# Identification of tryptophan 55 as the primary site of [$^3$H]nicotine photoincorporation in the γ-subunit of the *Torpedo* nicotinic acetylcholine receptor

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**Abstract [$^3$H]nicotine has been used as a photoaffinity agonist to identify amino acids within the *Torpedo* nicotinic acetylcholine receptor (nAChR) γ-subunit that contributes to the structure of the agonist binding site. UV irradiation (254 nm) of nAChR-rich membranes equilibrated with [$^3$H]nicotine results in covalent incorporation into α- and γ-subunits that is inhibitable by agonists and competitive antagonists, but not by non-competitive antagonists (Middleton, R.E. and Cohen, J.B. (1991) Biochemistry 30, 6887–6897). To identify sites of specific incorporation, SDS-PAGE and reversed-phase HPLC were used to isolate proteolytic fragments of [$^3$H]nicotine-labeled γ-subunit. Amino-terminal sequence analysis identified γTrp-55 as the major site of [$^3$H]nicotine photoincorporation in γ-subunit. Thus γTrp-55 is the first amino acid within a non-α-subunit to be identified by affinity labeling in direct contact with a bound agonist.**



*Key words:* Nicotinic acetylcholine receptor; Ligand binding domain; Photoaffinity labeling; Nicotine; Tryptophan

## 1. Introduction

The *Torpedo* nicotinic acetylcholine receptor (nAChR), a multi-subunit protein consisting of four homologous subunits arranged as a pentamer ($\alpha_2\beta\gamma\delta$) around a central pore, is the best characterized member of the ligand-gated ion channel protein superfamily (for review see [1,2]). Although initial studies indicated that the two acetylcholine binding sites were contained within the α-subunits, more recent data establish that the sites are localized at the α-γ- and α-δ-subunit interfaces. Affinity labeling studies with agonists and competitive antagonists identified amino acids within three distinct regions of *Torpedo* α-subunit primary structure which contribute to the binding sites (αTyr-93, αTrp-149, and αTyr-190 to αTyr-198), and mutational analyses have established their contributions to agonist and competitive antagonist binding (reviewed in [3]). For mouse muscle nAChRs, mutational analyses have identified amino acids differing between γ- and δ-subunits that account for the preferential binding of some ligands at the α-γ or α-δ site. With reference to the γ-subunit numbering, amino acids 116, 117 and 161 determine selectivity for metocurine and other small competitive antagonists [4], while 34, 111, and 174 confer selectivity for the peptide antagonist α-conotoxin M1 [5]. Amino acids at γ34 and 172 are the primary determinants of site selectivity for the agonist carbamylcholine, with some contribution from residues at 57 and 115 [6]. Additionally, the acidic side chain at positions γ174 and δ180 contribute to agonist and antagonist binding affinity [7]. Based upon affinity labeling of *Torpedo* nAChRs, γTrp-55 and δTrp-57 were identified as primary sites of photoincorporation of the competitive antagonist [$^3$H]*d*-tubocurarine (dTC) [8], with γTyr-111 and γTyr-117 identified as minor sites [9], while δAsp-180 was identified by chemical cross-linking from αCys-192 [10].

The agonist [$^3$H]nicotine can be used as a photoaffinity reagent, since UV irradiation of *Torpedo* nAChR-rich membranes equilibrated with [$^3$H]nicotine results in pharmacologically specific covalent incorporation in α- and γ-subunits. While the efficiency of photoincorporation is low ($<1\%$ of bound nicotine), both the concentration dependence of incorporation as well as its inhibition by agonists and antagonists establish that [$^3$H]nicotine is incorporated within the agonist binding site in the desensitized state of the nAChR [11]. Within α-subunit, the primary site of photoincorporation is αTyr-198, with additional labeling at αTyr-190 and αCys-192 and 193 [11]. In this work, we identify the primary site of [$^3$H]nicotine photoincorporation in the γ-subunit as γTrp-55.

## 2. Materials and methods

### 2.1. [$^3$H]nicotine photolabeling of nAChR-rich membranes

nAChR-rich membranes were isolated from the electric organs of *Torpedo californica* (Marinus, Westchester, CA) as described [11]. Membranes were stored in 38% sucrose/0.02% $NaN_3$ at −80°C and contained 1.3 to 1.4 nmol [$^3$H]acetylcholine (ACh) sites per mg of protein. For photolabeling, nAChR-rich membranes (~10 mg protein at 2 mg/ml) were equilibrated with 15 μM [$^3$H]nicotine (30 Ci/mmol, New England Nuclear) in 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM $NaPO_4$, pH 7.0 with 1 mM oxidized glutathione (an aqueous scavenger) and 30 μM proadifen (a desensitizing non-competitive antagonist). Carbamylcholine (carb, 300 μM) was added to some samples to define agonist inhibitable photoincorporation. Equilibrated samples were irradiated at 254 nm for 5 min and nAChR subunits were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [11].

### 2.2. Isolation of proteolytic digestion products of [$^3$H]nicotine-labeled γ-subunit

Isolated γ-subunit (70 μg) was digested with 0.25 U of endoproteinase Lys-C (Boehringer Mannheim) in 55–70 μl of 0.1 M Tris, pH 8.8, with 0.5 mM DTT, 0.5 mM EDTA, and 0.05% SDS at 25°C for 4 weeks with an additional 0.1 U of enzyme added after 3 weeks. Isolated γ-subunit (170 μg) was digested with 1.2 μg *Staphylococcal aureus* glutamyl endopeptidase (V8-protease, ICN Biochemical) in 285 μl 0.1 M Tris, pH 6.8, with 0.75 mM DTT, 0.75 mM EDTA, and 2% SDS for 12 h at 25°C. Endoproteinase Lys-C digests were

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*Abbreviations:* nAChR, nicotinic acetylcholine receptor; dTC, *d*-tubocurarine; ACh, acetylcholine; carb, carbamylcholine


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fractionated by SDS-PAGE using the tricine gel system (16.5% T/3% C) of Schägger and von Jagow [12], and the resulting gel was treated with Amplify (Amersham), dried, and exposed to x-ray film to define $^3$H distribution. The autoradiogram was used as a template to excise $^3$H bands which were diced and eluted as described [8]. V8-protease digests were fractionated by reversed-phase high performance liquid chromatography (HPLC) using a Vydac $C_4$ (214TP54) column at 0.5 ml/min with 20% acetonitrile/0.1% trifluoroacetic acid (TFA) as aqueous solvent and 80% isobutanol/20% acetonitrile, 0.1% TFA as organic solvent and system components as described previously [13]. HPLC fractions of interest were pooled, dried by vacuum centrifugation, and resuspended in 100 μl of 10 mM $NaPO_4$ buffer, pH 7.0 with 1 mM DTT, 1 mM EDTA, and 0.1% SDS. For deglycosylation, samples (10 μl) were adjusted to 20 μl in 200 mM $NaPO_4$, pH 7.0 with either 0.5% Lubrol-PX (Pierce) for N-glycanase (250 mU, Genzyme) or 1% SDS for endoglycosidase H (5 mU, Sigma). Samples were then incubated overnight at 25°C.

*2.3. Amino acid sequence analysis*

Sequence analysis was performed on an Applied Biosystems Model 470A protein sequencer with an inline Model 120A PTH-amino acid analyzer. Approximately 40% of each cycle was injected into the analyzer for amino acid identification and 60% (Fig. 1) or 40% (Fig. 2) was collected for scintillation counting. The actual cpm and pmol detected are reported. Samples for sequence analysis were loaded onto trifluoroacetic acid treated glass-fiber filters coated with 3 mg Polybrene. Cycle yields for PTH-amino acids were estimated from peak heights. Mass values from sequencing were fit (Sigma Plot, Jandel Corp.) to the equation $M_x = I_o R^x$ where $M_x$ is the amino acid mass in cycle $x$, $I_o$ is the initial mass of the peptide in pmol, and $R$ is repetitive yield.

## 3. Results

For preparative [$^3$H]nicotine photolabeling, nAChR-rich membranes, equilibrated with [$^3$H]nicotine, were irradiated at 254 nm for 5 min. Parallel reactions were carried out in the absence and presence of the agonist carbamylcholine to define agonist inhibitable photoincorporation. nAChR subunits were isolated from labeled membranes by preparative slab gel electrophoresis (−carb/+carb in $^3$H cpm/μg: α-subunit, 1390/135; β-subunit, 140/85; γ-subunit, 575/110; and δ-subunit, 170/75).

To identify the sites of specific [$^3$H]nicotine photoincorporation within γ-subunit, isolated γ-subunit was digested with endoproteinase Lys-C, and the digest was fractionated by SDS-PAGE. Based upon gel slice analysis (Fig. 1A), 65% of the specific $^3$H incorporation in γ-subunit was recovered in a single band of ∼22 kDa. Material contained within this band was eluted from the gel and characterized by amino-terminal sequence analysis (Fig. 1B). Prominent release of $^3$H (350 cpm) was seen in cycle 9, release that resulted from specific photolabeling of the agonist site since sequence analysis of the fragment isolated from nAChRs labeled in the presence of carb resulted in a >90% reduction of $^3$H release in that cycle. Analysis of the released PTH-amino acids revealed the presence of three peptides. The primary sequence began at γGlu-47 (31 pmol) with secondary sequences at γVal-273 (7 pmol) and Arg-148 from the β-subunit of the $Na^+/K^+$ ATPase (10 pmol), which is a known contaminant of *Torpedo* nAChR γ-subunit isolated by SDS-PAGE [13,14]. The release of $^3$H in cycle 9 would correspond to γTrp-55 in the primary sequence (γGlu-47), i.e. the amino acid that is the primary site of photoincorporation of [$^3$H]*d*-tubocurarine [8].

To determine whether γTrp-55 was the source of $^3$H release in cycle 9 of Fig. 1, the site of incorporation was also mapped by use of *S. aureus* V8-protease. Isolated [$^3$H]nicotine-labeled

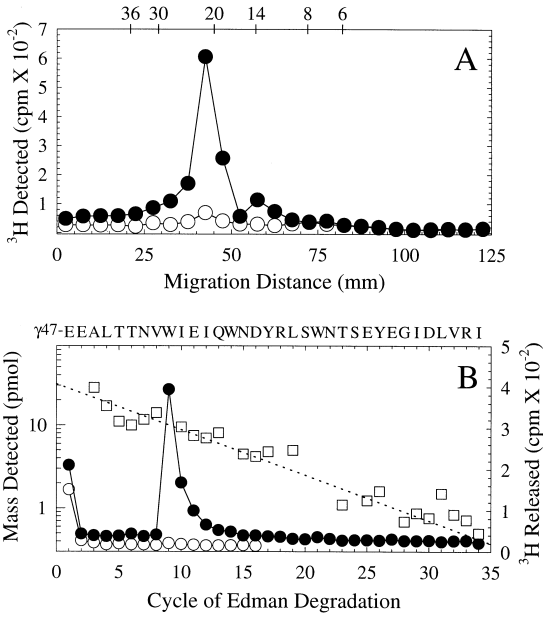


Fig. 1. Mapping of the primary site of [$^3$H]nicotine incorporation in γ-subunit by use of endoproteinase Lys-C. nAChR membranes (9 mg, 11 nmol ACh binding sites) were photolabeled with 15 μM [$^3$H]nicotine in the absence (−carb) or presence (+carb) of 300 μM carbamylcholine, and labeled γ-subunits (575 cpm/μg −carb, 110 cpm/μg +carb) were isolated by SDS-PAGE. Aliquots of γ-subunit (70 μg) were digested with endoproteinase Lys-C and subsequently fractionated by tricine SDS-PAGE (see Section 2). Panel A: $^3$H distribution as determined by liquid scintillation counting of 5 mm gel slices cut from individual lanes containing 5 μg aliquots (●, −carb, ○, +carb). The peak of $^3$H was centered at 22 kDa. Molecular mass standards (kDa): glyceraldehyde-3-phosphate dehydrogenase (36), carbonic anhydrase (30), soybean trypsin inhibitor (20), and myoglobin fragments (14, 8, 6). Panel B: $^3$H release profiles when the material eluted from that 22 kDa band was sequenced (●, −carb, 6000 cpm loaded, 2250 cpm left on the filter after 34 cycles of Edman degradation; ○, +carb, 2000 cpm loaded, 500 cpm left on the filter after 16 cycles of Edman degradation). For the −carb sample three peptides were present, beginning at γGlu-47 (□, $I = 31 \pm 3$ pmol, $R = 88 \pm 1\%$), γVal-273 ($I = 7 \pm 1$ pmol, $R = 89 \pm 1\%$), and $Na^+/K^+$ ATPase β-subunit Arg-147 ($I = 10 \pm 1$ pmol, $R = 85 \pm 2\%$). The dotted line represents a non-linear best fit of the mass values from the γGlu-47 peptide as described in Section 2. The +carb sample contained γGlu-47 ($I = 9 \pm 2$ pmol, $R = 90 \pm 3\%$), γVal-273 ($I = 5 \pm 1$ pmol, $R = 88 \pm 1\%$), and $Na^+/K^+$ ATPase β-subunit Arg-147 ($I = 5 \pm 1$ pmol, $R = 85 \pm 5\%$). Release of $^3$H in cycle 9 (350 cpm) was consistent with [$^3$H]nicotine incorporation into γTrp-55 from the peptide beginning at γGlu-47. Calculated incorporation for γTrp-55 was 24 cpm/pmol.

γ-subunit was digested overnight with *S. aureus* V8-protease, and the digest was then fractionated by reversed-phase HPLC (Fig. 2A and B). Two peaks of $^3$H were detected and the four fractions spanning each peak were pooled, concentrated, and sequenced. For peak I (∼19% organic) prominent release of $^3$H (230 cpm) was detected in cycle 7 (Fig. 2C). This sample contained γ-subunit fragments beginning at γVal-102 (39 pmol) and γAla-49 (7 pmol). Release in cycle 7 was consistent with photoincorporation at γTrp-55 within the γAla-49 peptide. For the material in peak II (∼22% organic from Fig. 2B) no significant $^3$H release was detected in 24 cycles of Edman degradation (i.e. <20 cpm above background). Two sequences were present, one beginning at the γ-subunit amino-terminus (γGlu-1, 3 pmol) and the other at γLeu-373 (4 pmol, data not shown). For the $^3$H-labeled γ-subunit fragment

in peak II, the site of $^3$H incorporation was characterized relative to the known sites of glycosylation within γ-subunit. *Torpedo* nAChR γ-subunit contains two sites of N-linked glycosylation, Asn-68 and Asn-141 [8,15]. Both can be deglycosylated by N-glycanase, which removes all N-linked carbohydrates [16], while Asn-68 is resistant and Asn-141 is sensitive to endoglycosidase H, which removes high-mannose and some hybrid N-linked carbohydrates [17]. Aliquots from peak II were treated with endoglycosidase H or N-glycanase and analyzed by tricine SDS-PAGE (Fig. 3). The untreated sample (Fig. 3A) had a relative molecular mass of 17 kDa, and this was not altered by treatment with endoglycosidase H (Fig. 3B), while deglycosylation with N-glycanase (Fig. 3C) resulted in a $^3$H band of ~8 kDa. Thus the $^3$H within peak II was associated with the carbohydrate at γAsn-68, consistent with $^3$H incorporation at γTrp-55 in the fragment beginning at γGlu-1.

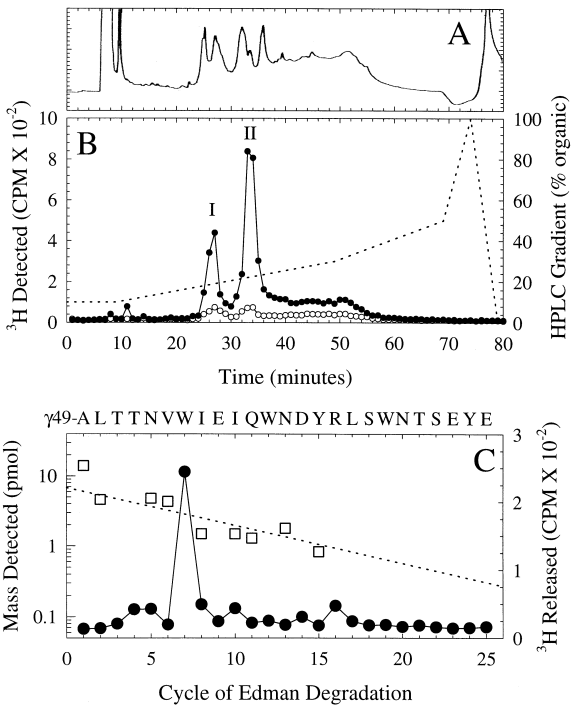


Fig. 2. Identification of sites of [$^3$H]nicotine incorporation in γ-subunit by use of *S. aureus* V8-protease. Aliquots of γ-subunit (170 μg, −carb, 730 cpm/μg; +carb, 144 cpm/μg) were digested with V8-protease and then fractionated by reversed-phase HPLC. Panel A: absorbance at 280 nm; panel B: $^3$H elution profiles with 25 μl aliquots of each 500 μl fraction counted (●, −carb, 122 000 cpm injected, 100% recovered; ○, +carb, 24 000 cpm injected, 91% recovered). The dotted line represents the % organic. Four fractions were pooled from peak I (20 300 cpm) and from peak II (42 700 cpm). Panel C: $^3$H release profile during sequence analysis of material from peak I (●, 2900 cpm loaded, 1030 cpm left after 25 cycles of Edman degradation). Two γ-subunit sequences were present beginning at Val-102 ($I = 39 \pm 3$ pmol, $R = 90 \pm 1\%$) and Ala-49 (□, $I = 7 \pm 1$ pmol, $R = 88 \pm 3\%$). The dotted line represents a non-linear best fit of the mass values from the γGlu-47 peptide as described in Section 2. Release of $^3$H in cycle 7 (224 cpm) was consistent with incorporation at γTrp-55 from the Ala-49 peptide, with a calculated incorporation of 78 cpm/pmol. Sequence analysis of material from peak II in panel B revealed fragments beginning at γGlu-1 ($I = 3.4 \pm 0.4$ pmol, $R = 94 \pm 1\%$) and γLeu-373 ($I = 4.5 \pm 0.5$ pmol, $R = 93 \pm 1\%$). Release of $^3$H was $< 20$ cpm (2800 cpm loaded and 2000 cpm left after 24 cycles of Edman degradation).

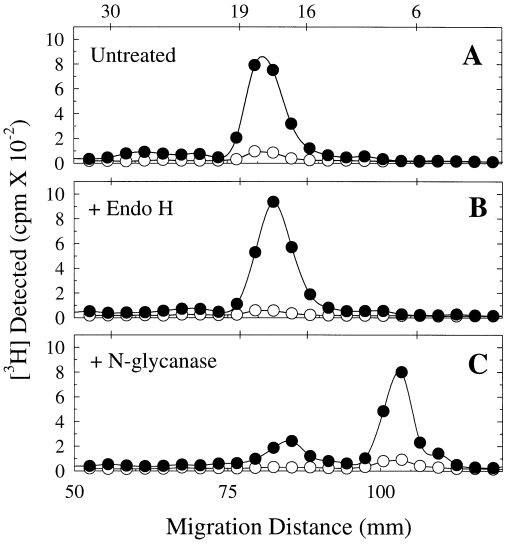


Fig. 3. Glycosidase sensitivity of [$^3$H]nicotine-labeled γ-subunit fragment produced by V8-protease. Aliquots (10 μl, ●, −carb; ○, +carb) of material from HPLC peak II of Fig. 2B were treated as described in Section 2 either without glycosidase (A), with endoglycosidase H (B) or with N-glycanase (C). The digests were then fractionated by tricine SDS-PAGE (10% T/3% C) and the $^3$H distribution was determined by liquid scintillation counting of the gel lanes cut into 3 mm slices. The apparent molecular mass of the $^3$H containing bands were 17 kDa for A and B and 8 kDa for C. Migration distances of molecular mass markers (kDa) are indicated for carbonic anhydrase (30), β-lactoglobulin (19), lysozyme (16), and bovine trypsin inhibitor (6). Only a portion of the gel is shown. The remainder of the gel lanes contained no slices $> 55$ cpm.

## 4. Discussion

The primary site of photoincorporation of [$^3$H]nicotine within the γ-subunit of the *Torpedo* nAChR was identified as γTrp-55 by sequence analysis of peptides isolated after subunit digestion by endoproteinase Lys-C, which cleaves after lysines, and by V8-protease, which cleaves after glutamic acids. The calculated incorporation in γTrp-55 (24 cpm/pmol, Fig. 1) was similar to the calculated specific incorporation in the γ-subunit (460 cpm/μg or 26 cpm/pmol). It is noteworthy that γTrp-55 is also the principle site of photoincorporation in γ-subunit of the competitive antagonist [$^3$H]*d*-tubocurarine [8]. Within α-subunit the principle sites of photoincorporation for both drugs are within the α190–198, but [$^3$H]nicotine reacts primarily with αTyr-198 while [$^3$H]*d*-tubocurarine reacts primarily with αTyr-190 [8,11].

With our lack of knowledge of the precise photochemistry of [$^3$H]nicotine, it is not possible to determine the orientation of nicotine within the agonist binding site, but it is likely that both αTyr-198 and γTrp-55 interact with the same part of the nicotine molecule that is photoactivated, which could be either the aromatic (pyridine) or aliphatic (pyrolidine) nitrogen. While it is possible that there are additional sites in γ-subunit where [$^3$H]nicotine is photoincorporated at much lower efficiency than in γTrp-55, prominent photoincorporation was not seen at γTyr-111 or 117, which contribute to antagonist binding [4,5]. When a gel band containing a fragment beginning at γVal-102 was sequenced (Fig. 2C), the observed $^3$H release at cycles 10 and 16, corresponding to γTyr-111 and

γTyr-117, establishes that any $^3$H incorporation would be at $< 5\%$ the efficiency of γTrp-55. Since there are no aromatic side chains in the primary structure near γLys-34, another agonist binding determinant, [$^3$H]nicotine probably could not photoincorporate into that region even if in proximity, while γTrp-170 is proximal to other agonist affinity determinants (γ172/174) [6,7] without evidence for [$^3$H]nicotine photoincorporation in that region.

Mutational analyses of residues homologous to *Torpedo* nAChR γTrp-55 in a neuronal nAChR have demonstrated effects on both agonist and antagonist binding [18], while substitutions at that position in $GABA_A$ receptor α1- and γ2-subunits affect agonist potency [19] and benzodiazepine binding [20], respectively. In *Torpedo* nAChR substitution of γTrp-55 by Leu reduces both ACh potency and the potency of *d*-tubocurarine (dTC) as an inhibitor [21], but direct analysis of dTC and ACh binding to this mutant establishes that dTC binding is the same as wild-type and that the weakening of dTC potency occurs because there is a major perturbation of the binding of agonist and the mechanism of channel gating [22]. The functional consequences of these substitutions establish that this position plays an important role in the conformational transitions leading to channel gating, but they do not distinguish between direct and indirect interactions with bound ligands. The photoincorporation of [$^3$H]nicotine into γTrp-55 reported here is the first evidence of direct agonist contact with an amino acid in a non-α-subunit.

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