−3461.
(41) Haskell-Luevano, C.; Lim, S.; Yuan, W.; Cone, R. D.; Hruby, V. J. Structure Activity Studies of The Melanotropin Antagonist SHU-9119 Modified at the 6, 7, 8 and 9 Positions. *Peptides* **2000**, *21*, 49−57.
(42) Kask, A.; Mutulis, F.; Muceniece, R.; Pähkla, R.; Mutule, I.; Wikberg, J. E. S.; Rägo, L.; Schiöth, H. B. Discovery of a Novel Superpotent and Selective Melanocortin-4 Receptor Antagonist (HS 024): Evaluation *In Vitro* and *In Vivo*. *Endocrinology* **1998**, *139*, 5006−5014.
(43) Li, S.-J.; Varga, K.; Archer, P.; Hruby, V. J.; Sharma, S. D.; Kesterson, R. A.; Cone, R. D.; Kunos, G. Melanocortin Antagonists Define Two Distinct Pathways of Cardiovascular Control by α- and γ-Melanocyte-Stimulating Hormones. *J. Neurosci.* **1996**, *16*, 5182−5188.
(44) Huang, Q.-H.; Entwistle, M. L.; Alvaro, J. D.; Duman, R. S.; Hruby, V. J.; Tatro, J. B. Antipyretic Role of Endogenous Melanocortins Mediated by Central Melanocortin Receptors During Endotoxin-Induced Fever. *J. Neurosci.* **1997**, *17*, 3343−3351.
(45) Chen, W.; Kelly, M. A.; Optimiz-Araya, X.; Thomas, R. E.; Low, M. J.; Cone, R. D. Exocrine Gland Dysfunction in MC5−R Deficient Mice: Evidence for Coordianted Regulation of Exocrine Gland Functions by Melanocortin Peptides. *Cell* **1997**, *91*, 789−798.
(46) Fan, W.; Boston, B. A.; Kesterson, R. A.; Hruby, V. J.; Cone, R. D. Role of Melanocortinergic Neurons in Feeding and the Agouti Obesity Syndrome. *Nature* **1997**, *385*, 165−168.
(47) Skuladotter, G. F.; Jonsson, L.; Skarphedinsson, J. O.; Mutulis, F.; Munceniece, R.; Raine, A.; Helgason, J.; Prusis, P.; Wikberg, J. F. S.; Schiöth, H. B. Long-Term Orexigenic Effect of a Novel Melanocortin 4 Receptor Selective Antagonist. *Br. J. Pharmacol.* **1999**, *126*, 27−34 and references therein.
(48) Wessells, H.; Fuciarelli, K.; Hansen, J.; Hadley, M. E.; Hruby, V. J.; Dorr, R.; Levine, N. Synthetic Melanotropic Peptide Initiates Erections in Men with Psychogenic Erectile Dysfunction: Double-Blind, Placebo Controlled Crossover Study. *J. Urology*. **1998**, *160*, 389−393.
(49) A reviewer raised a question about whether χ angle values will be as important in larger peptides as they seem to be in the smaller peptide ligands examined here. The answer is unclear, but based on first principles it is likely to be so in any case where a specific "epitope", whether continuous or discontinuous in sequence, is critical for intermolecular interactions that lead to biological activities. Since such noncovalent interactions seem to be universally used in biological systems, χ angles will be of critical importance to most biological functions.
(50) Two reviewers have raised the issue about how and whether exploring the shape of the ligand that binds to a GPCR provides any insight into behavior. From a reductionist point of view, there are indeed many difficulties in such a "simple" explanation. However, such a view seems too limiting. Of course, behavior cannot be understood without recognizing the importance of the entire system (ligand, receptor, intercellular machinery, cell, synapses, etc.), but a more interdisciplinary perspective is needed. What this work suggests is that behavioral responses in humans are an emergent property of the entire system, such that their manifestation can be dramatically changed by very modest changes in structure and topography of the ligand. This insight provides a very useful approach to developing ligands (drugs) that can modify undesirable behaviors. However, it also gives pause when one considers that we cannot predict what changes in structure will lead to what changes in behavior. Indeed, it is likely that some such changes will lead to behaviors that are unexpected and unacceptable. Thus, we have an ethical responsibility to freely admit that we cannot guarantee that we will obtain only beneficial ligands. Most importantly, we have to be eternally vigilant for undesirable ligands.

AR990063Q