# *Articles*

## Investigation of the Selectivity of Oxymorphone- and Naltrexone-Derived Ligands via Site-Directed Mutagenesis of Opioid Receptors: Exploring the 'Address' Recognition Locus

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The $\delta$-selective opioid antagonist naltrindole (NTI), as well as the $\kappa$-selective opioid antagonists norbinaltorphimine (norBNI) and 5′-guanidinonaltrindole (GNTI), are derived from naltrexone, a universal opioid antagonist. Previous studies have indicated that extracellular loop III is the key region for discrimination by naltrexone-derived selective ligands between the $\delta$, $\mu$, and $\kappa$ opioid receptor types. It has been proposed that selective ligands could bind to all three receptor types if the appropriate portions of the extracellular loops were eliminated. To investigate this possibility, several single-point mutant opioid receptors have been generated with the aim of conferring enhanced affinity of selective ligands for their nonpreferred receptor types. Mutations were made in all three types of opioid receptors with the focus on two positions at the extracellular end of transmembrane regions (TM) VI and VII. It was found that the $\delta$-selective NTI could bind both $\mu$ and $\kappa$ receptors with significantly enhanced affinity when an aromatic residue in TM VII was replaced with alanine ($\mu$[W318A] and $\kappa$[Y312A]). Similarly, $\kappa$-selective antagonists, norBNI and GNTI, showed enhanced affinity for the $\mu$[W318A] mutant and for both $\mu$ and $\delta$ receptors when a glutamate residue was incorporated into the extracellular end of TM VI ($\mu$[K303E] and $\delta$[W284E]). These results demonstrate that naltrexone-derived selective ligands achieve their selectivity via a combination of enhanced affinity of the address for a particular subsite along with loss of affinity due to steric interference at nonpreferred types. The results reveal key residues in the 'address' recognition locus that contribute to the selectivity of opioid ligands and support the hypothesis that recognition of the naltrexone moiety is essentially the same for all three receptor types.

### Introduction

Opioid receptors are the primary site of interaction of morphine and related opiate alkaloids as well as the various endogenous opioid peptides.[1] The cloning of the opioid receptors has revealed three gene products displaying pharmacological profiles consistent with traditional classification of $\mu$, $\kappa$, and $\delta$ receptor types.[2−8] It is now well-established that opioid receptors belong to the rhodopsin subclass within the superfamily of G protein-coupled receptors (GPCRs). Structurally, GPCRs are transmembrane proteins characterized by the presence of seven transmembrane (TM) helices that are clustered in the form of a bundle. Within the family of opioid receptors, there is a high degree of sequence homology within the transmembrane region, while there is significant divergence in the sequence of the N-terminus and extracellular loops (Figure 1).

The cloning of the opioid receptors has enabled two modes of investigation that greatly enhance the understanding of ligand−receptor interactions. The first is molecular modeling which can be used to generate protein models that can then be used for ligand docking experiments and identification of putative binding sites. The second mode is site-directed mutagenesis experiments that are useful for determining the role of particular segments or amino acid residues in ligand recognition. Together, these two tools combined with ligand design and synthesis afford a powerful combination aimed at elucidating the structural basis of molecular recognition at opioid receptors.

In the past, selectivity of opioid ligands has been rationalized in terms of the 'message−address' concept of Schwyzer[9] wherein the 'message' component of the ligand specifies primary receptor recognition and the 'address' portion confers selectivity by specific recognition at a particular receptor subsite.[10] Several selective opioid antagonists have been derived from a common pharmacophore. The $\delta$-selective antagonist, naltrindole (NTI), and the $\kappa$-selective antagonists, norbinaltorphimine (norBNI) and 5′-guanidinonaltrindole (GNTI), which are derived from the universal opioid antagonist, naltrexone (Figure 2), exemplify this concept. In NTI, the benzene moiety acts as a $\delta$ 'address'. Likewise, in the $\kappa$-selective antagonists, the presence of a second positively charged group has been shown to provide $\kappa$ selectivity.[11]

Various studies using site-directed mutagenesis have

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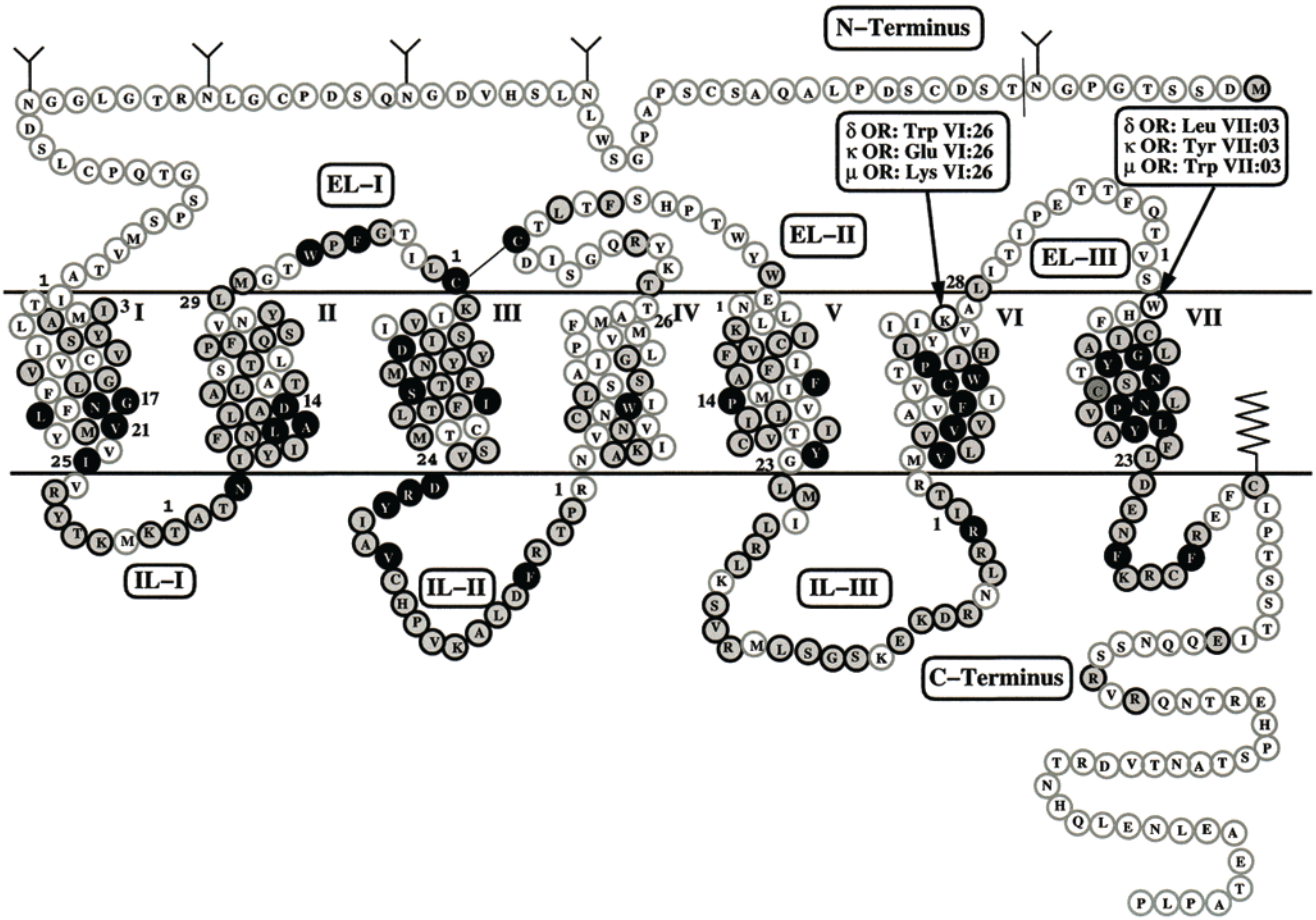


**Figure 1.** Serpentine model of the $\mu$ opioid receptor. Black lines represent the boundaries of the membrane. Circles contain the one-letter code for the given amino acid. Gray circles represent amino acids that are present in all three receptor types ($\mu$, $\delta$, and $\kappa$). Black circles represent residues highly conserved among the rhodopsin subclass of GPCRs. Each transmembrane region is indicated with a Roman numeral. The Arabic number in the membrane denotes the position of each residue from the N-terminal region of each helix using a generic numbering scheme (e.g. Trp VI:26 indicates the 26th position from the N-terminal end of TM VI) defined in the Experimental Section. Positions that were mutated in this study are indicated with arrows. IL = intracellular loop; EL = extracellular loop.

shown that extracellular loop III (EL-III) of the opioid receptor is the key region of discrimination between $\delta$, $\mu$, and $\kappa$ receptors for NTI, norBNI, and GNTI.[12−17] For example, binding studies of both $\mu/\kappa$[12] and $\delta/\kappa$[13] chimeric opioid receptors revealed that the presence of EL-III from the $\kappa$ receptor was the only requirement for high-affinity binding of norBNI. Likewise, the $\delta$-selective NTI showed high affinity for $\delta/\kappa$[13] and $\delta/\mu$[14] chimeric opioid receptors as long as EL-III from the $\delta$ receptor was present. Our previous receptor modeling studies led to the proposal of a specific docking mode for naltrexone as well as the selective naltrexone-derived antagonists.[18] In this docking mode, the 'address' (or selectivity-conferring) portions of the selective ligands were oriented toward EL-III of the receptor model.

In this report, particular amino acid residues have been mutated in order to assess the impact of these changes on binding affinity of selective oxymorphone- and naltrexone-derived ligands. The mutant receptors generated in this study focus on positions that were identified as interacting with the putative 'address' portions of NTI and norBNI in the prior modeling study.[18] The primary goal of the mutagenesis was to generate mutations that give selective ligands enhanced affinity for their *nonpreferred* wild-type receptors. This was believed to be possible based on several working hypotheses: (1) nonselective ligands such as naltrexone bind in a highly homologous pocket in the transmembrane region of the receptor in a similar fashion in all three receptor types; (2) recognition of the antagonist pharmacophore of the selective naltrexone-derived ligands is the same as that for naltrexone; and (3) selectivity can, in part, be conferred by a mechanism of exclusion whereby particular regions (or individual amino acid residues) of the receptor prevent ligand docking to the binding pocket.[19]

### Design Rationale for Mutant Receptors

Candidate sites for mutagenesis were selected based on two criteria. First, the amino acid side chain would need to be proximal to the 'address' portion of the selective ligand as specified by docking modes proposed previously.[18] Second, any such position must vary among the three opioid receptor types. Only two positions (TM VII:03 and TM VI:26, see the Experimental Section for a definition of the GPCR numbering scheme) fulfill both criteria. Our previous docking studies have suggested that a leucine residue at position VII:03, which is unique to the $\delta$ receptor, was positioned to interact with the 'address' portion of NTI.[18] A sequence alignment of opioid receptors in this region shows that

**Table 1.** Inhibition Constants of Opioid Agonist and Antagonist Ligands[a]

| | naltrexone | | NTI | | norBNI | | GNTI | | oxymorphone | | SIOM | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| receptor | $K_i$, nM | $\Delta K_i$[b] | $K_i$, nM | $\Delta K_i$ | $K_i$, nM | $\Delta K_i$ | $K_i$, nM | $\Delta K_i$ | $K_i$, nM | $\Delta K_i$ | $K_i$, nM | $\Delta K_i$ |
| $\mu$ | 1.6 ± 0.2 | | 34.4 ± 0.6 | | 56.6 ± 6.6 | | 22.5 ± 3.9 | | 17.4 ± 1.3 | | 88.4 ± 11.7 | |
| $\mu$-W318A | 2.5 ± 0.4 | −1.6 | 0.36 ± 0.01 | 96 | 0.39 ± 0.01 | 144 | 0.50 ± 0.30 | 45 | 23.1 ± 1.3 | −1.3 | 3.9 ± 0.6 | 23 |
| $\mu$-K303E | 2.0 ± 0.2 | −1.3 | 13.5 ± 0.6 | 2.5 | 0.22 ± 0.14 | 257 | 0.09 ± 0.09 | 254 | 26.2 ± 0.8 | −1.5 | 154 ± 1 | −1.7 |
| $\delta$ | 44.7 ± 3.1 | | 0.19 ± 0.07 | | 41.5 ± 13.0 | | 46.2 ± 5.1 | | 730 ± 151 | | 4.1 ± 1.0 | |
| $\delta$-W284E | 55.7 ± 6.2 | −1.2 | 1.82 ± 0.37 | −9.4 | 5.2 ± 2.7 | 7.8 | 1.1 ± 0.6 | 42 | 610 ± 72 | 1.2 | 46.6 ± 2.0 | −11 |
| $\kappa$ | 4.2 ± 0.4 | | 25.5 ± 4.6 | | 0.24 ± 0.06 | | 0.18 ± 0.05 | | 208 ± 36 | | 488 ± 90 | |
| $\kappa$-Y312A | 12.7 ± 3.9 | −3.1 | 1.23 ± 0.31 | 21 | 0.07 ± 0.02 | 3.2 | 0.03 ± 0.01 | 6 | 435 ± 19 | −2.1 | 55 ± 12 | 8.8 |
| $\kappa$-E297A | 1.8 ± 0.7 | 2.3 | 42.0 ± 9.8 | −1.6 | 1.39 ± 0.50 | −5.7 | 3.9 ± 0.6 | −22 | 95 ± 17 | 2.2 | 292 ± 72 | 1.7 |

[a] The standard error of the mean is shown with each value. [b] Fold increase (+) or decrease (−) of $K_i$ values relative to that of the wild-type.

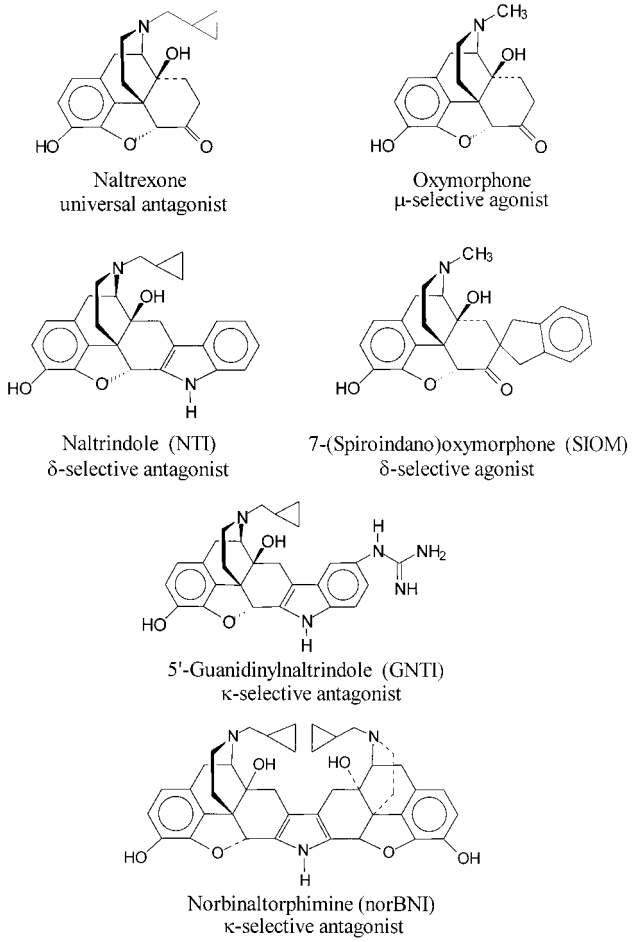


**Figure 2.** Opioid ligands used in this study.

this position is occupied by a tryptophan residue in the $\mu$ receptor and a tyrosine in the $\kappa$ receptor (Figure 1). Since the modeling suggested that these side chains could interfere with NTI binding to the receptor, these residues were mutated to alanine in the $\mu$ and $\kappa$ receptors in an effort to see if removal of the side chains would enable high-affinity binding of NTI to these receptors. Alanine was selected instead of leucine to determine if it is the absence of the aromatic side chain rather than the presence of leucine at position VII:03 that enables high-affinity NTI binding. In addition, the $\delta$-selective agonist 7′-spiroindanooxymorphone (SIOM)[20] was tested against the mutant receptors to see if it gave a pattern of results similar to that of the antagonist NTI. For the $\kappa$-selective antagonists norBNI and GNTI, two mutants were constructed that incorporate a glutamate residue into the $\mu$ and $\delta$ receptors at position VI:26. Mutation $\mu$[K303E], which incorporates this glutamate into the $\mu$ receptor, has been shown previously to give enhanced affinity to both norBNI and GNTI.[15] A glutamate residue has now been incorporated into the $\delta$ receptor at the same position to test whether these $\kappa$-selective antagonists show enhanced affinity at this mutant $\delta$ receptor.

## Results

Results of displacement binding studies are expressed as $K_i$ values and are summarized in Table 1. The most striking feature of the displacement binding results was the enhanced affinity of the address-containing ligands (NTI, SIOM, GNTI, and norBNI) for $\mu$[W318A]. Both $\delta$ and $\kappa$ antagonists, along with the $\delta$-selective agonist SIOM, displayed significantly enhanced affinity for this mutant $\mu$ receptor. Indeed, each of these ligands had an affinity for the mutant $\mu$ receptor that was very close to that of its preferred wild-type. Though not as pronounced, a similar pattern of results exists for $\kappa$[Y312A] in which Y312A occupies the same location (position VI:03) as W318A in the $\mu$ receptor. Again, significant enhancements in affinity occur for $\delta$-selective ligands NTI and SIOM at this mutant $\kappa$ receptor. The $\kappa$-selective antagonists, norBNI and GNTI, also displayed higher affinities at the Y312A mutant than at the $\kappa$ wild-type. On the other hand, the nonselective naltrexone showed similar affinity for both $\mu$[W318A] and $\kappa$[Y312A] compared to the $\mu$ and $\kappa$ wild-types.

Mutant $\mu$[K303E] exhibited enhanced binding for $\kappa$ antagonists similar to that reported previously.[15] In particular, $\kappa$-selective antagonists norBNI and GNTI bound to this mutant receptor with an affinity similar to that of the wild-type $\kappa$ receptor. Incorporation of a glutamate into the same position (VI:23) in the $\delta$ receptor ($\delta$[W284E]) gave enhanced affinity for norBNI and GNTI. However, the increase in affinity was significantly greater for GNTI than for norBNI. Furthermore, it is noteworthy that both the $\delta$-selective agonist and antagonist (SIOM and NTI) showed a decrease in affinity at $\delta$[W284E]. Both naltrexone and oxymorphone displayed affinities similar to those of the $\mu$ and $\delta$ wild-type receptors when a glutamate was introduced into position VI:26 of the $\mu$ and $\delta$ receptors, respectively.

## Discussion

Previously, a specific receptor docking mode for naltrexone and the selective antagonists derived from naltrexone has been proposed based on modeling of the

opioid receptors and analysis of site-directed mutagenesis studies.[18] A key feature of this docking mode is that the naltrexone-derived moiety binds within the transmembrane region in a similar fashion in all three receptor types. With the naltrexone-derived pharmacophore docked into the transmembrane pocket, the 'address' moieties of the selective ligands extend into a region between the top of TM helices VI and VII for all three receptor types.

Furthermore, the modeling studies have suggested that specific residues at the top of TM VI and VII may form key interactions with the 'address' moieties of the selective ligands.[18] The mutant receptors generated in this study have focused on the two positions (TM VII:03 and TM VI:26) that were believed to be interacting with the 'address' moieties of NTI and norBNI, based on the docking modes proposed for these ligands. The goal of the present mutagenesis was to produce mutations that would enhance the affinity of selective ligands for their nonpreferred types. At the same time, it would be expected that the binding mode of naltrexone would be essentially unchanged at mutant receptors since, according to the model, it does not possess an 'address' that is proposed to interact with the positions that have been mutated.

Two principal mechanisms acting in concert contribute to the selectivity at opioid receptors. The first involves an attraction of the 'address' moiety of the ligand by a complementary residue or group of residues to afford high affinity at the target receptor. The second mechanism is one of exclusion wherein steric hindrance of the ligand at the address recognition locus prevents high affinity to nontarget receptors.[19] The result of such a combination of exclusion and attraction plays a key role in conferring the selectivity to ligands such as NTI and norBNI. The results obtained in this study enable a qualitative assessment of how enhanced affinity and exclusion mechanisms contribute to the selectivity of a class of naltrexone-derived opioid antagonists.

**Selectivity of NTI and SIOM.** Ligand structure–activity relationships (SAR) suggest that when the indolic benzene moiety is placed on an opiate pharmacophore, it enhances affinity for the $\delta$ receptor while inhibiting binding to $\mu$ and $\kappa$ receptors.[20] This is apparent in the relative affinities of naltrexone and NTI for the three opioid receptor types (Table 1). Similarly, addition of a $\delta$ address onto oxymorphone to afford SIOM (Figure 2) causes an increase in affinity at the $\delta$ receptor and a reduction in affinity at non-$\delta$ receptors.

Mutations in both the $\mu$ and $\kappa$ receptors at TM VII:03 involve the replacement of a bulky aromatic group (Trp318 in $\mu$ and Tyr312 in $\kappa$) with alanine. Both NTI and SIOM showed substantially improved affinities at $\mu$[W318A] compared to the $\mu$ wild-type receptor. Similarly, removal of the tyrosine (Y312) from TM VII:03 in the $\kappa$ receptor ($\kappa$[Y312A]) gave enhanced affinity for both selective compounds. These results are consistent with the hypothesis that the presence of the large aromatic side chain at this position inhibits the docking of the 'address' portion of these ligands. These results emphasize the contribution of an exclusion mechanism in conferring the $\delta$ selectivity of both compounds.

That the 'address' recognition locus of the receptors is sensitive to the mutational changes is supported by the fact that only minor changes in affinity were observed for naltrexone and oxymorphone which do not project into the 'address' recognition locus. In this regard, naltrexone and oxymorphone serve as ligand structural controls, since they possess all key elements of the selective ligands with the exception of the 'address' component. Since the affinities of naltrexone and oxymorphone were relatively unchanged (Table 1), it appears that the 'address' moieties of the selective ligands are primarily responsible for the observed affinity changes.

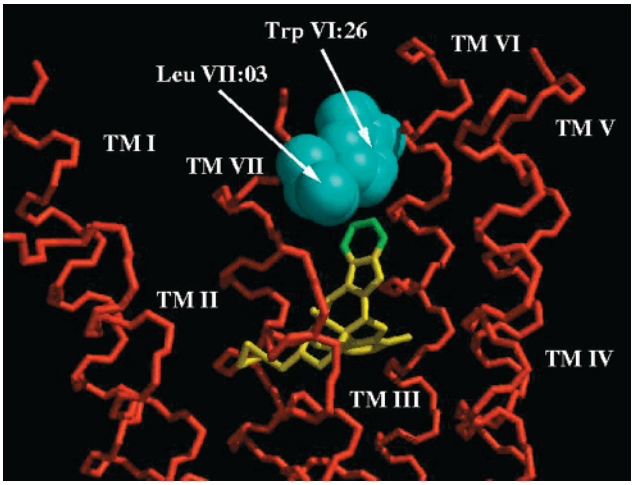


**Figure 3.** NTI docked into a $\delta$ opioid receptor model. The indolic benzene moiety or 'address' is shown in green. Backbone atoms of the transmembrane α-helices are shown in orange. Space-filling representations for the side chain atoms of Leu VII:03 and Trp VI:26 are shown in cyan.

Both NTI and SIOM show about a 10-fold reduction in affinity for the $\delta$ receptor mutant $\delta$[W284E]. It is possible that incorporation of a negative charge into this hydrophobic portion of the $\delta$ receptor causes unfavorable interactions with the 'address' of these ligands or disrupts the favorable interactions at the address recognition locus. In this regard, previous studies have shown that the presence of a tryptophan at TM VI:26 in the $\delta$ receptor plays a role in selective NTI binding.[16] The $\delta$[W284A] mutant receptor showed a 5-fold reduction in affinity for NTI. Since the decrease in NTI affinity was even greater (9-fold) for the $\delta$[W284E] mutant, it is apparent that the presence of the glutamate as well as the absence of the tryptophan are both responsible for the reduced affinity.

In summary, it is evident that both NTI and SIOM possess $\delta$ selectivity as a result of a combination two factors. First, inclusion of the indolic benzene affords high-affinity binding to $\delta$ receptor 'address' recognition locus. This is a clear case of enhanced affinity. In contrast, the presence of bulky aromatic groups at position TM VII:03 in the $\mu$ and $\kappa$ receptors prevents docking of the 'address' at these receptors and thereby augments $\delta$ selectivity. The reduced steric bulk in the $\mu$[W318A] and $\kappa$[Y312A]mutants contributes to reducing nonbonded interactions with the indolic benzene moiety of NTI and results in enhanced binding affinity at these receptors. A representation of this concept is shown in Figure 3 which illustrates the naltrexone-derived pharmacophore of NTI is bound within the transmembrane region and out of contact with residues at positions VI:26 and VII:03, while the indolic 'address' projects

directly into them. When Leu300 (TM VII:03) of the $\delta$ receptor is replaced with a large aromatic group, the 'address' of NTI encounters unfavorable steric interactions as in the case of the wild-type $\mu$ and $\kappa$ receptors.

**Selectivity of NorBNI and GNTI.** The molecular basis of selectivity for $\kappa$-selective antagonists norBNI and GNTI for $\kappa$ receptors has been investigated extensively.[11,12,15,17] In the case of norBNI, several studies have highlighted the importance of Glu297 (position VI:26) in enabling the $\kappa$ selectivity of this antagonist.[12,17] Replacement of the acidic side chain Glu297 with a basic lysine (E297K) was shown to cause a substantial reduction in norBNI affinity for the $\kappa$ receptor. This work was extended in another report where it was shown that GNTI, as well as norBNI, displayed $\kappa$-like affinity for the $\mu$ receptor when a glutamate residue was incorporated into the top of TM VI at position VI:26 of the $\mu$ receptor.[15] In the present report, we have further extended the investigation of the basis of selectivity by incorporating a glutamate residue in position VI:26 of the $\delta$ receptor as well as the $\mu$ receptor. In addition, a $\kappa$ receptor mutant was generated (E297A) wherein the glutamate side chain was replaced by an alanine.

In the case of the $\mu$ receptor, incorporation of a glutamate into position VI:26 causes a dramatic enhancement of affinity for both norBNI and GNTI (Table 1). These results are consistent with those seen previously.[15] When a glutamate is introduced at the same position of the $\delta$ receptor, affinities for both norBNI and GNTI are enhanced relative to that for the $\delta$ wild-type. However, the magnitude of the enhancement for both compounds is significantly less than that observed for the corresponding mutant $\mu$ receptor. Also, the level of enhancement for GNTI (42-fold) is notably greater than that seen for norBNI (8-fold) at the $\delta$ mutant (W284E). The affinity of norBNI for the $\delta$ receptor may be limited by the fact that it is derived from naltrexone, which has relatively lower affinity at the $\delta$ receptor. In contrast, GNTI is derived from the $\delta$-selective NTI and thus is able to achieve a stronger increase in affinity when the appropriate 'address' recognition epitope is incorporated into the $\delta$ receptor.

The role of the glutamate in position VI:26 of the $\kappa$ receptor was further studied by its replacement with an alanine residue ($\kappa$[E297A]). In general, the mutation had little effect on the nonselective and $\delta$-selective ligands. The effect of removal of the glutamate on norBNI binding was surprisingly small (6-fold reduction in affinity) while somewhat greater for GNTI (22-fold). Reductions in affinity at this mutant receptor have been reported previously.[17] As in the case of the $\delta$ receptor, it appears that GNTI is more sensitive to the presence (or absence) of a negative charge at this position, in that GNTI showed a greater enhancement of affinity for $\delta$[W284E] and a greater decrease in affinity at $\kappa$[E297A] when compared with norBNI. This could be the result of a greater degree of hydrogen bonding in the case of GNTI, which contains a guanidinium group, relative to that of the protonated N17′ group of norBNI.

The enhanced affinity of $\kappa$-selective antagonists for $\mu$[W318A] was somewhat surprising. Relative to the wild-type $\mu$ receptor, norBNI displayed a greater than 100-fold enhancement in affinity for $\mu$[W318A] while GNTI showed a significant but smaller increase in affinity. This result is particularly interesting since the 'address' of both norBNI and GNTI is positively charged and its interaction with Glu297 (TM VI:26) has been shown to be important for selective binding. The results with $\mu$[W318A] show that these antagonists can tolerate the presence of a lysine (potentially positively charged) at position VI:26 (in the wild-type $\mu$ receptor) while still displaying relatively high affinity. The increased space at the top of TM VII created by the removal of the tryptophan would enable greater steric freedom for the protonated N17′ address of norBNI to avoid electrostatic repulsion by the $\epsilon$-ammonium group of lysine. This steric freedom could be especially important in the case of norBNI which has its N17′ 'address' conformationally immobilized.

## Conclusions

The results presented in this study have revealed that the interactions between the 'address' of selective ligands and the address recogntion locus of the three types of opioid receptors give rise to selectivity through the interplay of two factors. The first is the enhanced affinity of the 'address' moiety for a particular subsite on its preferred receptor type. The second is a loss of affinity due to exclusion of the ligand from the binding pocket residues in the region of the address recognition locus of nonpreferred receptor types. In this light, the $\delta$ selectivity of NTI is the net result of enhanced affinity of the 'address' for the $\delta$ receptor and reduced affinity for $\mu$ and $\kappa$ receptors. This reduced affinity at $\mu$ and $\kappa$ receptors can be attributed to the presence of a bulky aromatic amino acid at position VII:03 of these receptors. The case of the $\kappa$-selective antagonists, norBNI and GNTI, is similar in its sensitivity to mutations at positions VII:03 and VI:26. On the other hand, the selectivity of norBNI and GNTI appeared to be dominated by favorable interactions of their cationic 'address' moieties with a glutamate at position VI:26. Nevertheless, norBNI appears to be less sensitive to the presence of glutamate at TM VI:26 than GNTI and this glutamate does not appear to be essential for norBNI binding when steric constraints within the 'address' recognition locus of the $\mu$ receptor are reduced by replacement of tryptopan with alanine (W318A).

Overall, the results support a simple and coherent model for the docking orientation of naltrexone and its derivatives into the opioid receptors. The central hypothesis of this model is that recognition of the naltrexone moiety, both by itself and as a component of a selective ligand, is similar in all three receptor types. The fact that selective ligands can be made to bind all three receptor types when small changes are made supports this hypothesis. This model should be useful in selective ligand design. If the hypothesis that naltrexone binds in a similar orientation in all three receptor types is correct, then the location in the receptor of synthetic additions to the naltrexone scaffold can be predicted. Indeed, the experiments performed in this study were suggested by previous modeling that was based on this concept. This combination of molecular modeling and site-directed mutagenesis can be used to inform ongoing ligand design efforts.

## Experimental Section

**Numbering of Residues.** Residues are numbered according to their position in the primary amino acid sequence for a given receptor type. Mutants are identified as (wild-type residue)(residue number)(substituted residue). Specific residues are also identified by their aligned position in a generic GPCR alignment where the Roman numeral indicates the transmembrane region and the Arabic numeral denotes the position from the N-terminal end of the helix.[22] For example, $\mu$ residue 303, $\delta$ residue 284, and $\kappa$ residue 297 are all located at position VI:26.

**Ligands.** The nonselective opioid antagonist [$^3$H]diprenorphine (specific activity, 21 Ci/mmol) was supplied by NIDA Drug Supply Program. Naltrexone and oxymorphone were obtained from Mallinckrodt. NTI, norBNI, SIOM, and GNTI were synthesized in our laboratory.

**Site-Directed Mutagenesis.** Rat $\kappa$, $\mu$, or mouse $\delta$ opioid receptor cDNA was subcloned into pcDNA3 (Invitrogen).

Point mutations were introduced in the opioid receptor genes by polymerase chain reaction (QuikChange site-directed mutagenesis kit, Stratagene). Primers were designed to incorporate a particular restriction site that can be used for screening of mutant DNA. Mutations were then confirmed by DNA sequencing.

**Transient Transfection.** HEK-293 cells in DMEM (Gibco, BRL) supplemented with 10% bovine calf serum (Hyclone) and 1% penicillin/streptomycin (Gibco, BRL) were maintained at 37 °C and in 5% $CO_2$. Cells were seeded at 16% for 24 h prior to transfection. Fresh medium was added 2 h prior to transfection. Cells were transfected with plasmid DNA (20 $\mu$g/100 mm plate) of either wild-type or mutant receptor cDNA using the calcium phosphate precipitation method.[23] Medium was changed 5 h after transfection. Transfected cells were harvested 60−72 h after tranfection for binding studies.

**Receptor Binding Assays.** Sixty to 72 h after transfection, HEK cells were washed three times with 25 mM HEPES buffer (pH 7.4) and resuspended with 8−12 mL of 25 mM HEPES/100 mm plate. Saturation binding assays were determined using [$^3$H]diprenorphine with concentrations ranging from 25 to 2000 pM. All displacement binding studies were carried out with 50−200 pM [$^3$H]diprenorphine depending on the $K_d$ value of the particular receptor. Nine concentrations of competing ligands were used in the [$^3$H]diprenorphine displacement analysis. All binding assays were performed in duplicate. Reported $K_i$ values represent an average of at least three independent determinations. Nonselective binding was determined in the presence of 1 $\mu$M naltrexone or 1 $\mu$M NTI in the case of the $\delta$ receptor. Assays were incubated at room temperature for 90 min in a total binding volume of 0.5 mL and were terminated by filtration through a Whatman GF/B filter that had been presoaked in 0.25% poly(ethylenimine) immediately prior to filtration. Filters were washed three times with 4 mL of ice-cold 25 mM HEPES buffer, and scintillation counting was performed with a Beckman 3801 LS scintillation counter. Protein concentrations were determined by the method of Bradford.[24] Raw binding data was analyzed with RADLIG and LIGAND (G. A. McPherson, Biosoft, Cambridge, U.K.). Inhibition constants ($K_i$) were detemined from $IC_{50}$ values with the Cheng−Prusoff equation.[25] In all cases, the data was fit to a simple one-site model and reported changes in affinity were based on parallel shifts in concentration curves.

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