The Nociceptin Pharmacophore Site for Opioid Receptor Binding Derived from the NMR Structure and Bioactivity Relationships*

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Nociceptin, a 17 amino acid opioid-like peptide that has an inhibitory effect on synaptic transmission in the nervous system, is involved in learning, memory, attention, and emotion and is also implicated in the perception of pain and visual, auditory, and olfactory functions. In this study, we investigated the NMR solution structure of nociceptin in membrane-like environments (trifluoroethanol and SDS micelles) and found it to have a relatively stable helix conformation from residues 4–17 with functionally important N-terminal residues being folded aperiodically on top of the helix. In functional assays for receptor binding and calcium flux, alanine-scanning variants of nociceptin indicated that functionally important residues generally followed helix periodicity, consistent with the NMR structural model. Structure-activity relationships allowed identification of pharmacophore sites that were used in small molecule data base searches, affording hits with demonstrated nociceptin receptor binding affinities.

Nociceptin is a 17 amino acid opioid-like peptide that was identified as a natural ligand of the orphan opioid receptor ORL1 (also referred to as OP4) (1). Reinscheid et al. (2) called the same peptide orphanin FQ to signify that it is a ligand for ORL1 with an N-terminal phenylalanine and a C-terminal glutamine. Nociceptin signaling through ORL1 elicits many of the same responses induced by opioid signaling through the opioid receptors. Nociceptin causes inhibition of adenyl cyclase (1, 2), activation of potassium channels (3–8), inhibition of calcium channels (9–11), mobilization of intracellular calcium (10), and activation of mitogen-activated protein kinase (12–14). These effects indicate that, similar to opioids, nociceptin has an inhibitory effect on synaptic transmission in the nervous system, acting to reduce the secretion of neurotransmitters. Consistent with its cellular effects, nociceptin inhibits the release of glutamate (5, 15–17), γ-Aminobutyric acid (5), acetylcholine (18, 19), tachykinin (20, 21), and noradrenaline (22) neurotransmitters. Based on their distribution in the brain and spinal cord, nociceptin and its receptor may be involved in a wide range of functions, including learning, memory, attention, and emotion. Nociceptin is also implicated in various sensory processes such as perception of pain, visual, auditory, and olfactory functions.

Nociceptin is related to dynorphin A, a peptide 17-mer ligand of the a-opioid receptor. Dynorphin A also binds ORL1 but with 100-fold lower affinity than nociceptin (23). Orphanin FQ2 is another biologically active peptide 17-mer processed from the same nociceptin precursor, prenociceptin (1, 2, 24). Fig. 1 shows the amino acid sequences of orphanin FQ2, nociceptin, and dynorphin A. Although nociceptin and dynorphin A are the most homologous of these three peptides (particularly at the N and C termini and being polycationic), there are clear differences in amino acid sequence that should affect conformation and receptor binding as noted above. For example, dynorphin A contains a conformationally constraining proline residue and has mid-segment cationic residues sequentially out of sync relative to nociceptin. Knowledge of the structures of these peptides would be highly informative and could aid in understanding their functions. However, both dynorphin A and nociceptin have been structurally elusive. In previous biophysical studies, dynorphin A (25) and nociceptin (26) show little tendency to form well defined structures in water and in other polar solvents.

Because these peptides function by interacting with a membrane receptor, possibly by first interacting with membrane lipids (27), we investigated the NMR solution conformation of nociceptin in a relatively low dielectric environment, i.e. 30% (v/v) trifluoroethanol (TFE) and water, and in the presence of SDS micelles. Both systems are often employed in NMR investigations to mimic membrane-like environments. Here, we demonstrate that nociceptin forms a relatively well defined helix structure in aqueous solution with SDS micelles. In addition, the measurement of receptor binding and function using nociceptin and numerous amino acid-substituted variants allowed structure-activity relationships to be derived. Results are discussed in terms of explaining differences in activities and receptor binding among opioid peptides. Overall, this work aids in the design of small molecules that can mimic the structure and function of nociceptin.

MATERIALS AND METHODS

Peptide Preparation—Peptides were synthesized using a Milligen/ Biosearch 9600 peptide solid-phase synthesizer using fluorenylmethoxy carbonyl chemistry. Lyophilized crude peptides were purified by preparative reversed-phase high performance liquid chromatography on a C18 column with an elution gradient of 0–60% acetonitrile with 1 The abbreviations used are: TFE, trifluoroethanol; NOE, nuclear Overhauser effect; PFG, pulsed field gradient; HTS, high-throughput screening; ROE, rotating-frame Overhauser effect.
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**Fig. 1. Peptide sequences.** Amino acid sequences are shown for nociceptin, orphanin FQ2, and dynorphin A.

0.1% trifluoroacetic acid in water. Purity and composition of the peptides were verified by high performance liquid chromatography (Beckman Model 6300), amino acid analysis, and mass spectrometry.

**NMR Measurements**—For NMR measurements, freeze-dried peptide was dissolved in water (H₂O or D₂O), in 30% (v/v) TFE/water, or in 10 mM SDS/water. These aqueous solutions also contained 10 mM potassium phosphate and KCl. Peptide concentration varied in the millimolar range. The pH value was adjusted to 5.5 by adding milliliter quantities of NaOD or DCl to the peptide sample. NMR spectra were acquired on a Varian UNITY Plus-600 NMR spectrometer.

Two-dimensional homonuclear magnetization transfer (HOHAHA) spectra, obtained by spin-locking with a MLEV-17 sequence (29) with a mixing time of 60 ms, were used to identify spin systems. NOE spectra were acquired on a Varian Unity-Plus 600 NMR spectrometer.

**RESULTS AND DISCUSSION**—As part of defining the best solution conditions for solution structure studies of nociceptin, PFG NMR self-diffusion measurements were performed in water (D₂O), in a water/TFE mixture, and in the presence of SDS micelles over the concentration range of 0.1–4 mM. Standard settings were used for the flexible searching. The Unity data base was created from the command line starting from a Concord-generated three-dimensional structural data file.

Three-dimensional Data Base Searching—Three-dimensional Flex searches were performed with Unity 4.2 (Tripos, Inc., St. Louis, MO) using the Directed Tweak algorithm. Three point queries were defined for explicit atom types within the peptide pharmacophore using distance constraints with tolerances of ±1 Å. Standard settings were used for the flexible searching. The Unity data base was created from the command line starting from a Concord-generated three-dimensional structural data-base.

**Binding of Peptides to the Nociceptin Receptor (ORL1).**—The nociceptin receptor binding assay measures the binding of [³⁵S]-Tyr¹-nociceptin (2200 Ci/mmol, New England Nuclear) to human ORL1 on HEK-293 cell membranes. HEK-293 cell membranes from cells stably expressing ORL1 were prepared as described previously (35) with the exception that the binding buffer used was 50 mM Tris-Cl, pH 7.8, 5 mM MgCl₂, and 1 mM EGTA. ORL1 membranes were diluted in binding buffer such that 25 μl containing 1 μg of membrane was added to each well of a standard 96-well microtiter plate in binding buffer. Peptides were diluted at 50% of the indicated concentration, and 1 μl was added to each well. [³⁵S]-Tyr¹(Nociceptin was added at a final concentration of 0.5 μM so that the total reaction volume was 50 μl. Plates were incubated for 2 h at room temperature, and the reactions were filtered over GF/C Filterplates (PerkinElmer Life Sciences) prewetted in 0.03% polyethyleneimine in a Filtermate 196 apparatus (Packard). Plates were washed six times with binding buffer in the fraction collector apparatus and then dried under vacuum for 1–2 h. 25 μl of Microscan 20 (Packard) per well was added to solubilize bound radioactivity. The plates were then sealed and counted on a Packard Top Count to determine radioactivity bound to the membranes. The curves were fitted, and Kᵢ values were determined using GraphPad Prism software (version 3.0).

Calcium Flux—Although ORL1 is coupled to the G protein, G, and thus does not normally elicit a calcium response, we were able to pretreat cells with the muscarinic receptor agonist carbachol, upon which cells were “sensitized” and able to elicit a subsequent calcium response upon nociceptin administration. This technique has previously been described for ORL1 specifically (9) and for G-coupled receptors in general (36).

HEK-293 cells expressing ORL1 receptor were plated at a density of 5 × 10⁴ cells/well in a total volume of 50 μl onto 96-well plates. Two days later, cells were prepared for assay using the FLIPR calcium assay kit (Molecular Devices) according to manufacturer’s directions with the exception that the volume of dye mixture added to each well was 50 μl instead of 100 μl. Cells were then treated with 100 μM carbachol delivered in a total volume of 100 μl. After base line was reestablished, cells were challenged with varying concentrations of nociceptin or a nociceptin variant. Data points were collected at one second for 120 s and then every 3 s for 30 s for a total collection time of 150 s after both the first and second additions.

The calcium flux as a function of nociceptin administration was calculated and divided and the drop occurring from carbachol administration to normalize within individual wells. This produced a “percent of control” or “% of response” for each dose. The experiment was repeated six times, and the high and low values for each concentration were dropped. The data were used to generate EC₅₀ curves using GraphPad Prism, version 3.0 software.

Three-dimensional Data Base Searching—Three-dimensional Flex searches were performed with Unity 4.2 (Tripos, Inc., St. Louis, MO) using the Directed Tweak algorithm. Three point queries were defined for explicit atom types within the peptide pharmacophore using distance constraints with tolerances of ±1–2 Å. Standard settings were used for the flexible searching. The Unity data base was created from the command line starting from a Concord-generated three-dimensional structural data-base.
effect is attributable to self-association of the peptide, and the magnitude drop in the diffusion coefficient is consistent with nociceptin forming dimers. As will be evident later, this may be attributable to the folded structure of nociceptin, which is an amphipathic helix. Dimer formation may be mediated through interactions of the hydrophobic faces of two molecules of the peptide. Above 5 mM, changes in the diffusion coefficient are explainable by changes in solution viscosity, allowing one to conclude that further aggregation does not occur up to at least 14 mM. The dashed line in this plot indicates changes in the diffusion coefficient expected solely for increases in solution viscosity as peptide concentration is increased. Although this phenomenon is interesting, NOESY/ROESY data acquired in water at any concentration of peptide were of little use in terms of structure elucidation. As discussed in the following section, NOEs (ROEs) were few and resonances were generally broadened, probably the result of monomer-dimer exchange.

In TFE/water, nociceptin behaves similarly (Fig. 2). The presence of 30% TFE in water increases solution viscosity and by itself accounts for the observation of overall smaller diffusion coefficients. However, the shape of curve does parallel that observed in water alone. The abrupt 10% drop in diffusion coefficients at 2 mM peptide is again consistent with dimer formation. Nevertheless, as in water, NOESY data were of limited use in terms of structure elucidation. As discussed in the following section, NOEs (ROEs) were few and resonances were generally broadened, probably the result of monomer-dimer exchange.

In SDS, it is apparent that nociceptin interacts with micelles. At peptide concentrations of 0.1 mM and at 1 mM, the observed diffusion coefficient of about $7 \times 10^{-6}$ cm$^2$/s at 25 °C for nociceptin is close to the diffusion coefficient reported for SDS micelles alone ($1 \times 10^{-6}$ cm$^2$/s at 25 °C (38), $0.85 \times 10^{-6}$ cm$^2$/s at 37 °C (39), and $1.19 \times 10^{-6}$ cm$^2$/s at 37 °C (40)), indicating that nociceptin is binding to SDS micelles. This diffusion coefficient is consistent with the presence of $\sim 50$–100 SDS molecules/micelle and $\sim 1$–2 molecules of nociceptin bound/micelle. In the high concentration range (2–12 mM), diffusion coefficients are only somewhat smaller than those observed for monomeric nociceptin in water (Fig. 2). This minimal difference reflects a weighted average of free and micelle bound nociceptin with most of the peptide in the free state. At a concentration of 2 mM peptide and higher, the 2.5-fold shift in diffusion coefficient between SDS and water is consistent with $\sim 10$% or less of the nociceptin being bound at any given time.

**NMR Conformational Analysis—**In water alone, nociceptin at any concentration investigated (0.1–14 mM), even under conditions favoring the dimer state, shows mostly random coil chemical shifts (41) and only a few conformationally meaningful NOEs/ROEs. This is consistent with what was reported previously for nociceptin dissolved in water (26). Moreover, resonances in both TOCSY (Fig. 3A) and ROESY (Fig. 4, A and B) spectra are generally broader than they are in TFE (data not shown) or in the presence of SDS micelles (Figs. 3B and 4, C and D).

However, in aqueous TFE, nociceptin at 2 (monomer state) or 5 mM (dimer state) demonstrates some conformational preference. At either concentration, the observation of a series of NH-NH NOEs running from 600 MHz $^1$H NMR TOCSY spectra are shown for nociceptin in water (A) and in the presence of SDS micelles (B). Peptide concentration was 3 mM in 10 mM potassium phosphate, 10 mM SDS, pH 5.5, and 25 °C. Spectra were accumulated with 8192 data points over 6,000 Hz sweep width and were processed with 1 Hz line broadening.

In the presence of SDS micelles, nociceptin exhibits a significantly larger number of NOEs (compare Fig. 4, A and B with C and D, respectively). This is best evident at nociceptin concentrations above $\sim 2$ mM where resonance broadening is not of concern as it is at lower concentration. Combined with the results from the diffusion data, this observation is consistent with the idea that nociceptin interacts with SDS micelles in relatively fast exchange between free and bound states on the
NMR chemical shift time scale. In this regard, it is most likely that we are observing transferred NOEs (44) as the result of this interaction and exchange equilibrium. Therefore, for NMR structural analysis, NOESY data were acquired at a nociceptin concentration of 3 mM to take advantage of the transferred NOE effect and to work with the peptide mostly in the free state where resonance broadening is not problematic.

Nociceptin exhibits considerably more stable helical structure in the presence of SDS micelles than in TFE or in water. This is evidenced by differences in chemical shifts of resonances from the peptide compared with those observed in aqueous TFE (Table I) as well as by comparison of TOCSY (HO-HAHA) spectra of the peptide in water and in SDS micelles (Fig. 3). Most NH and αCH resonances shift upfield, and residue Gly³ αCH₂ and some βCH₂ resonances become either non-degenerate or better separated (Table I and see Fig. 3, Thr⁵ and Ser¹⁰). The Gly³ αCH resonances, nearly degenerate in TFE, become separated by 0.25 ppm and shifted upfield by 0.68 ppm in SDS micelles (Table I) (not in field in Fig. 3). In contrast, residue Gly⁶ αCH₂ resonances are downfield shifted by 0.87 ppm from TFE (Table I) and by 0.65 ppm from water (Fig. 3). These observations are probably due to ring current shifting because of proximity to residues Phe¹ and/or Phe⁴ and indicate structural stabilization and possible positioning of the phenylalanine ring either above the Gly³ αCH₂ group or on edge with the Gly⁶ αCH₂ group. Other similar chemical shift differences listed in Table I can be found when comparing nociceptin in water and in SDS micelles (see Fig. 3).

Also in SDS micelles, nociceptin exhibits numerous structurally informative NOEs as exemplified in Fig. 4, which shows αH-NH and NH-NH regions from NOESY data acquired on nociceptin in the presence of SDS micelles. Fig. 5 summarizes these and other observed NOEs. The pattern of NOEs indicates the formation of a relatively good helix conformation (39) from approximately Ala⁷ to Gln¹⁷, and three αH-NH i,i+2 NOEs at the N terminus are suggestive of multiple turn or 3₁0 helix.

Conformational Modeling—Conformational modeling was performed using NOE data acquired for the peptide in the presence of SDS micelles. A total of 140 NOE distance con-
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The thickness of the bar is proportional to the magnitude of the NOE.

FIG. 5. Summary of NOEs. Observed NOEs are summarized for nociceptin in SDS. The thickness of the bar is proportional to the magnitude of the NOE.

Constraints were derived from the analysis of NOESY spectra. These include 68 intraresidue, 24 sequential, 20 medium-range (\(i - j\) \(\leq 5\)) and 28 long-range (\(i - j\) \(\geq 5\)) constraints. In addition, a total of eight hydrogen bonds could be identified by inspection of initial structures and from NH temperature factors and/or long-lived backbone NHs (Thr7, Lys5, Ser10, Ala11, Arg12, Leu14, Ala15, and Asn16), giving rise to 16 hydrogen bond distance constraints. Therefore, the total number of experimentally derived constraints was 156, giving an average of 13 constraints/residue.

Initially, 100 structures for nociceptin were calculated as described under "Materials and Methods." From the Accept routine in X-PLOR, 19 structures were selected and superimposed (backbone Cα atoms) in Fig. 6, left panel. These structures showed no NOE violations greater than 0.5 Å and satisfy experimental constraints quite well. An analysis of these structures shows a well-formed helix running from residue 4 through residue 17 with a less structurally defined N terminus. For these 19 structures, the best atomic r.m.s. differences with respect to the mean coordinate positions were found for residues 4–16 (0.71 \(\pm\) 0.08 Å for backbone N, Cα, and C atoms and 1.4 \(\pm\) 0.4 Å for all of the heavy atoms and \(\phi\) and \(\psi\) angular order parameters all \(>0.8\)).

The six lowest energy structures from these 19 are also shown superimposed in the to Fig. 6, middle panel. Structural statistics are summarized in Table II. This set of structures defined our pharmacophore model discussed below and is illustrated at the right in Fig. 6. The surface of one face of the helix is highly positively charged with two pairs of Arg-Lys residues (Arg8/Lys9 and Arg12/Lys13) lying below the functionally important N-terminal phenylalanines (Phe8 and Phe11). It should be noted that when comparing all 19 accepted structures, Phe8 was positioned within 2 Å of Phe8 in 16 of these 19 structures, even though the N terminus was more internally mobile than the remainder of the peptide. This observation increased confidence in the pharmacophore model (Fig. 6, right panel) used in our small molecule data base search described previously.

Opioid Receptor Binding and Calcium Release—Structure is only one part of defining structure-activity relationships for a peptide. A receptor binding or functional assay is the other requisite part. Table III shows the results for ORL1 receptor binding (\(K_i\) values) and calcium release (EC_{50} values) for nociceptin and 30 single and double amino acid-substituted variants. The first 18 rows provide results from alanine scanning (or glycine when an alanine was already present), whereas the last 13 rows show results from various other substitutions to better discern effects from changes in charge, polarity, and side-chain composition. Representative binding curves of alanine and glycine substitutions are shown in Fig. 7A, whereas binding curves from more conservative substitutions at critical residues are shown in Fig. 7B. The ability of the different substituted peptides to bind to ORL1 and to function as agonists as assessed by calcium release correlate well with a correlation coefficient of 0.84 (Fig. 7C).

Alanine substitutions at positions 1 and 4 produced some of the most dramatic decreases in receptor binding as well as signaling as measured by calcium release (Table III and Fig. 7A). This was anticipated because previous studies reported that these residues are in the N-terminal “address” portion of the molecule known to be critical for ORL1 receptor binding (45–47). However, it was surprising that other alanine-substituted variants also produced relatively large effects. In particular, the substitution of Gly8 for alanine (G2A) produced the largest single drop in binding affinity, twice that of substitution of Phe1. One explanation for this is induced conformational changes in the peptide when alanine is present at position 2 as opposed to the greater freedom of rotation around the peptide bond provided by the glycine residue. Another sensitive position is at Arg8 where substitution with alanine led to a significant decrease in binding affinity (Fig. 7A) and signaling. Substitution of neighboring Ala7 with glycine also caused a substantial reduction in binding affinity. Modest effects were also noted with substitutions of Ala11 and Arg12 on receptor binding and of Ser10 and Ala13 on signaling. In addition, the substitutions at C-terminal residues Leu14, Ala15, and Asn16, which have been reported to be dispensable for signaling, produced modest 2–3-fold increases in receptor binding affinity, whereas signaling remained unaffected by substitutions at Ala15 and Asn16.

Substitution of Thr4 with leucine (Fig. 7B) or serine was better tolerated than substitution with alanine, suggesting that side-chain bulkiness and/or structure-inducing properties were important to the bioactive conformation of the peptide. Similarly, the replacement of Ala2 with leucine, norleucine, or serine produced little if any change on receptor binding (Fig. 7B).
receptor binding and signaling (Fig. 7).

7B) and only a modest change in functional activity with the exception of norleucine, which demonstrated a 12-fold decrease in magnitude closer to the C terminus, consistent with the substitution with the polar yet neutral citrulline moiety.

The substitution of the basic arginine with lysine at position 8 was not well tolerated in terms of both receptor binding and signaling, whereas the substitution of Arg\(^4\) with citrulline also had a negative effect on receptor binding but showed a much more modest effect on signaling (Table III). Apparently, the presence of an unbranched butyl group at position 7 has a negative effect on function but not on receptor binding. At position 11, the addition of a β-hydroxy group (serine) at position 11 was well tolerated, whereas the replacement of the alanine with bulkier leucine or norleucine residues had a modest negative effect on receptor binding and signaling (Fig. 7B and Table III).

The substitution of the basic arginine with lysine at position 8 was not well tolerated in terms of both receptor binding and signaling, whereas the substitution of Arg\(^4\) with citrulline also had a negative effect on receptor binding but showed a much more modest effect on signaling (Table III and Fig. 7B). A different trend was noted for the arginine at position 12 where the substitution with alanine had a modest effect on receptor binding but essentially no effect on signaling. Substitution with lysine or citrulline had essentially no effect on binding or function. Simultaneous substitution of both arginines at positions 8 and 12 with lysine produced a peptide that behaved similarly to the R8A substitution alone with respect to its ability to signal, i.e., calcium release was reduced to a similar extent (Table III and Fig. 7B). This is in contrast to the behavior of these peptides in the receptor binding assay where lysine substitution at both Arg\(^4\) and Arg\(^12\) produced a molecule that bound with an affinity closer to that of the wild type peptide (Fig. 7B). Taken together, these findings suggest that the presence of positive charges at positions 8 and 12 is not the sole determining factor for potency. The hydrogen-bonding potential of arginine compared with that of lysine may, at least partly, help explain this observation.

**TABLE III**

<table>
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<tr>
<th>Peptide</th>
<th>(K_i)</th>
<th>(K_i/\langle\text{EC}_{50}\rangle) WT</th>
<th>(\text{EC}_{50})</th>
<th>(\text{EC}<em>{50}/\langle\text{EC}</em>{50}\rangle) WT</th>
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<td>F1A</td>
<td>91</td>
<td>127</td>
<td>1443</td>
<td>601</td>
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<td>191</td>
<td>265</td>
<td>1394</td>
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</tr>
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<td>3881</td>
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<td>66</td>
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<td>16</td>
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<tr>
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<td>50</td>
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<td>69</td>
<td>29</td>
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<td>5.4</td>
<td>53.5</td>
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</table>

Note that this comparison is made using data from Table III converted to their natural logarithms, making the 5–10% experimental error in these values less meaningful to this comparison. It should also be emphasized that the larger the bar is in Fig. 8, the more reduced is either ORL1 binding or signaling relative to native nociceptin. Although both receptor binding and calcium release data show this trend, it is more evident with the receptor binding data. Starting at Phe\(^4\) and moving toward the C terminus, substitutions made at every \(i + 3\) or \(i + 4\) residue have a deleterious effect on activity. This effect diminishes in magnitude closer to the C terminus, consistent with residues at the N terminus being most important for binding of nociceptin to ORL1.

The nociceptin peptide structure-function relationships described here allow for refinement of a pharmacophore model based on nociceptin residues important for binding and signaling through ORL1. Such a model is expected to aid in identifying novel non-peptide modulators of ORL1 as potential drug candidates. The full pharmacophore site for nociceptin modeled in Fig. 6 has the phenyl ring of Phe\(^4\) on top of that for Phe\(^4\). The first turn of the helix places Phe\(^4\) over Ala\(^7\) and Arg\(^12\) followed by the second turn of the helix with another Ala-Arg pair, Ala\(^11\) and Arg\(^12\). This fold causes the two pairs of alanine-arginine residues, both of which appear to be crucial to activity, to lie on top of one another. This finding suggests that nociceptin when folded in this fashion interacts with ORL1 via both hydrophobic residues and positively charged residues. As mentioned above, it also appears that the hydrogen bonding potential of the arginine guanidinium group and not only its positive charge plays a role in the activity of nociceptin. Whereas both the R8K and R8Cit variants show significant losses in their receptor binding activities, calcium release is relatively less affected by substitution with the polar yet neutral citrulline moiety.

There is clearly a relationship between receptor binding and signal transduction. However, the correlation shown in Fig. 7C is not absolute. In fact, the substitution of one residue may have a greater effect on signal transduction than on receptor binding. To visualize this simplistically, we plotted the ratio of the signaling to binding values in Fig. 8C. The larger and more positive this ratio is, the larger the negative effect of the substitution with the polar yet neutral citrulline moiety.

Nevertheless, as expected, larger ratios are noted at the N-terminal address portion of nociceptin because function is expected to be impacted by the decrease in ligand affinity. However, even within this region, it appears that the largest effects are found with Thr\(^5\) and Gly\(^6\), suggesting that these two residues may be somewhat more crucial to signal transduction.
than to receptor binding relative, for example, to Phe$^3$ and Phe$^4$. What is most surprising in this plot is that residues Ser$^{10}$, Leu$^{14}$, and Gln$^{17}$ also display a strong capacity to affect signal transduction. This analysis suggests that, for example, the side-chain hydroxyl group of Ser$^{10}$ may play a role in signaling but not in ORL1 binding. It may be that the methylene portion of the serine C$_{\alpha}$H$_2$OH group is sufficient for ORL1 binding but that the OH group, perhaps through hydrogen bond formation with some group on the receptor, is crucial to signaling. It has been reported that these C-terminal residues are dispensable for nociceptin function (27, 47); however, when viewed in terms of the signaling-to-binding ratio, it appears that the nociceptin C terminus does play some role in ORL1 signaling. We also observed that the activity profile for these residues also shows (i+3, i+4) helix periodicity. This perspective on these data may aid in designing nociceptin mimetics as agonists or as antagonists. For example, an antagonist would be excellent at receptor binding but poor at signal transduction.

Comparison to Related Peptides—As shown in Fig. 1, dynorphin A and orphanin FQ2 are homologous to nociceptin. To test the intrinsic tendency of dynorphin A to assume helical conformations, Spadaccini et al. (25) studied the conformational properties of dynorphin A over a range of solution conditions including methanol, acetonitrile, Me$_2$SO and mixtures of organic solvents in water. In most of these solutions, the dynorphin A peptide showed conformational flexibility comparable to that observed in water alone, i.e., no stable structure. However, in Me$_2$SO/water (80:20), some structural stabilization was noted from Arg$^7$ to Gln$^{17}$. Nevertheless, the peptide demonstrated little regular structure. Amodeo et al. (48) investigated the NMR solution structure of orphanin FQ2 and concluded similarly that it too possesses a helix conformation through the body of the peptide. Obviously, receptor binding and function are somewhat different for each of these peptides. One may conclude that not only the amino acid sequence but also the spatial relations of residues account for different activities relative to nociceptin. In a helix conformation, amino acid residues would be presented to the receptor differently in each of these homologous peptides. This, in turn, would define different pharmacophore sites in each peptide.
Small Molecule Data Base Search Using the Pharmacophore Model—To demonstrate that our pharmacophore model, which is based on the NMR-derived structure of the peptide and structure-activity relationship information, could lead to hits with demonstrated nociceptin receptor binding affinities, a three-point pharmacophore query was constructed. This query contained side-chain functionalities from the Phe¹ aromatic, the Phe³ aromatic, and the Arg⁶ terminal nitrogen of the guanidinium group. These three amino acid residues were clearly important for binding and function. The pharmacophore query was used to search a data base comprised of 250,000 small molecule structures. From this search, 940 unique compounds were obtained that fit the model. These compounds were tested in duplicate in the receptor binding assay at a concentration of 10 μM. Of these compounds, 37 inhibited the binding of labeled [125I]-nociceptin by 50% or more, which represents a hit rate of 3.9%. Random high-throughput screening (HTS) of a subset of the compound library at a screening concentration of 16 μM yielded 1,139 hits (defined as inhibition of 50% or more) of 188,787 compounds tested, a hit rate of 0.6%. Thus, the use of our pharmacophore model to select compounds increased the hit rate by nearly 7-fold.

Although random HTS yielded 1,102 more active compounds (a total of 1,139 hits) than were identified by the structure-based pharmacophore-screening paradigm (37 hits), it is more important to realize that the result of virtual screening using the pharmacophore model was a very small subset of the compound library (940 compounds of more than 250,000) and a much larger percentage of this compound subset (940 compounds) was hits. By definition, most of the random HTS hits should have been missed by the pharmacophore screening method because only a small portion of the larger library fit the pharmacophore model (940 compounds of a screening library of 188,787 compounds or 0.5%). In addition, the random HTS hits were distributed among a wide variety of structural types and probably reflect many different binding modes, whereas the NMR structure-derived pharmacophore yielded compounds that were similar in structure and probably were able to bind to ORL1 in a similar way as the native peptide. A retrospective analysis of the random (HTS) screening data showed that 357 of the 940 compounds selected by the pharmacophore paradigm had been previously screened. Of these, 357, 94 were hits (>50% inhibition) in the original HTS assay, representing a 26% hit rate by this measure. This also means that 94 of 1139 random screening hits or a respectable 8% also fit the pharmacophore model, a much higher success rate than the random (HTS) hit rate of 0.6%. When the 940 pharmacophore-based compounds were screened together for the purpose of evaluating the pharmacophore model, a slightly lower hit rate was found (37 of 940 or 0.9%). This is in line with the historical behavior of HTS screening in which it is common to have a 50% reduction in screening hits when the original hits are re-screened for confirmation.

It should also be mentioned that the spatial relationship between various pharmacophore elements in the nociceptin structure is essential to the success of this approach. It is not sufficient to merely choose aromatic compounds with positive charges. A large number of compounds in the screening library possess both aromatic rings and positively charged amines, and if these were the only requirements for successful inhibition of nociceptin binding, the hit rate from random HTS would have been much higher than 0.6%. As it turns out, the three-dimensional arrangement of the Phe¹ and Phe³ aromatics and the Arg⁶ terminal nitrogen in the pharmacophore model seems to mimic the binding behavior of nociceptin quite acceptably. These results indicate that a pharmacophore-based selection process is advantageous in screening compounds and can significantly reduce the time and effort expended to find lead compounds. This can be of great value for peptide receptors where initial small molecule leads may be difficult to find.

Conclusions—This study has demonstrated that structure-activity relationships in nociceptin, deduced using NMR-derived structures and functional assays, can lead to identification of a discrete pharmacophore site. The approach has been validated by using elements from this pharmacophore site in a three-dimensional data base search for small molecule hits with proven affinity for the nociceptin receptor. This approach should aid in the discovery and design of a clinically useful and pharmaceutically acceptable compounds that target the nociceptin pathway.

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The Nociceptin Pharmacophore Site for Opioid Receptor Binding Derived from the NMR Structure and Bioactivity Relationships
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