

A Perturbed pK_a at the Binding Site of the Nicotinic Acetylcholine Receptor: Implications for Nicotine Binding

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The nicotinic acetylcholine receptor (nAChR) is essential to synaptic transmission and is implicated in learning and memory, as well as a variety of neurological disorders including Alzheimer's disease, Parkinson's disease, and schizophrenia.¹ On binding acetylcholine (ACh) this integral membrane receptor undergoes a conformational change that permits cations to pass through a central pore, thereby converting a synaptic chemical signal to an electrical signal. The receptor comprises five subunits. The two ACh binding sites have been localized to the $\alpha\delta$ and $\alpha\gamma$ interfaces, on the basis of extensive biochemical studies¹ and the crystal structure of ACh binding protein (AChBP), a soluble protein homologous to the extracellular domain of the nAChR.²

We have previously used unnatural amino acid mutagenesis (nonsense suppression) techniques to incorporate tyrosine derivatives with tethered quaternary ammonium groups (quats; e.g., TyrO3Q, Figure 1) at the agonist binding site.^{3,4} These yielded constitutively active receptors (i.e. receptors that open in the absence of ACh), providing valuable geometrical information about the agonist binding site. Most agonists and antagonists of the nAChR (other than ACh), including nicotine, contain protonatable amines rather than quats, and it is generally assumed that the protonated, cationic species is the active form of the ligand. If so, one expects tethered amine analogues of TyrO3Q to produce pH-sensitive receptors, structures that are constitutively active only when the tethered group is protonated. We now report that the tethered amines TyrO3S and TyrO3T (Figure 1) indeed show a strong increase in constitutive activity at low pH. Titration of the amine side chain provides a probe of the local pK_a at the receptor binding site. We find that the phenomenological pK_a 's of these tethered amines are far lower than their values in free solution.

The syntheses of TyrO3P and TyrO3S in appropriate forms for nonsense suppression were straightforward. Both the α -N of the amino acid and the side-chain amine were protected as nitroveratryloxycarbonyl groups. The tertiary amine of TyrO3T cannot be protected as an amide. Interestingly, the *N*-nitroveratryl (NV) side-chain protecting group, which has been used successfully in many similar contexts, was not viable for TyrO3T because of inefficient photodeprotection of the quat group. Other studies with simpler model compounds confirm that nitrobenzyl-type photocleavage reactions are not efficient when converting a quaternary ammonium to a tertiary amine. We have found, however, that the dimethoxycoumarin (DMCm) group is an effective photocleavable protecting group for tertiary amines (see Supporting Information).

The tethered amine unnatural amino acids were incorporated into nAChR expressed in *Xenopus laevis* oocytes using now well-established protocols.⁵ Channel activity was monitored with standard two-electrode, voltage-clamp electrophysiology. Along with the

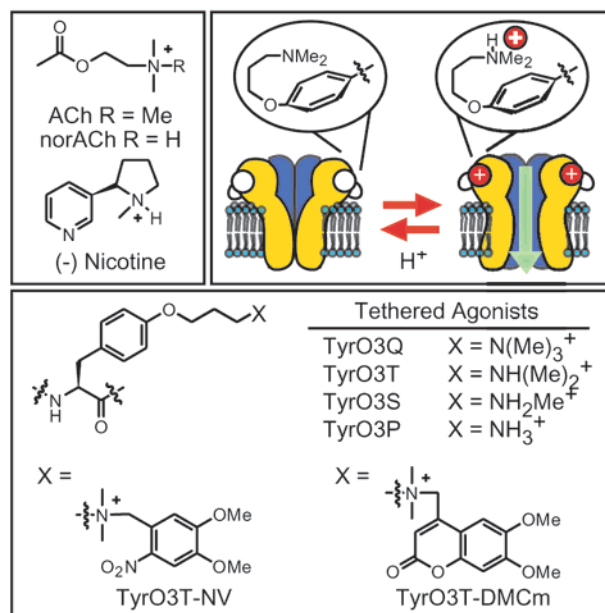


Figure 1. (Top left) ACh, norACh, and nicotine. (Top right) Incorporation of TyrO3T at $\alpha 149$ yields a receptor that is constitutively active at low pH. (Bottom) Tethered agonist unnatural amino acids.

constitutive (standing) current, responses to added ACh, known channel-blockers such as TMB-8 (8-(diethylamino)octyl trimethoxybenzoate) and QX-314 (lidocaine *N*-ethyl bromide), or agonist-free solutions of various pH were measured.

The response of the wild-type nAChR to saturating concentrations of ACh shows a small inherent dependence on pH, with conductance being maximal at pH 7.5 and falling at lower and higher pH values (see Supporting Information for examples of primary electrophysiological data).⁷ In sharp contrast, receptors that contain TyrO3T at position 149 of the α subunit, ($\alpha 149$) showed a systematic increase in constitutive current as the pH was lowered.⁶ These currents can be blocked by TMB-8, showing that they arise from the opening of nAChRs. In an important control, we find that the pH dependence of the constitutive current of the system with the tethered quat, TyrO3Q, mirrors that of wild-type receptor. These observations establish that the increase in blockable current observed for TyrO3T at lower pH is due to the specific protonation of the side-chain amine. TyrO3S constitutive currents also increase with decreasing pH. However, receptors containing TyrO3P showed no constitutive activity, even at pH 5.5. Perhaps the protonated TyrO3P lacks the steric bulk to activate the receptor. Note that attempts to incorporate Lys at $\alpha 149$ led to no surface nAChR expression; conventional mutagenesis does not permit these studies (see Supporting Information).

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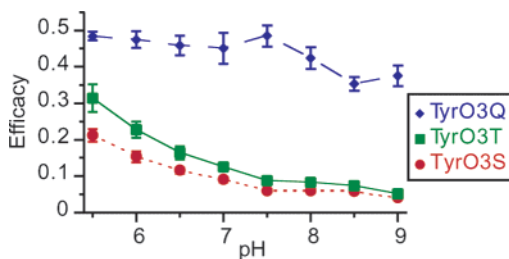


Figure 2. Tethered agonists at $\alpha 149$: tethered agonist efficacy as a function of solution pH. ACh efficacy = 1 at all pHs.

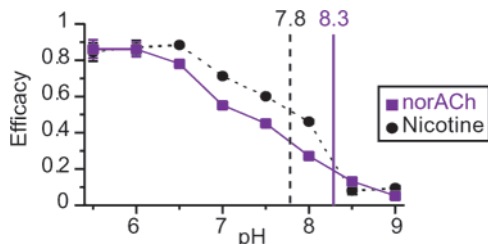


Figure 3. Tertiary agonists: norACh and nicotine efficacy as a function of solution pH. ACh efficacy = 1 at all pHs.

We define the *efficacy* of a tethered agonist as the ratio of the constitutive current that can be blocked by TMB-8 to the maximum current induced by saturating concentrations of ACh (corrected for basal conductance changes with pH). By considering only constitutive current that can be blocked, we eliminate any background (non-nAChR specific) leak current. The efficacies of TyrO3Q, TyrO3T, and TyrO3S at $\alpha 149$ are shown in Figure 2. TyrO3Q's efficacy, as expected, is insensitive to pH changes, when corrected for basal pH dependence. As described above, TyrO3T and TyrO3S exhibit strong titration behavior when incorporated at $\alpha 149$.

These adjusted measurements of receptor activation provide a straightforward way to assay the degree of protonation of a tethered amine at the binding site. Thus Figure 2 gives a phenomenological pK_a for the side chain. Since $pH \leq 5$ compromises the oocyte membrane integrity,⁸ we are unable to study the presumed plateau at low pH values. Nevertheless, it is clear that the side-chain pK_a of TyrO3T is ≤ 6 when incorporated at $\alpha 149$ of the nAChR, substantially shifted from its value in free solution (~ 9.3 , measured for *N,N*-dimethylaminopropanol).⁹ pK_a shifts of this magnitude are unprecedented. For example, Lys 115 in *Clostridium acetobutylicum* acetoacetate decarboxylase has an effective pK_a of 6.0, shifted by 4.5 units.¹⁰ Our results suggest that the agonist binding site of the nAChR is relatively hydrophobic, consistent with the fact that the binding site is primarily formed by aromatic residues.

Nicotine ($pK_a = 7.8$)¹² is, of course, a noted tertiary amine agonist of the nAChR, and we have measured the pH-dependent nicotine efficacy in the present context (Figure 3). Efficacy is computed for nontethered agonists by normalizing the current from applications of saturating agonist concentrations to the maximal ACh-induced current. Interestingly, there is no pK_a shift for nicotine; the phenomenological pK_a is not measurably different from the solution pK_a of the drug. In contrast, norACh ($pK_a = 8.3$),¹¹ the

closest possible protonatable analogue of ACh, shows a pK_a shift of ~ 1 unit, noticeable, although not as large as for the tethered amines.

These differences are interpreted as follows. We assume that only the cationic forms of agonists can activate the receptor. For the amine tethers and norACh, the protonatable amine can equilibrate with the medium when at the agonist binding site and the local microenvironment of the protein produces a pK_a shift. For nicotine, however, no pK_a shift is seen because the protonated amine does not equilibrate with the medium once it has bound to the receptor. The degree of receptor activation is then dependent only on the amount of protonated nicotine available to bind to the receptor, and the pH dependence of activation mirrors nicotine's normal pK_a .

This is the second recent line of evidence from our labs that indicates a difference between the binding modes of ACh and nicotine. We have recently shown that the potent cation- π interaction observed between ACh and $\alpha Trp149$ ³ is not evident for nicotine.¹³ Taken together, these data strongly suggest that pharmacophore models for the muscle-type nAChR should be expanded to include two distinct agonist binding modes: an ACh-like mode and a nicotine-like mode.

Acknowledgment. We thank Dr. Lintong Li for her help. This work was supported by the National Institutes of Health (NS-34407 and NS-11756).

Supporting Information Available: Procedures for the syntheses of the tethered agonists and their ¹H NMR, ¹³C NMR, and MS data; protocols for unnatural amino acid mutagenesis and oocyte electrophysiology have been published and are summarized; examples of primary electrophysiological data (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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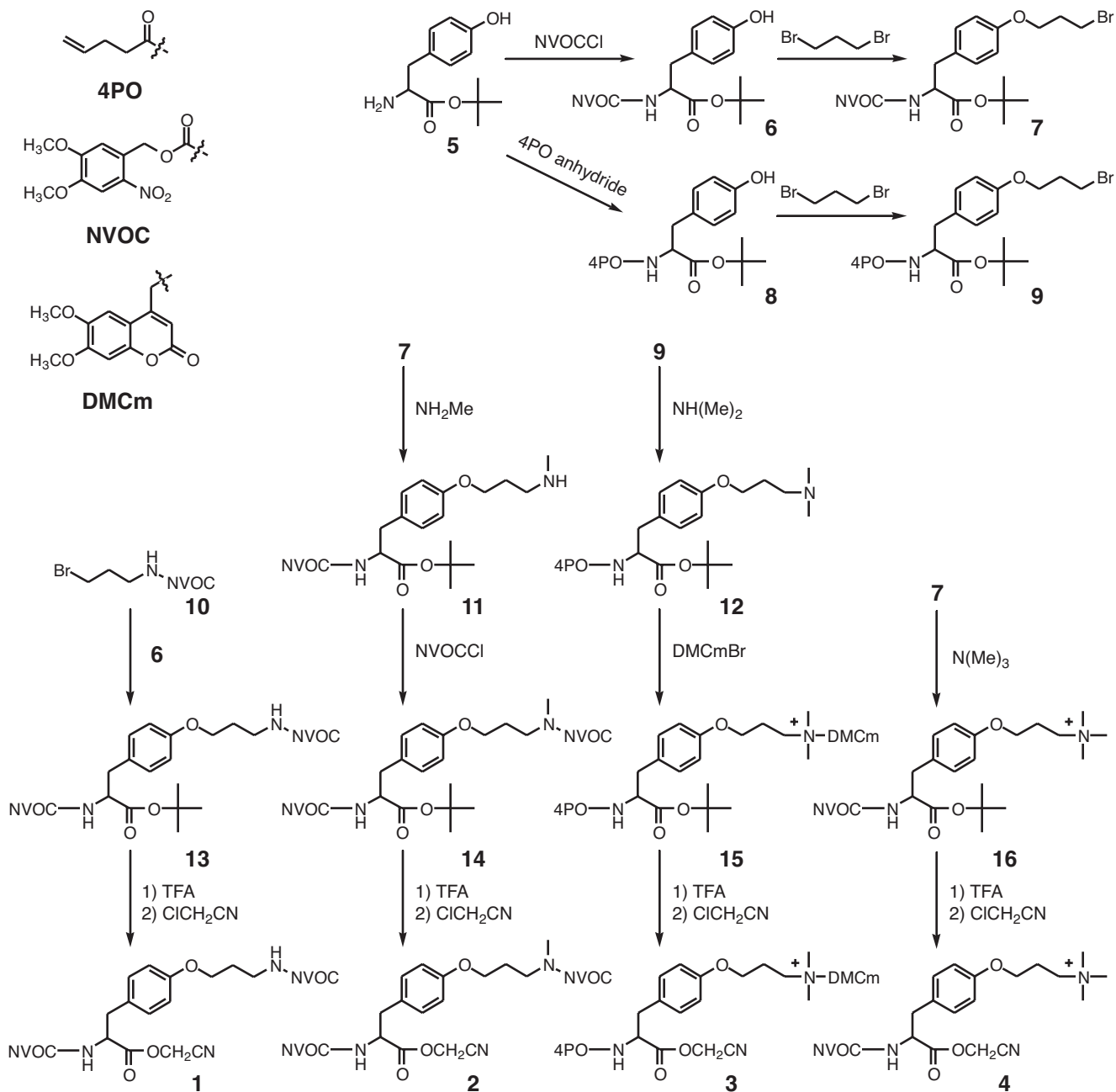
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Supporting Information for:
A Perturbed pK_a at the Binding Site of the Nicotinic Acetylcholine Receptor:
Implications for Nicotine Binding

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Scheme A. Syntheses of TyrO3P, TyrO3S, TyrO3T, and TyrO3Q precursors; compounds **1**, **2**, **3**, and **4** are prepared for coupling to dCA and subsequent enzymatic ligation to tRNA. The synthesis of **4** has been published previously.¹

Experimental Procedures

General. Reagents were purchased from Aldrich, Sigma, or other commercial sources. TMB-8 was purchased from RBI (Natick, MA). ACh chloride and QX-314 were purchased from Sigma. Anhydrous THF and methylene chloride were obtained from J. T. Baker solvent kegs; anhydrous DMF (Puris) was obtained from Fluka. Flash chromatography was on 230-400 mesh silica gel with the solvent indicated. All NMR shifts are reported as δ ppm downfield from TMS. ^1H NMR and ^{13}C NMR spectra were recorded at 300 MHz in CDCl_3 or CD_3CN using a Varian QE-300 spectrometer. Electrospray (ESI) ionization and matrix-assisted laser-desorbed ionization (MALDI) quadrupole mass spectrometry was performed at the Caltech Protein/Peptide Micro Analytical Laboratory or at the Caltech Division of Chemistry and Chemical Engineering Mass Spectrometry Facility. Nitroveratryloxycarbonyl chloride (NVOCCl), NVOC-Tyrosine *t*-butyl ester (NVOC-Tyr-*Or*Bu, **6**), and NVOC-Tyrosine-*O*-propylbromide *t*-butyl ester (NVOC-TyrO3Br-*Or*Bu, **7**) were prepared as previously described.¹

N-NVOC-Bromopropylamine (10). 0.15 g 3-bromopropylamine were dissolved in 15 mL *p*-dioxane and 15 mL water. 0.11 g Na_2CO_3 were dissolved in 15 mL water. 3-bromopropylamine solution was combined with the Na_2CO_3 solution. 0.19 g NVOC-Cl were completely dissolved in 25 mL dioxane, and were added to the other mixture and stirred for 1 h. The reaction mixture was extracted with 3 X 25 mL CH_2Cl_2 , the organic layers were combined, dried with sodium sulfate, and run on a column in methylene chloride to give 0.25 g (37%) of product. ^1H NMR (CDCl_3): δ 7.66 (s, 1H), 6.97 (s, 1H), 5.46 (s, 2H), 5.16 (br s, 1H), 3.95 (s, 3H), 3.92 (s, 3H), 3.43 (t, J=6.3 Hz, 2H), 3.36 (td, J=6.3 Hz, 6.3 Hz, 2H), 2.07 (m, 2H); ^{13}C NMR (CDCl_3): 155.7, 153.2, 147.8, 127.7, 110.1, 107.9, 63.5, 56.3, 56.3, 39.4, 32.3, 30.6.

NVOC-TyrO3P(NVOC)-*Or*Bu (13). To a mixture of 0.12 g NVOC-Tyr-*Or*Bu and 0.166 g Cs_2CO_3 (two equivalents) dissolved in 10 mL DMF were added 0.096 g *N*-NVOC-bromopropylamine in 13 mL of anhydrous DMF, under Ar. After the reaction was stirred for 1.5 hours, the reaction mixture was extracted using 3 X 25 mL methylene chloride, the organic layers were combined, dried with sodium sulfate, and run on a flash column in 1:1 petroleum ether / ethyl acetate to give 75 mg (38%) of product. ^1H NMR (CDCl_3): δ 7.70 (s, 1H), 7.49 (s, 1H), 7.08 (d, J=4.2 Hz, 2H), 7.00 (s, 1H), 6.97 (s, 1H), 6.81 (d, J=4.2 Hz, 2H), 5.52 and 5.47 (AB, J=11.4 Hz, 11.4 Hz, 2H), 5.50 (s, 2H), 5.41 (d, 1H), 5.26 (br s, 1H), 4.52 (m, 1H), 4.02 (t, J=5.7 Hz, 2H), 4.98 (s, 3H), 4.98 (s, 3H), 4.98 (s, 3H), 4.98 (s, 3H), 3.44 (dt, J=6.3 Hz, 6.3 Hz, 2H), 3.05 (m, 2H), 2.02 (m, 2H), 1.44 (s, 9H); ^{13}C NMR (CDCl_3): 170.4, 157.4, 155.7, 155.0, 153.4, 153.2, 147.7, 147.5, 141.0, 139.2, 130.2, 128.1, 128.0, 114.2, 114.0, 109.9, 109.5, 107.9, 107.9, 82.3, 65.6, 63.6, 63.4, 56.4, 56.3, 56.1, 55.2, 38.7, 37.2, 29.3, 27.9.

NVOC-TyrO3P(NVOC)- OCH_2CN (1). 0.075 g NVOC-Tyr-O3P(NVOC)-*Or*Bu were dissolved in 5 mL methylene chloride. 3 mL TFA were added to the mixture, using a glass pipette. The reaction was stirred for 1 hour. Volatiles were removed on the vacuum pump with a dry ice/acetone trap. 10 mL anhydrous DMF, 5 mL chloroacetonitrile, and 1 mL diisopropylethylamine (DIPEA)

were added to the flask, under argon. After the reaction was stirred for 3 hours, the volatiles in the mixture were removed on the vacuum pump with a dry ice/acetone trap. The reaction mixture was run on a silica column in 1:1 petroleum ether / ethyl acetate to give 53 mg (72%) product. ^1H NMR (CDCl_3): 7.70 (s, 1H), 7.70 (s, 1H), 7.07 (d, J=4.2 Hz, 2H), 7.01 (s, 1H), 6.94 (s, 1H), 6.85 (d, J=4.2 Hz, 2H), 5.53 and 5.50 (AB, J=14.7 Hz, 14.8 Hz, 2H), 5.50 and 5.47 (AB, J=15.1 Hz, 14.7 Hz, 2H), 5.41 (s, 1H), 5.28 (d, J=6.3 Hz, 1H), 4.78 and 4.70 (AB, J=21.3 Hz, 21.3 Hz, 2H), 4.69 (m, 1H), 4.03 (t, J=5.7 Hz, 2H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.44 (dt, J=6.3 Hz, 6.3 Hz, 2H), 3.10 (m, 2H, J=6.3 Hz, 6.3 Hz), 2.02 (m, J=6.3 Hz, 2H); ^{13}C NMR (CDCl_3): 170.2, 157.8, 155.7, 155.0, 153.3, 153.2, 147.8, 147.6, 141.0, 139.4, 130.0, 127.9, 127.4, 126.7, 114.6, 113.5, 110.0, 109.8, 107.9, 65.6, 64.0, 63.4, 56.4, 56.2, 56.3, 56.1, 54.8, 48.9, 38.6, 36.9, 29.7, 29.2.

NVOC-TyrO3S-*Or*Bu (11). 268 mg NVOC-TyrO3Br-*Or*Bu were dissolved in 20 mL anhydrous THF under Ar in a 3-neck round-bottom flask with a CO_2 (s) / acetone condenser. This was cooled to -20 °C in a 30% KCl / ice bath and then NH_2Me gas was bubbled through the solution until the drip rate from the condenser tip was about 2 s⁻¹. The setup was allowed to warm to RT and then the reaction mixture was rotoevaporated to ensure removal of the dissolved NH_2Me . Chromatographic purification was achieved by elution of the starting material in CH_2Cl_2 followed by collection of the product in CH_2Cl_2 with 5% triethylamine. 164 mg of flaky yellow solid (67%) were obtained in this manner. ^1H NMR (CDCl_3) δ 7.67 (s, 1H), 7.09 (d, J = 8.5 Hz, 2H), 6.96 (s, 1H), 6.81 (d, J = 8.5 Hz, 2H), 5.81 (d, J = 8.1 Hz, 1H), 5.52 and 5.44 (AB, J = 15.2 Hz, 2H), 4.49 (m, 1H), 3.99 (t, J = 6.3 Hz, 2H), 3.95 (s, 3H), 3.93 (s, 3H), 3.03 (m, 2H), 2.75 (t, J = 6.9 Hz, 2H), 2.44 (s, 3H), 1.95 (m, 2H), 1.43 (s, 9H)

NVOC-TyrO3S(NVOC)-*Or*Bu (14). 65 mg Na_2CO_3 (2 equiv.) were dissolved in 15 mL water and added to a solution of 164 mg NVOC-TyrO3S-*Or*Bu in 15 mL *p*-dioxane. 168 mg NVOC-Cl (2 equiv.) in 15 mL *p*-dioxane were added to this and the reaction was stirred overnight. The product was purified by flash chromatography in 1:1 EtOAc / petroleum ether, giving 104 mg (44%) of a dark yellow solid. ^1H NMR (CDCl_3) δ 7.72 (s, 1H), 7.68 (s, 1H), 7.07 (d, J = 8.4 Hz, 2H), 7.03 (s, 1H), 7.01 (s, 1H), 6.79 (d, J = 8.4 Hz, 2H), 5.56 and 5.51 (AB, J = 15.0 Hz, 2H), 5.47 (br s, 2H), 5.35 (d, J = 7.5 Hz, 1H), 4.51 (m, 1H), 3.96 (br t, J = 6.3 Hz, 2H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.52 (t, J = 12.5 Hz, 2H), 3.05 (m, 2H), 3.00 (d, J = 17.5 Hz, 2H), 2.05 (m, 2H), 1.44 (s, 9H)

NVOC-TyrO3S(NVOC)- OCH_2CN (2). After 64 mg NVOC-TyrO3S(NVOC)-*Or*Bu were stirred with 1.00 mL trifluoroacetic acid for 1 hr. in 10 mL CH_2Cl_2 , the reaction mixture was pumped on for 2 h. with a dry ice / acetone trap. The crude amino acid was coevaporated with toluene and redissolved in 10 mL DMF under Ar for esterification. 0.50 mL chloroacetonitrile was added with 0.10 mL DIPEA and the mixture was stirred at RT. The next morning, the volatiles were removed under vacuum and the mixture was run on a column in 3:1 petroleum ether / EtOAc. 24 mg of an orange solid were obtained in a 40% yield. ^1H NMR (CDCl_3) δ 7.70 (s, 1H), 7.67 (s, 1H), 7.07 (d, J = 8.4 Hz, 2H), 7.03 (s, 1H), 6.99 (s, 1H), 6.81 (d, J = 8.4 Hz, 2H), 5.55 and 5.49 (AB, J = 15.1 Hz, 2H), 5.47 (br d, J = 15.1 Hz, 2H), 5.33 (d, J = 7.5 Hz, 1H), 4.98 (br s, 2H), 4.38 (m, 1H), 3.97 (br t, J = 6.3 Hz, 2H), 3.94 (s, 3H), 3.94 (s, 3H), 3.94 (s, 3H), 3.94 (s, 3H),

3.56 (t, J = 10.5 Hz, 2H), 3.04 (m, 2H), 2.98 (d, J = 16.0 Hz, 2H), 2.08 (m, 2H), 1.46 (s, 9H)

4PO-Tyr-OtBu (8). 148 mg Na₂CO₃ (1.4 equiv.) were stirred with 237 mg Tyr-OtBu in 25 mL H₂O and 20 mL *p*-dioxane. 256 μL 4-pentenoic anhydride (4P) anhydride, 2.1 equiv., Aldrich), dissolved in 5 mL *p*-dioxane, were added. The reaction was quenched after 1 hr. with 25 mL each CH₂Cl₂ and 1 M NaHSO₄. The aqueous layer was extracted with 3 X 25 mL CH₂Cl₂ and purified on a column in 3:1 petroleum ether / EtOAc. 249 mg of an oily yellow solid obtained in a 73% yield. ¹H NMR (CDCl₃) δ 6.90 (d, J = 8.4 Hz, 2H), 6.68 (d, J = 8.5 Hz, 2H), 6.21 (d, J = 6.6 Hz, 1H), 5.66 (m, 1H), 4.94 (d, J = 18.0 Hz, 1H), 4.89 (d, J = 11.2 Hz, 1H), 4.66 (m, 1H), 2.90 (m, 2H), 2.18 (m, 2H), 2.21 (m, 2H), 1.37 (s, 9H) ¹³C NMR (CDCl₃): 171.6, 171.2, 155.6, 139.3, 130.5, 128.5, 115.5, 114.5, 82.6, 53.4, 37.5, 35.7, 32.6, 28.2.

4PO-TyrO3Br-OtBu (9). 750 mg Cs₂CO₃ (2 equiv.) were combined with 2.00 mL 1,3-dibromopropane in 20 mL dry DMF in a flame-dried flask under Ar. 249 mg 4PO-Tyr-OtBu were dissolved in 10 mL DMF under Ar, added to the Cs₂CO₃ slurry through a septum, and stirred overnight. The reaction mixture was stirred 10 min. with 30 mL water and extracted with 3 X 25 mL CH₂Cl₂. Flash chromatography of the combined organics with 3:1 petroleum ether / EtOAc gave 157 mg of sticky yellow solid, a 47% yield. ¹H NMR (CDCl₃) δ 7.00 (d, J = 8.9 Hz, 2H), 6.75 (d, J = 8.7 Hz, 2H), 6.24 (d, J = 6.8 Hz, 1H), 5.68 (m, 1H), 4.96 (d, J = 17.9 Hz, 1H), 4.91 (d, J = 11.3 Hz, 1H), 4.67 (m, 1H), 3.99 (t, J = 5.8 Hz, 2H), 3.51 (t, J = 6.6 Hz, 2H), 2.94 (m, 2H), 2.27 (m, 2H), 2.23 (m, 2H), 2.20 (m, 2H), 1.36 (s, 9H) ¹³C NMR (CDCl₃): 171.8, 171.1, 157.8, 137.1, 130.7, 128.7, 115.7, 114.5, 82.4, 65.5, 53.8, 37.5, 35.9, 32.7, 30.4, 29.7, 28.3.

4PO-TyrO3T-OtBu (12). 236 mg 4PO-TyrO3Br-OtBu were dissolved in 25 mL dry THF in a 3-neck round-bottom flask with a dry ice / acetone condenser under Ar. The setup was cooled in ice and NH(Me)₂ gas was bubbled through the yellow solution until it was dripping vigorously from the condenser tip. The cooling apparatus was maintained for 2 hrs. and then the reaction mixture was allowed to warm to RT. After rotoevaporation to remove the volatile components, the mixture was chromatographed. Starting material was eluted with EtOAc and then the product was collected with 5% triethylamine in CH₂Cl₂, giving 210 mg (96%) of a sticky yellow solid. ¹H NMR (CDCl₃) δ 7.01 (d, J = 8.0 Hz, 2H), 6.79 (d, J = 8.0 Hz, 2H), 5.95 (d, J = 7.2 Hz, 1H), 5.76 (m, 1H), 5.02 (d, J = 17.7 Hz, 1H), 4.97 (d, J = 12.3 Hz, 1H), 4.71 (m, 1H), 3.96 (t, J = 6.3 Hz, 2H), 3.00 (m, 2H), 2.44 (t, J = 7.5 Hz, 2H), 2.35 (t, J = 9.0 Hz, 2H), 2.20 (s, 6H), 1.93 (m, 2H), 1.35 (s, 9H), 1.27 (m, 2H) ¹³C NMR (CDCl₃): 171.3, 170.6, 157.7, 136.7, 130.2, 127.8, 115.3, 114.1, 82.0, 66.0, 56.3, 53.4, 45.5, 37.0, 35.6, 29.3, 27.9, 27.5.

4PO-TyrO3T(DMCm)-OtBu (15). 762 mg **12** and 675 mg dimethoxycoumarinmethylbromide (DMCmBr) were stirred overnight in 200 mL CH₃CN at 60 °C under Ar. After rotoevaporation, the reaction mixture was purified by flash chromatography on silica gel. First, starting material was eluted using 1:1 petroleum ether / EtOAc with 5% MeOH, then product was eluted with 7:1:1:1 EtOAc / MeOH / AcOH / H₂O. 857 mg of an intensely yellow acetate salt were obtained in a 67% yield. ¹H NMR (CDCl₃) δ 7.58 (s, 1H), 7.05 (d, J = 9.4 Hz, 2H), 6.86 (s, 1H), 6.78 (d, J = 9.6 Hz, 2H), 6.63 (s, 1H), 6.11 (d, J = 7.4 Hz, 1H), 5.79 (m, 1H), 5.20 (br s, 2H), 5.04 (d, J = 18.0 Hz, 1H), 4.99 (d, J = 10.0 Hz, 1H), 4.72 (m, 2H), 4.05 (t, J = 8.2 Hz, 2H), 3.96 (s, 3H), 3.94 (s, 3H), 3.88 (br

t, 2H), 3.30 (s, 6H), 3.01 (m, 2H), 2.35 (t, J = 7.8 Hz, 2H), 2.31 (m, 2H), 2.28 (m, 2H), 2.05 (s, 3H), 1.43 (s, 9H); ¹³C NMR (CDCl₃): 177.1, 171.6, 170.6, 159.6, 156.8, 153.6, 149.9, 147.0, 142.0, 136.6, 130.4, 128.9, 119.8, 115.4, 114.0, 111.1, 105.9, 100.1, 82.3, 64.0, 63.1, 62.2, 56.9, 56.4, 53.5, 51.0, 37.1, 35.5, 29.3, 28.0, 23.2, 22.1.

4PO-TyrO3T(DMCm)-OCH₂CN (3). 857 mg 4PO-TyrO3T(DMCm)-OtBu were dissolved in 25 mL methylene chloride with 5 mL TFA. When reaction was complete, volatiles were removed on the vacuum pump with a dry ice / acetone trap. 15 mL anhydrous ClCH₂CN and 1 mL triethylamine were added to the flask, under argon. After stirring overnight, the volatiles in the mixture were removed on the vacuum pump with a dry ice/acetone trap. The reaction mixture was run on a column in 10:1:1:1 EtOAc / MeOH / AcOH / H₂O. acetate. Collected fractions were rotovapped to dryness, redissolved in minimal CH₂Cl₂, and extracted against H₂O to remove triethylamine salt. 651 mg of an acetate salt, a 78% yield. ¹H NMR (CDCl₃) δ 7.73 (s, 1H), 6.98 (d, J = 8.5 Hz, 2H), 6.79 (s, 1H), 6.70 (d, J = 8.5 Hz, 2H), 6.65 (s, 1H), 6.11 (d, J = 7.3 Hz, 1H), 5.77 (m, 1H), 5.44 (br s, 2H), 5.03 (d, J = 15.0 Hz, 1H), 4.97 (d, J = 10.5 Hz, 1H), 4.81 (m, 2H), 4.04 (t, J = 6.8 Hz, 2H), 4.02 (s, 3H), 4.00 (s, 3H), 3.96 (t, J = 6.7 Hz, 2H), 3.42 (s, 6H), 3.07 (m, 2H), 2.42 (m, 2H), 2.36 (m, 2H), 2.31 (m, 2H), 2.02 (s, 3H), 1.43 (s, 9H); ¹³C NMR (CDCl₃): 177.1, 172.2, 170.2, 159.5, 157.9, 163.6, 149.8, 147.1, 141.8, 136.6, 130.1, 128.0, 120.2, 115.4, 114.5, 114.0, 111.0, 106.2, 100.0, 64.1, 63.5, 62.1, 57.6, 56.4, 53.2, 50.5, 48.8, 36.6, 25.0, 29.2.

General procedure for coupling of cyanomethyl esters to dCA. The dinucleotide was prepared as previously reported² with a few modifications: 1) the dinucleotide coupling, oxidation, and deprotection with *p*-toluenesulfonic acid were done in one pot; 2) the desalting of dCA was accomplished by redissolving it in Millipore water, freezing, and lyophilization to obtain a fluffy material; 3) the tetrabutylammonium salt of the dinucleotide was formed by mixing the proper amount 1 M N(*n*-Bu)₄OH in MeOH with a solution of dCA in water. Freezing and lyophilization provided a white fluffy solid which was then stored at -80 °C.

NVOC-TyrO3P(NVOC)-OdCA. The synthesis of NVOC-TyrO3P(NVOC)-OdCA is described as a general procedure. 23 mg of dCA (tetrabutylammonium salt) were dissolved in 0.5 mL anhydrous DMF, and were stirred with 53 mg NVOC-TyrO3P(NVOC)-OCH₂CN (four equivalents) dissolved in 0.5 mL anhydrous DMF in a 10 mL pear-shaped flask, under argon. After 4 hours, a small amount of tetrabutylammonium acetate was added using a metal spatula. The reaction was monitored by analytical HPLC, using a Waters NOVA-Pak C₁₈ (150 x 3.9 mm) reverse-phase column with a gradient from 25 mM NH₄OAc (pH 4.5) to CH₃CN. When the reaction was judged complete after 24 hours, the mixture was purified by semi-preparative HPLC with a Waters NOVA-Pak C₁₈ (300 x 7.8 mm) using a similar gradient. The appropriate fractions were combined, frozen, and lyophilized overnight. To remove ammonium ions, which inhibit T4 RNA ligase in the ligation of the product to tRNA, the product was redissolved in 10 mM acetic acid, frozen, and lyophilized again. This yielded 5.6 mg (6%; yields can be as low as 1%) of the desired product as a pale yellow solid. Small amounts of material were quantified by their UV-Vis spectra in solution of 10 mM acetic acid, assuming ε₃₅₀ = 6336 M⁻¹ per nitroveratryl group. ESI⁻MS: calculated for 755.7; found [M+Na]⁺: 778.4.

NVOC-Tyr-O3S(NVOC)-dCA. Coupling procedure differed in that $N(n\text{-Bu})_4\text{OAc}$ salt was added to a cloudy mixture of dCA in 500 μL DMF until dissolution was complete and then this was combined with a 500 μL solution of the cyanomethyl ester in DMF. Reaction was complete after 2 h. 2 mg obtained in a 5% yield. from 24 mg of cyanomethyl ester and 32 mg dCA. ESI⁺-MS: calculated for $\text{C}_{52}\text{H}_{63}\text{N}_{12}\text{O}_{27}\text{P}_2^+$: 1349.3 ; found $[\text{M}+\text{H}]^+$: 1349.3

4PO-TyrO3T(DMCm)-dCA. Prepared by the general coupling procedure. After 36 h., reaction appeared to have reached steady-state at ~ 60% completion. 6 mg obtained in a 31% yield. from 16 mg of cyanomethyl ester and 20 mg dCA. Quantification was performed assuming $\epsilon_{350} = 13,000 \text{ M}^{-1}$ for the coumarin group. ESI⁺-MS: calculated for $\text{C}_{50}\text{H}_{63}\text{N}_{10}\text{O}_{20}\text{P}_2^+$: 1185.4 ; found $[\text{M}+\text{H}]^+$: 1185.4

Note: 4PO-TyrO3T(DMCm)-dCA was originally synthesized with a nitroveratryl (NV) protecting group on its side-chain amine. However, even after 1 h of photolysis under the above conditions, no cleavage of the NV group was observed, as detected by tandem HPLC electrospray MS (positive ion, in 25 mM NH_4OAc). Photolysis of the model compound *N*-NV-3-dimethylaminopropanol also failed, even after 1 h of irradiation. Deprotection of the DMCm-protected 3° amine, on the other hand, was complete after 5 - 10 min.

Unnatural amino suppression in *Xenopus* oocytes. The site-directed mutagenesis of the nAChR TAG mutants, gene construction and synthesis of suppressor tRNA and ligation of aminoacyl-dCA to tRNA have been described previously². Plasmid DNAs were linearized with NotI, and mRNA was transcribed using the Ambion (Austin, TX) T7 mMACHINE Kit.

Oocytes were removed from *Xenopus laevis* as described⁴ and maintained at 18 °C, in ND96 solution (96 mM NaCl/2 mM KCl/1.8 mM CaCl_2 /1 mM MgCl_2 /5 mM HEPES/2.5 mM sodium pyruvate/0.5 mM theophylline/10 g/ml Gentamycin, pH 7.5, with NaOH). Before microinjection, the NVOC-aminoacyl-tRNA was deprotected by irradiating the sample for 5 or 10 min. with a 1000 W Hg/Xe arc lamp (Oriel) operating at 400 W equipped with WG-335 and UG-11 filters (Schott). 4PO-protected tRNA-aa was mixed 1:1 with a solution of saturated I_2 in water and allowed to sit for ten minutes at room temperature. Each oocyte was injected with a 1:1 mixture of deprotected aminoacyl-tRNA (25-50 ng) and mRNA (12.5–18 ng of total at a concentration ratio of 20:1:1:1 for $\alpha:\beta:\gamma:\delta$ subunits) in a volume of 50 nL.

Electrophysiological recordings. Voltage-clamped electrophysiological recordings were carried out 24-72 hours after injection. Whole-cell currents from oocytes were measured using a Geneclamp 500 amplifier and pCLAMP software (Axon Instruments, Foster City, CA) in the two-electrode voltage-clamp configuration. Microelectrodes were filled with 3 M KCl and had resistances ranging from 1.0 to 2.5 M Ω . Oocytes were continuously perfused with a nominally Ca^{2+} -free bath solution consisting of 96 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , and 5 mM HEPES (pH 7.5). Microscopic ACh-induced and TMB-8 or QX-314-blocked currents were recorded in response to bath application of ACh and TMB-8 at a holding potential of -80 mV. Low (5.5 - 6.5) and high (8.5 and 9.0) pH solutions were of the same composition as Ca^{2+} -free bath with MES (low) or CHES (high) substituted for HEPES buffer. To ensure that changes in buffer were not responsible for the observed changes in channel conductance, recordings were taken at pH 7.0 and 8.0 in HEPES alongside recordings in MES and CHES.

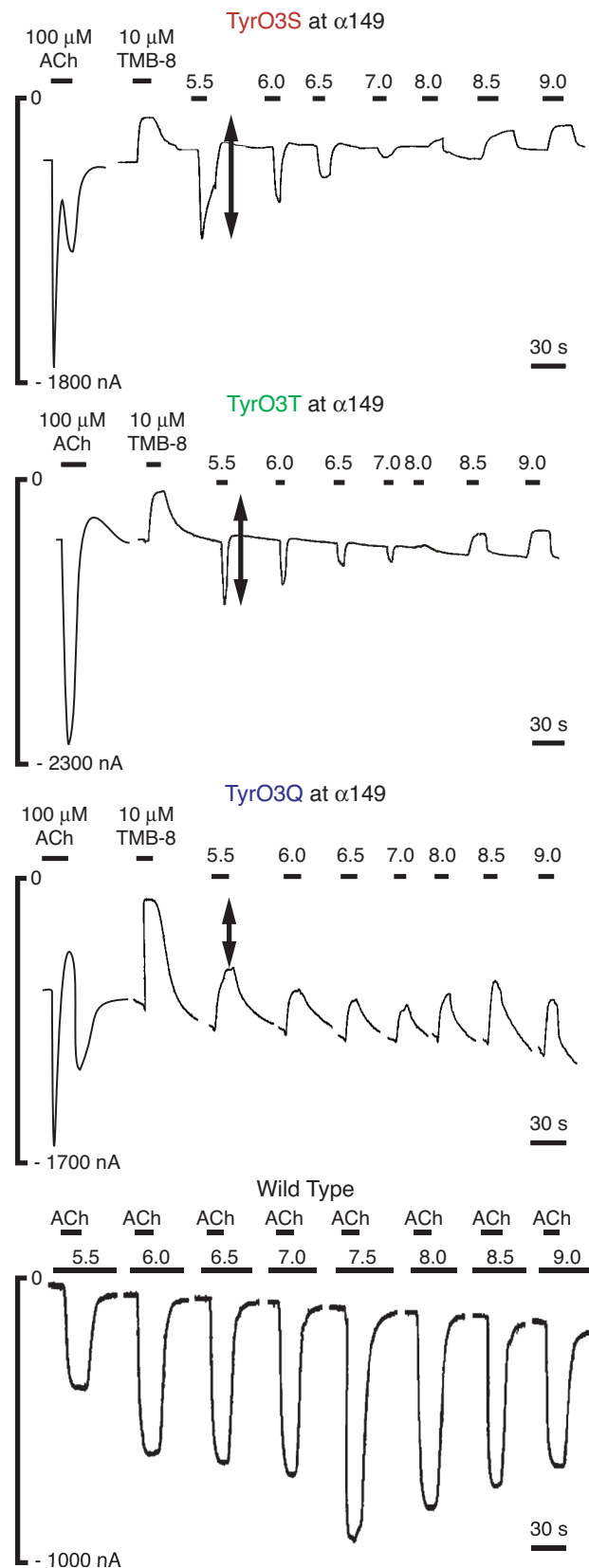


Figure A. Primary Electrophysiological Data: TyrO3S, TyrO3T, and TyrO3Q at $\alpha 149$: Tethered agonist responses to ACh, TMB-8, and agonist-free solutions of varying pH. Wild Type nAChR responses to 10 μM ACh at differing pHs. Upper bars indicate agonist or blocker application. Lower bars indicate application of solution of given pH. Arrows indicate blockable constitutive currents.

Primary Electrophysiological Data: Examples of electrophysiological recordings for TyrO3S, TyrO3T, and TyrO3Q suppressed at α 149 are shown in Figure A. For each tethered agonist, there is a substantial standing current even at pH 7.5, which can be blocked by TMB-8. As seen in previous tethered agonist studies, added ACh causes an increase in current (followed by desensitization), indicating that the tethers are "partial agonists." For TyrO3S and TyrO3T, application of low pH agonist-free solutions potentiate constitutive activity by protonating the tether. For TyrO3Q, which is always charged, changes in constitutive activity with pH merely mirror the inherent changes in channel conductance as seen for wild type responses to 10 μ M ACh at various pHs (Figure A, bottom).

TyrO3P: The primary tether incorporates and gives functional nAChRs, as seen by the response to ACh, but shows no constitutive activity, even at pH 5.5. (Figure B) It would be surprising that the tether remains unprotonated, as the pK_a of aminopropanol is 10.2, higher than the 3° amine.⁵ TyrO3P may lack the steric bulk to activate the receptor.

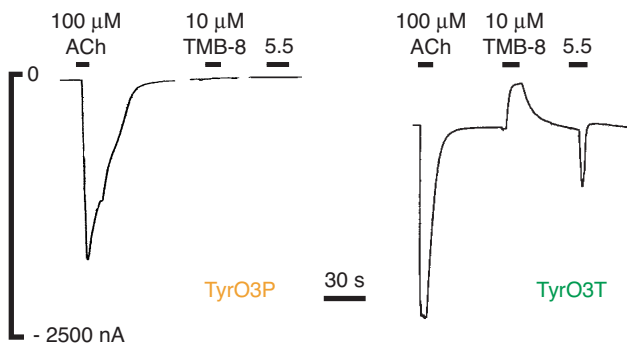


Figure B. TyrO3P shows no constitutive activity. TyrO3P and TyrO3T at α 149, their current responses to ACh, TMB-8, and pH 5.5.

α 149 Lys mutant: No ACh-induced or TMB-8-blockable currents were observed, and fluorescent labelling studies with tetramethylrhodamine-conjugated bungarotoxin (BuTx-TMR, an antagonist) showed no surface expression of α 149 Lys mutant nAChRs in oocytes. Six days following mRNA injection, oocytes were incubated (60 min, 4°C) in ND96 solution containing BuTx-TMR (100 nM) and bovine serum albumin (5 mg/ml). After three washes with ND96 the fluorescence intensity of the animal pole was determined using an inverted epifluorescent microscope (IX-70 FLA; Olympus Corp.) equipped with a photomultiplier tube (R928P; Hamamatsu Photonics) attached to the side port. This microscope (described previously, Li *et al.*, 2000) was fitted with an oil-immersion objective of 40X, NA 1.35.

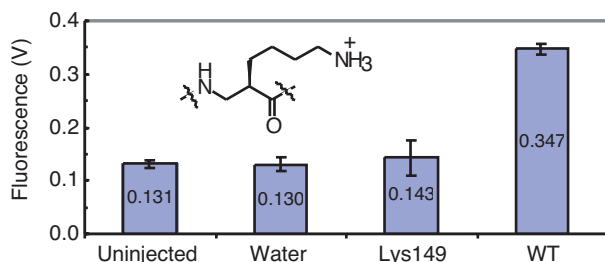


Figure C. Fluorescent labelling of α 149 Lys mutant nAChRs.

Tethered agonists at sites α 93 and γ 55/ δ 57. As stated earlier, the efficacy of a tethered agonist can be evaluated in terms of the ratio of the constitutive current that can be blocked by TMB-8 to the maximum current induced by saturating concentrations of ACh. An examination of the efficacies of TyrO3S, TyrO3T, and TyrO3Q at α 93 and γ 55/ δ 57 (Figures D and E) shows that all three tethers are less potent at these sites than at α 149. TyrO3T and TyrO3Q curves are similar in shape to the α 149 curves. Surprisingly, although TyrO3S yields constitutive activity at all three sites, it cannot be potentiated by pH at α 93 and γ 55/ δ 57.

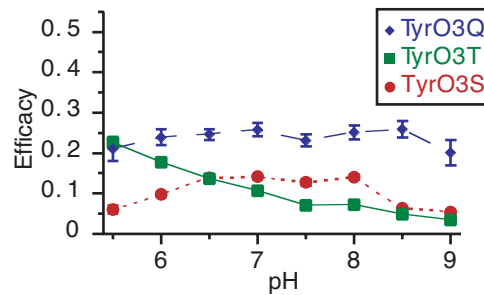


Figure D. α 93: Efficacy as a function of solution pH for tethered agonists at α 93. Shown on the same scale as Figure 2 in main text.

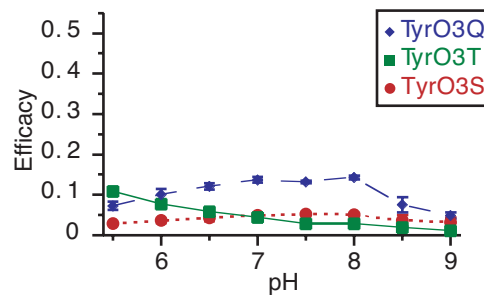


Figure E. γ 55/ δ 57: Efficacy as a function of solution pH for tethered agonists at γ 55/ δ 57. Shown on the same scale as Figure 2 in main text.

TyrO3t-Bu, an uncharged analog of TyrO3Q in which the quaternary nitrogen has been replaced by a carbon, also gave weakly constitutively active receptors at α 93 and γ 55/ δ 57 (but not at α 149). TyrO3t-Bu showed no pH-dependent potentiation over background. This indicates that charge may be less of a factor at these positions than at α 149, and may help to explain the puzzling TyrO3S behavior.

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