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Interaction of *d*-tubocurarine analogs with mutant 5-HT_3 receptors

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Abstract

d-Tubocurarine is a potent competitive antagonist of both the muscle-type nicotinic acetylcholine receptor (AChR) and the serotonin type-3 receptor ($5HT_3R$). We have previously used a series of structural analogs of *d*-tubocurarine to demonstrate that the ligand-binding domains of both receptors share common structural features. We have now extended these studies to examine the interaction of a series of *d*-tubocurarine analogs with $5HT_3Rs$ containing mutations at either of two residues within the ligand-binding domain of the receptor (W90F and R92A). The W90F mutation results in an approximately 2–4-fold decrease in the affinity of the analogs relative to wild-type receptors, while the R92A results in an approximately 8–10-fold increase in affinity. However, since the effect of a given mutation is more or less equivalent for all analogs, neither residue W90 nor R92 is likely to make a specific interaction with *d*-tubocurarine itself. Rather, these two residues are likely to play a role in determining both the geometry of the binding site, as well as the overall environment that a ligand encounters in the binding site.

Keywords: 5-HT; 5-HT_3 receptor; *d*-tubocurarine analogs; Mutagenesis

1. Introduction

The serotonin type 3 receptor (5-HT_3R) is a member of the ligand-gated ion channel gene family, which includes the muscle and neuronal nicotinic acetylcholine receptors (AChR), the glycine receptor (GlyR), and the γ-aminobutyric acid type A ($GABA_AR$) receptor (Karlin and Akabas, 1995; Ortells and Lunt, 1995; Unwin, 1993). The receptor consists of two different subunits, termed 5-HT_{3A} and 5-HT_{3B} (Davies et al., 1999; Dubin et al., 1999; Hanna et al., 2000; Maricq et al., 1991). Expression of the 5-HT_{3A} subunit alone results in the appearance of 5-HT-gated ion channels with ligand specificity similar to that of native $5-HT_3Rs$, but with smaller-than-normal single-channel conductance than the native receptor. Expression of the 5-HT_{3B} subunits alone does not produce 5-HT-gated channels. However, co-expression of the 5-HT_{3A} and 5-HT_{3B} subunits results in expression of receptors with the pharmacological and ion permeation properties of native receptors (Davies et al., 1999; Dubin et al., 1999).

Like all members of the family, the $5HT_3R$ subunits exhibit a large degree of sequence similarity to the AChR (Davies et al., 1999; Dubin et al., 1999; Hanna et al., 2000; Maricq et al., 1991). Chimeras consisting of the *N*-terminal domain of the α7 neuronal AChR and the *C*-terminal region of the 5-HT_{3A} subunit form functional ligand-gated ion channels with pharmacological specificity of the AChR and permeability properties of receptors composed of 5-HT_{3A} subunits (Eisele et al., 1993), suggesting that the two receptors also have quite similar structures and signal transduction properties.

A large number of chemical labeling and mutagenesis studies have been carried out to determine the structural features of the ligand-binding site of the AChR , and details concerning the structure of the site(s) for agonists and competitive antagonists are beginning to appear (for reviews see Arias, 1997; Corringer et al., 2000; Karlin, 2002). On the other hand, far less is known about the structure of the ligand-binding domain of the 5-HT_3R (for a review see Reeves and Lummis, 2002). Over the past few years, we have used information obtained from the (relatively) well-studied AChR to guide structure–function studies on the $5HT_3R$. One ligand that the two receptors have in common is the competitive antagonist *d*-tubocurarine, which has nanomolar affinity for mouse

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(Papineni and Pedersen, 1997) and *Torpedo* (Pedersen and Cohen, 1990) nicotinic ACh receptors. While *d*-tubocurarine has micromolar affinity for human (Bufton et al., 1993) and guinea pig (Newberry et al., 1991) 5-HT_3Rs, it has nanomolar affinity for mouse and rat 5-HT_3Rs (Lummis et al., 1990; Newberry et al., 1991; Peters et al., 1990). Analysis of the interactions of a number of *d*-tubocurarine analogs with the AChR and homomeric 5-HT_{3A} receptors demonstrated that structure–activity relationships for the interaction of *d*-tubocurarine with the two types of receptors are more or less identical (Papineni and Pedersen, 1997; Pedersen and Papineni, 1995; Yan et al., 1998), suggesting that the structure of the ligand-binding sites of the two receptors are similar. In line with this finding, we subsequently showed that a tryptophan residue in the 5-HT_{3A} subunit (W90) that is homologous to tryptophans in the γ (γW55) and δ (δW57) subunits of the AChR known to be important for *d*-tubocurarine interaction with the AChR (Chiara and Cohen, 1997) is also important for *d*-tubocurarine interaction with the 5-HT_3R. Replacement of this residue with phenylalanine (W90F) resulted in receptors with a tenfold lower sensitivity to *d*-tubocurarine and other antagonists such as granisetron, but had no effect on the interaction of agonists with the receptor. In addition, replacement of a nearby residue (R92) by alanine (R92A) resulted in receptors with a reduced affinity for serotonin and granisetron, and an increased affinity for *d*-tubocurarine (Yan et al., 1999).

While these data demonstrate that the region around W90 is important for receptor–*d*-tubocurarine interactions, other regions of the receptor are also important for the interaction of *d*-tubocurarine with the 5-HT_3 receptor. Hope et al. (1999) have shown that mutations of amino acids in the 5-HT_{3A} subunit around position 225 also affect the affinity of *d*-tubocurarine for 5-HT_3Rs, indicating that multiple regions of the receptor are involved in the interaction of *d*-tubocurarine (and by extension, other ligands) with the receptor.

In this study, we examined the effects of these two mutations (W90F and R92A) on the interaction of a number of *d*-tubocurarine analogs with the receptor. We find that the analogs are similarly affected by the two mutations. The lack of a selective effect of either mutation on the interaction of the analogs with the receptor suggests that while these two residues do play an important role defining the environment of the ligand-binding site, neither is likely to make a specific interaction with *d*-tubocurarine itself.

2. Materials and methods

2.1. Molecular biology and transfection

A full-length cDNA clone corresponding to the 5-$HT_{3A(b)}$ form (Hope et al., 1993) of the receptor was isolated from a neuroblastoma N1E-115 cell line cDNA library as previously described (Yan et al., 1999) and subcloned into vector pCI (Promega, Madison, WI). Site-directed mutagenesis was carried out using the QuickChange system (Stratagene, La Jolla, CA) as described previously (Yan et al., 1999). The nomenclature used to describe mutants is amino acid in wild-type/position/substitution; e.g., W90F. Because the amino terminus of the mature 5-HT_{3A} subunit is unknown, the amino acid numbering system here includes the signal sequence and has the initial methionine as position 1. Please note that the numbering system for the 5-HT_{3A} receptor used in a previous study from this laboratory has changed somewhat so that the W90F and R92A mutations in this study correspond to the W89F and R91A mutations in the original study (Yan et al., 1999), respectively. Cultures of tsA201 cells, a derivative of the widely used HEK 293 cell line, were maintained in DMEM medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin. Cultures at 30–40% confluence were transfected with 20 μg receptor cDNA per 100 mm dish using the calcium phosphate technique (Wigler et al., 1979). After 12 h exposure to the DNA/calcium phosphate solution, the medium was replaced with fresh medium and the cells were allowed to grow for another 24–36 h prior to use. Maximal expression was obtained 36–72 h after transfection.

2.2. Ligand binding assays

Transfected cells were scraped from dishes, washed once with phosphate-buffered saline and resuspended and homogenized in 2.5 ml 154 mM NaCl, 50 mM Tris-HCl, pH 7.4 per 100 mm dish. The homogenate was then used in binding assays or frozen until needed. We observed no change in either ligand affinity or B*max* values after freezing.

Membranes were incubated for 2 h at 37°C in a total volume of 0.5 ml 154 mM NaCl, 50 mM TrisHCl, pH 7.4 containing the appropriate concentrations of antagonist and radioligand ([^{3}H] granisetron; New England Nuclear, 85 Ci/mmol). Binding was terminated by rapid vacuum filtration onto GF/B filters that had been pretreated with 50 mM TrisHCl, pH 7.4, 0.2% polyethyleneimine, and the filters were washed with 10 ml cold 50 mM TrisHCl, pH 7.4 per sample. Nonspecific binding was defined as that binding not displaced by 100 μM *m*-chlorophenyl biguanide. Each data point in the figures represents the mean ± SEM of 3 determinations. IC_{50} values for various antagonists were determined by fitting the data to Eq. (1) using a Levenberg–Marquardt algorithm in a commercially available software package for Macintosh computers (Igor Pro, WaveMetrics, Oswego, OR):

$$\theta = (1 + ([I]/IC_{50})^n)^{-1} \quad (1)$$

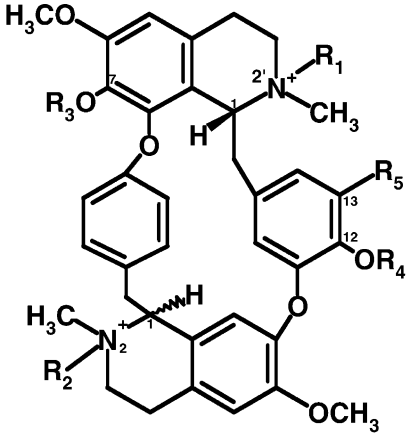
where θ is the fractional amount of [^{3}H] granisetron bound in the presence of the antagonist at concentration [I] compared to that in the absence of antagonist, IC_{50} is the concentration of antagonist at which $\theta = 0.5$, and n is the apparent Hill coefficient. K_i values were calculated from the IC_{50} values and the K_d for [^{3}H] granisetron using the Cheng–Prusoff relation (Cheng and Prusoff, 1973) (Eq. (2).):

$$K_i = \frac{IC_{50}}{1 + ([L]/K_d)} \quad (2)$$

where [L] is the concentration of [^{3}H] granisetron used to determine the IC_{50} value in the experiment and K*d* is the dissociation constant for [^{3}H] granisetron. For the Cheng–Prusoff relation to be applicable, the Hill coefficient for the IC_{50} curve must be equal to 1. In our experiments, all Hill coefficients were not statistically different from unity at a 95% confidence level (data not shown). In this study, all experiments were carried out with a [^{3}H] granisetron concentration equal to its experimentally determined dissociation constant for the particular receptor (WT: 1.5 nM; W90F: 11 nM; R92A: 7.5 nM (Yan et al., 1999)), meaning that the IC_{50} values were twice the K_i.

2.3. d-Tubocurarine analogs

The structures of the *d*-tubocurarine analogs used in this study are shown in Fig. 1. Two of the compounds were obtained commercially: *d*-tubocurarine (Sigma, St. Louis, MO) and metocurine (Diosynth, Inc., Chicago, IL) and the others were obtained from Dr. Steen Pedersen of Baylor University (Papineni and Pedersen, 1997; Pedersen and Papineni, 1995). Purity of all compounds was checked by HPLC both before use and after prolonged incubation with the assay buffers.



R_1	R_2	R_3	R_4	R_5	Stereo Isomer 1	1'	NAME
CH_3	H	H	H	H	S	R	*d*-Tubocurarine
H	H	H	H	H	S	R	Tubocurine
CH_3	CH_3	H	H	H	S	R	Chondocurarine
H	H	CH_3	CH_3	H	S	R	O,O-dimethyl-tubocurine
CH_3	CH_3	CH_3	H	H	S	R	7'-O-methyl-chondocurarine
CH_3	CH_3	H	CH_3	H	S	R	12'-O-methyl-chondocurarine
CH_3	CH_3	CH_3	CH_3	H	S	R	Metocurine
CH_3	H	H	H	I	S	R	Iodo-*d*-tubocurarine
CH_3	H	H	H	Br	S	R	Bromo-*d*-tubocurarine
CH_3	H	H	H	SO_3^-	S	R	Sulfo-*d*-tubocurarine

Fig. 1. Structures of the *d*-tubocurarine analogs used in this study.

3. Results

Previously, we had examined the interaction of a series of *d*-tubocurarine analogs (Fig. 1) with wild-type $5HT_3$Rs and showed that the structure–activity relationships for the interactions of these analogs for the $5HT_3$R were similar to those for the AChR (Yan et al., 1998). In this report, we have extended these studies to include receptors with mutations at two positions which are located in one of the portions of the $5HT_3$R ligand binding site, W90F and R92A (Yan et al., 1999). The affinities of *d*-tubocurarine and the series of analogs for wild-type, W90F, and R92A receptors were determined by competitive inhibition of [^{3}H] granisetron binding (Table 1). Fig. 2 shows inhibition data for *d*-tubocurarine for the three receptor types. It can be seen that the W90F mutation increases the IC_{50} for *d*-tubocurarine relative to wild-type, while the R92A mutation decreases the IC_{50} relative to wild-type.

The effects of these two mutations on the interaction of *d*-tubocurarine with the receptor are seen for the other analogs as well. Fig. 3 shows the effects of the mutations on the interaction of sulfo-*d*-tubocurarine, which contains a sulfonate group at position 13', with the receptors. Although the affinity of this analog with the wild-type receptor is approximately 100-fold lower than that of *d*-tubocurarine, the two mutations have the same relative effect on affinity — W90F decreases affinity, while R92A increases the affinity.

Fig. 4 shows the pK_i values for all ten compounds (*d*-tubocurarine and nine analogs) for the mutant receptors plotted against their affinities for the wild-type receptor. As is the case for the interaction of the analogs with wild-type receptors (Yan et al., 1998), the affinities for the mutants span an approximately 100-fold concentration range (60–180-fold, depending on the receptor). In addition, the effect of the mutations on each analog are more or less the same; e.g., the analogs exhibit an increased affinity for the R92A receptor relative to wild-type receptors, while the analogs have reduced affinity for the W90F receptors. Finally, the rank order of affinities of the compounds for the two mutant receptors are similar to each other, as well as to that observed for wild-type receptors, suggesting that the structure–activity relationships are identical for all three receptors. This information can provide some insight into the roles that W90 and/or R92 may play in the interaction of *d*-

Table 1
Affinity of *d*-tubocurarine analogs towards 5-HT_3Rs

Compound	WT pK ± S.D.	W90F pK_i ± S.D.	R92A pK_i ± S.D.
d-tubocurarine	6.57 ± 0.02	6.05 ± 0.04∗	7.41 ± 0.11∗
metocurine	4.93 ± 0.02	5.42 ± 0.04	5.87 ± 0.10#
tubocurine	6.67 ± 0.02	6.02 ± 0.03∗	7.69 ± 0.07∗
chondocurarine	6.34 ± 0.02	6.03 ± 0.02∗	7.00 ± 0.02∗
O,O-DMTC	5.33 ± 0.05	5.07 ± 0.05∗	6.33 ± 0.04∗
7'-OMCC	6.73 ± 0.04	6.42 ± 0.05∗	6.96 ± 0.17
12'-OMCC	4.96 ± 0.02	5.06 ± 0.04	6.36 ± 0.06∗
iodo–*d*-tubocurarine	5.74 ± 0.01	5.37 ± 0.05∗	6.89 ± 0.05∗
bromo–*d*-tubocurarine	5.76 ± 0.01	5.41 ± 0.02∗	7.43 ± 0.07∗
sulfo–*d*-tubocurarine	4.75 ± 0.02	4.13 ± 0.08∗	6.04 ± 0.07∗

Estimates of pK_i values were calculated from experimentally determined pIC_{50} values for the inhibition of [^{3}H] granisetron binding to wild-type or mutant receptors as described in the Methods section. Errors represent the error determined by the Levenberg-Marquardt regression routine used in the fitting. Values for the mutant receptors marked with ∗ are statistically different from wild-type at a 95% confidence level using Student's *t* test, while those marked with # are statistically different from wild-type at a 90% confidence level.

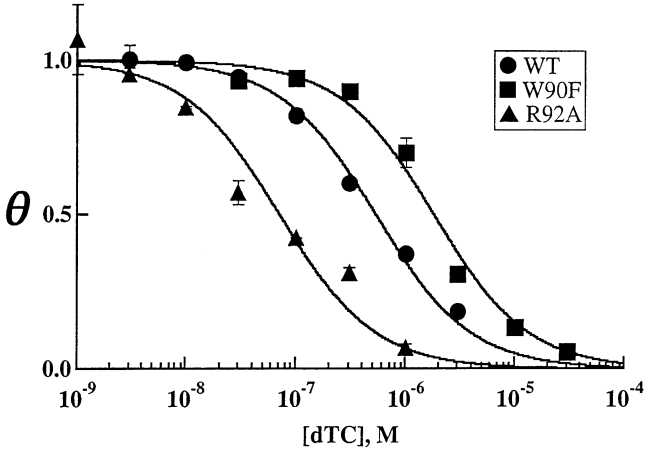


Fig. 2. The W90F and R92A mutations have different affects on *d*-tubocurarine affinity. The concentration dependences of inhibition of [^{3}H] granisetron binding by *d*-tubocurarine to wild-type (●), W90F (■), and R92A (▲) homomeric 5HT_3Rs are shown. The solid curves are drawn according to Eq. (1) with IC_{50} values of 530 nM (wild-type), 1800 nM (W90F), and 71 nM (R92A). Note that the W90F mutation decreases *d*-tubocurarine affinity, while the R92A mutation increases affinity.

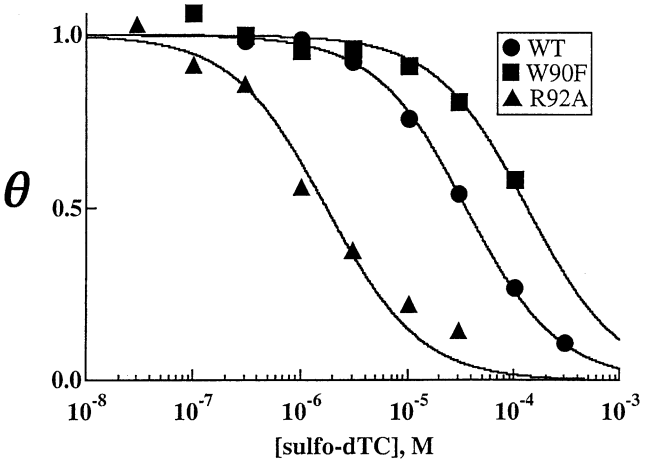


Fig. 3. Mutations have effects on a charged analog similar to that of *d*-tubocurarine. The concentration dependences of inhibition of [^{3}H] granisetron binding by the negatively charge sulfo–*d*-tubocurarine to wild-type (●), W90F (■), and R92A (▲) homomeric 5HT_3Rs are shown. The solid curves are drawn according to Eq. (1) with IC_{50} values of 35,290 nM (wild-type), 132,900 nM (W90F), and 1750 nM (R92A). Note that once again, the W90F mutation reduces the affinity of the competitor, while the R92A mutation increases it.

tubocurarine (and perhaps other ligands) with the 5HT_3R.

4. Discussion

We have measured the affinity of *d*-tubocurarine and a number of analogs for two mutant homomeric 5HT_3Rs in order to examine the types of interactions that these competitive antagonists make with the ligand-binding site of the receptor. In a previous study, we showed that the structure–activity relationship for the interaction of these antagonists with the wild-type 5HT_3R were similar to that for muscle-type nicotinic acetylcholine receptors (Yan et al., 1998), strongly suggesting that the ligand-binding sites of both receptors share common structural features. Subsequent to that, we showed that mutation of a tryptophan in the 5HT_3R (W90) that is homologous to tryptophans in the muscle-type AChR that are known to interact with *d*-tubocurarine (γW55, δW57) reduced the affinity of *d*-tubocurarine and a number of antagonists for the 5HT_3R (Yan et al., 1999). In support of the idea that W90 plays a role in ligand–receptor interactions, Spier and Lummis (Spier and Lummis, 2000) have shown that replacement of W90 by serine (W90S) does not interfere with surface expression of the receptor, but completely abolishes both [^{3}H]granisetron and [^{3}H] *m*-chlorophenylbiguanide binding.

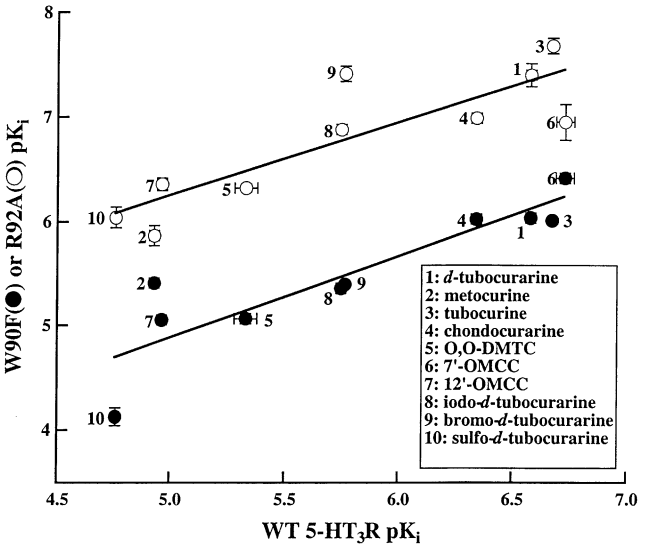


Fig. 4. Comparison of the affinities of the *d*-tubocurarine analogs for wild-type and mutant $5HT_3$Rs. pK_i values for the analogs interacting with the W90F (●) and the R92A (○) mutants (Table 1) were compared with those for the wild-type receptor. The solid lines represent linear fits to the data. The r^2 values for the fits were 0.79 and 0.74 for W90F vs wild-type, and R92A vs wild-type, respectively.

In this study, we have extended these studies to include analysis of the interaction of the analogs with two mutant receptors that have been shown to have altered affinity for *d*-tubocurarine. One of these mutations (W90F) reduces the affinity for *d*-tubocurarine, while the other (R92A) increases the affinity. These two residues are found in a region of the $5HT_3$R that we have shown forms a β-strand structure (Yan et al., 1999), and is homologous to the "Loop D" region of the AChR binding site (Corringer et al., 2000). In support of the notion that this region plays a role in ligand–receptor interaction, the recent determination of the structure of the acetylcholine binding protein (AChBP) from *Lymnea* (Brejc et al., 2001) demonstrates that the homologous region in the AChBP is in a β-strand conformation and forms part of the so-called "complementary face" of the ligand-binding domain. Thus, mutations at these sites might be expected to affect ligand–receptor interactions.

Both mutations affect the interaction of *d*-tubocurarine and the analogs with the receptor, but in different fashions. The W90F mutation, which reduces the affinity for *d*-tubocurarine and other antagonists (Yan et al., 1999), reduces the affinity of most of the analogs to a similar extent. The fact that the analogs are affected to more or less the same extent suggests that while this residue may play a role in determining the environment of the ligand-binding site, it probably does not make a specific interaction with *d*-tubocurarine at the 2, 2', 7', 12', or 13'positions of the molecule (the positions that are modified in the analogs). If W90 did make specific interactions with one of these portions of the molecule, then one might expect to see a differential effect of the W90F mutation on the affinities of one or more analogs. For example, if W90F interacted directly with position 13'on *d*-tubocurarine, one might expect to see a differential effect of the mutation on the affinities of those analogs modified at that position (iodo–*d*-tubocurarine, bromo–*d*-tubocurarine, sulfo–*d*-tubocurarine) relative to analogs modified at other positions. Instead, we observe more or less the same effect: a 2–4-fold reduction in affinity for each analog relative to wild-type receptors. This suggests strongly that W90 does not make a specific interaction with at least those portions of the *d*-tubocurarine molecule that have been modified.

Two of the analogs (metocurine and 12'*O*-methyl-chondrocurine) show a slight increase in affinity towards the W90F receptors rather than the decrease observed for the other analogs. Both analogs are methylated at the 2 and12' positions, which might suggest that there may be a specific interaction between the 2 and/or 12' portion of *d*-tubocurarine and W90. However, other analogs have the same substitutions (2 position: chondocurine and 7'-*O*-methyl-chondocurine; 12' position: *O,O*-dimethyl-tubocurine) but do not show an increase in affinity. This suggests that substitutions at positions 2 and/or 12' are not responsible for the observed difference between the effects of the W90 mutation on the interaction of metocurine and 12'-*O*-methyl-chondocurine with the receptor compared to the other analogs. While we are unsure as to why these two analogs behave differently with respect to the effect of the W90F mutation, our original conclusion that W90 does not make a specific interaction with at least those portions of the *d*-tubocurarine molecule that have been modified is probably correct.

Although the R92A mutation results in an increase in affinity for *d*-tubocurarine and the other analogs relative to wild-type, the fact that once again all analogs are affected more or less to the same extent relative to wild-type receptors indicates that R92 also does not make specific high-affinity interactions with the substituents at the 2, 2', 7', 12', or 13' positions of *d*-tubocurarine. The increase in affinity could be due to either the removal of the positive charge of the arginine side chain and/or a steric effect due to replacement of the bulky guanidinium side chain (volume = 140.5 Å^3) of arginine with the smaller methyl group side chain (volume=28.7 Å^3) of the alanine (Zamyatnin, 1984). If the presence of a positive charge in the binding site created an environment that reduced the affinity of *d*-tubocurarine, then substitution of alanine would remove the polarity/electrostatic effect and result in an increased affinity. Alternately, if the bulky arginine side chain affected the positioning of the large *d*-tubocurarine molecule in the binding site preventing optimal orientation within the ligand-binding site, then removal of the bulky side chain might allow a better fit of *d*-tubocurarine within the binding site. In

either case, the affinity would increase after the R92A mutation.

While the data at hand do not make it possible to unequivocally distinguish between an electrostatic versus steric mechanism for the increase in affinity, the evidence suggests that the increase in affinity is not likely due to an electrostatic effect. If the presence of a positive charge in the binding site might create an electrostatic repulsion between the guanidinium and one of the two charged nitrogens in *d*-tubocurarine, then removal of the positive charge in the binding site might be expected to increase the affinity of *d*-tubocurarine for the binding site. While this is observed, the fact that the affinities for two other ligands (serotonin and granisetron) which also contain "equivalent" positively charged nitrogens (from a pharmacophore standpoint (Hibert et al., 1990)) are decreased by removal of the guanidinium side chain (Yan et al., 1999), opposite to what might be expected. In addition, sulfo–*d*-tubocurarine is negatively charged at position 13', and one might expect that if the positive charge on R92 were important in *d*-tubocurarine–receptor interaction, that (a) sulfo–*d*-tubocurarine would have a higher affinity than *d*-tubocurarine, and (b) removal of the positive charge through the R92A mutation would result in a decrease in the affinity of sulfo–*d*-tubocurarine. The fact that neither of these two are observed once again suggests that in the case of *d*-tubocurarine–receptor interactions, the positive charge of R92 does not play an important role in determining affinity, and that the increase in affinity seen after the R92A mutation is due to replacing a bulky side chain with a smaller one, allowing the large *d*-tubocurarine molecule to "fit" better in the ligand-binding site of the mutant receptor.

The data presented in this study suggest that neither W90 nor R92 (and by inference the rest of this particular loop of the ligand-binding site) appear to form specific interactions with *d*-tubocurarine. In support of this, examination of the high-resolution structure of the AChBP (Brejc et al., 2001) shows that HEPES (which co-crystallized in the ligand-binding site and makes the cation-π interactions with aromatic residues in the binding site expected of AChR ligands) does not appear to make any close contacts with residues in the β-strand containing the homologous tryptophan residue (W B53 in the AChBP structure). Rather, this particular region of the protein appears to form part of the bottom and side of the cleft of the binding site cavity in the AChBP. Given the great deal of sequence homology between the $5HT_3R$ and the AChBP, the two binding sites probably have similar geometries, and mutations in this region could affect ligand binding by altering the walls of the cavity, thus affecting how the ligand fits within the binding site. Expansion of this type of study to mutations in other portions of the putative binding domain and other ligands can help to define the actual interactions that a ligand makes with the ligand-binding site and thus provide useful information for the design of potent and selective pharmacological agents.

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