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Supporting Information

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Fluorescent Epibatidine Agonists for Neuronal and Muscle-type Nicotinic Acetylcholine Receptors[**]

Jörg Grandl, Elias Sakr, Florence Kotzyba-Hibert, Florian Krieger, Sonia Bertrand, Daniel Bertrand, Horst Vogel, Maurice Goeldner*[*] and Ruud Hovius*

Synthesis of fluorescent EPBs

[*] Dr. J. Grandl[+], Prof. H. Vogel, Dr. R. Hovius
Laboratoire de Chimie Physique des Polymères et Membranes
Ecole Polytechniques Fédérale de Lausanne (EPFL)
1015 Lausanne (Switzerland)
Fax: (+41) 21 693 61 90
E-mail: Horst.Vogel@epfl.ch

Dr. E. Sakr[+], Dr. F. Kotzyba-Hibert, Dr. F. Krieger, Prof. M. Goeldner
Laboratoire de Chimie Bioorganique UMR 7175 LC1 CNRS
Faculté de Pharmacie, Université Louis Pasteur Strasbourg
BP 24, 67401 Illkirch Cedex (France)
Fax: (+34) 390 24 43 06
E-mail: Goeldner@bioorga.u-strasbg.fr

S. Bertrand, Prof. D. Bertrand
Département des Neurosciences
Centre Médical Universitaire
1221 Genève (Swizerland)

[+] Equally contributing authors

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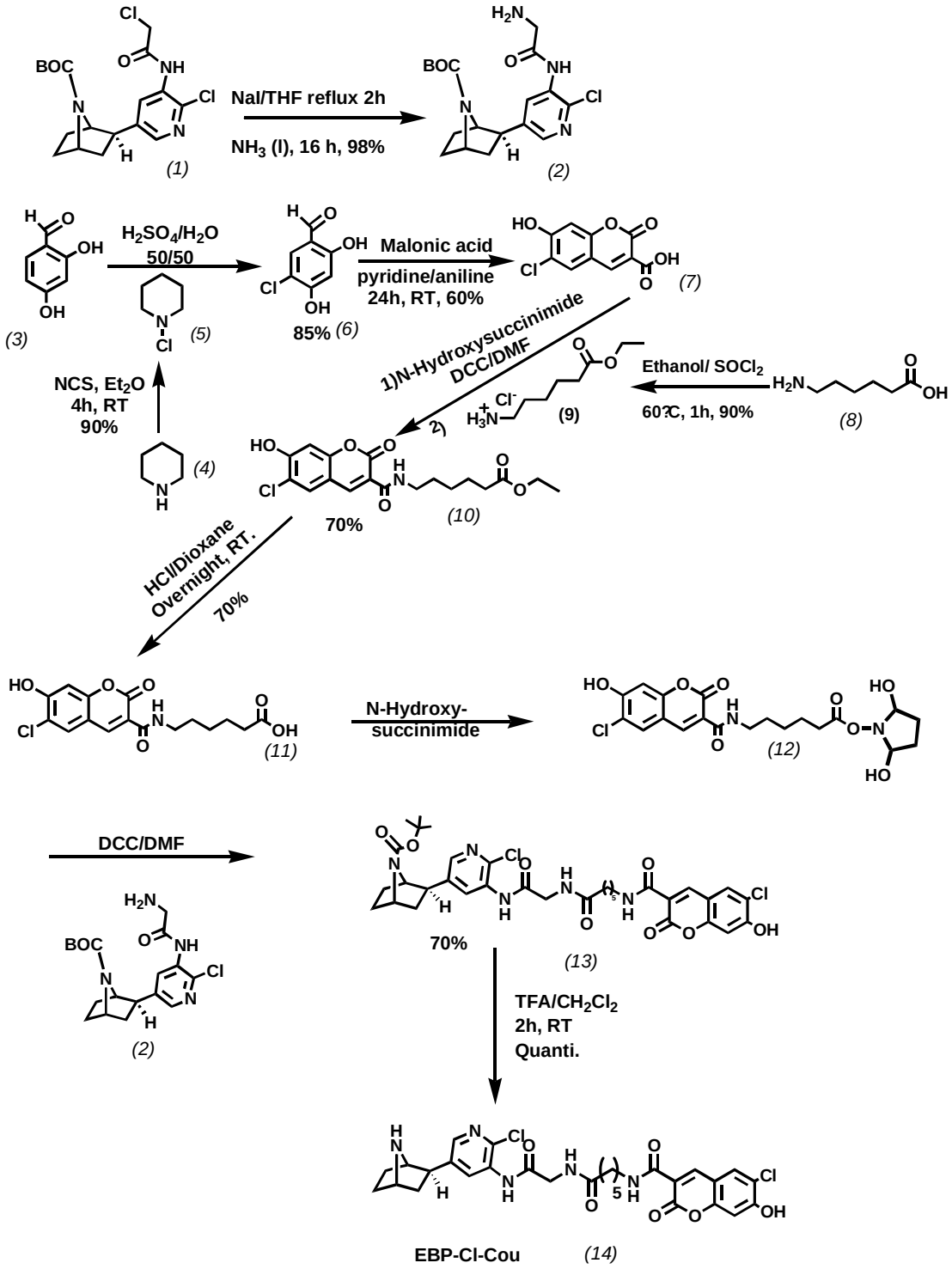
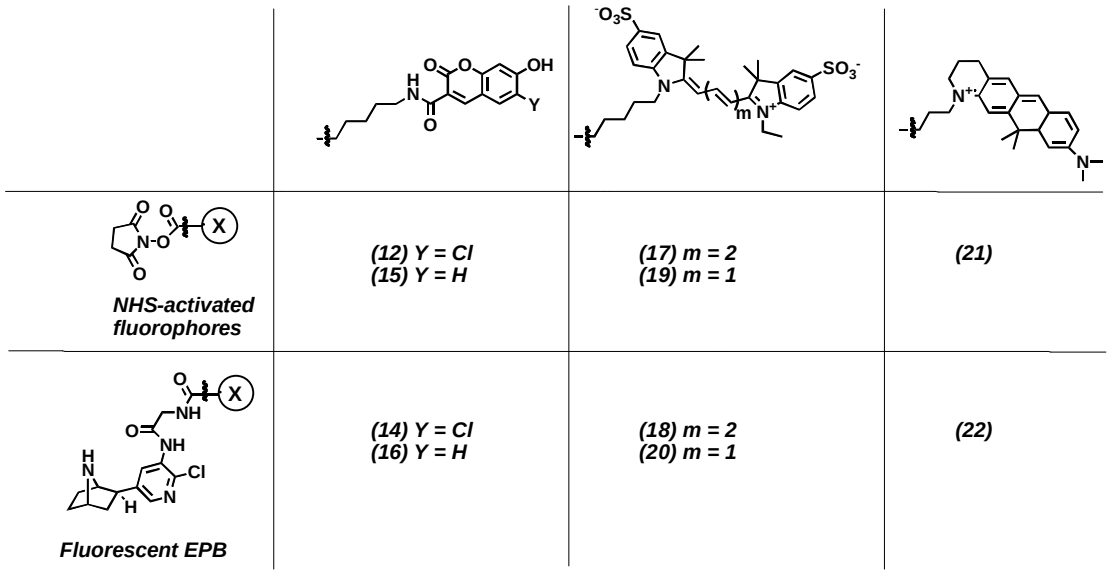


Scheme 1. Overview of the synthesis of EPB-Cl-Cou ***(14)***.

Solvents were dried prior to use according to standard procedures: CH_2Cl_2 and diethylether were distilled under N_2 over calcium hydride; tetrahydrofurane was distilled under N_2 over Na. All solvents were removed by evaporation under reduced pressure. Thionyl chloride and trifluoroacetic acid were distilled freshly before use. Silica gel 60 (40-63 µm) (Merck) was used for column chromatography. NMR spectra

were recorded on Bruker DPX200 or DPX300 spectrometers. The δ (ppm) scale was in reference with the deuterated solvent. The signals are reported as s (singulet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet) and br (broad). Coupling constants (J) are reported in Hertz. UV spectra were recorded on a double beam Uvikon XL spectrophotometer. Mass spectra were recorded on Applied Biosystems Mariner system 5155 and MICROTOF Bruker Daltonique using electrospray ionisation (ESI-TOF). Determinations of purity by HPLC were performed with Waters 600 pump and Waters 2996 photodiode array detector using a Zorbax reversed-phase SB C-18 column (150 mm X 4.6 mm) fitted with a bondasorb C-18 pre-column.

Scheme 1 illustrates the reactions explained below to synthesize EPB-Cl-Cou ***(14)*** as atypical example of a fluorescent EPB. EPB-Cou ***(16)*** was made as ***(14)***, but using the N-hydroxysuccinimide ester of 7-hydroxy-coumarin-3-carboxylic acid[1] ***(15)*** instead of ***(12)***. Commercially available NHS-activated Cy3, Cy5 (both Amersham) and Atto610 (Fluka) were coupled to ***(2)*** as indicated in Scheme 1 of the main text, and Scheme 2 below.



Scheme 2. Activated fluorophores used for the synthesis of fluorescent EPBs.

(±)-7-*tert*-Butoxycarbonyl-2-exo-(2'-chloro-3'-(2-chloroacetyl)amino-5'-pyridinyl)-7-azabicyclo[2,2,1]heptane *(1)*

105 mg (0.542 mmol) of N-Boc-7-azabicyclo[2,2,1]hept-2-ene[2] in DMF (2 mL) was added to a solution of 200 mg (0.784 mmol) of 3-amino-2-chloro-5-iodo-pyridine[3], 90 mg (1.07 mmol) of potassium formate, 40 mg (0.14 mmol) of tetrabutylammonium

chloride and 4 mg (0.017 mmol) of $Pd(OAc)_2$ in 2 mL DMF. After 2 hours at 100°C, the reaction mixture was cooled, hydrolyzed by a saturated aqueous solution of $NaHCO_3$ and extracted with ethyl acetate. The combined organic extracts were dried with Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel matrix using 80% hexane/EtOAc as eluent to yield after solvent evaporation (86 mg, 50%) a solid, which was dissolved in anhydrous CH_2Cl_2 (2.5 mL). 39 µL (0.26 mmol) of triethylamine was added under N_2. The reaction mixture was cooled to 0°C and chloroacetyl chloride (31 µL, 0.39 mmol) was added drop-wise. The reaction mixture was stirred at RT for 2h, then neutralized with an aqueous solution of $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic extracts were dried with Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel using 80% heptane/EtOAc to obtain ***(1)*** as a colourless oil (93 mg 85 %).

^{1}H NMR ($CDCl_3$): δ (ppm) 1.43 (s, 9H), 1.44- 1.62 (m, 2H), 1.75-1.86 (m, 3H), 2 (dd, J = 9 ; 12.4 Hz, 1H), 2.88 (dd, J = 5.2 ; 9 Hz,1H), 4.22 (br s, 3H), 4.38 (br s, 1H), 8.12 (s,1H), 8.62, (s,1H), 8.83 (s, 1H, amine NH). 13C NMR ($CDCl_3$): δ (ppm) 28.67 (3C), 29.18, 30.07, 40.50, 43.32, 45.76, 56.46, 62.27, 80.29, 128.31, 130.84, 138.72, 141.93, 143.79, 155.61, 164.75

(±)-7-tert-Butoxycarbonyl-2-*exo*-(2'-chloro-3'-(2-aminoacetyl)amino-5'-pyridinyl)-7-azabicyclo[2.2.1]heptane *(2)*

Sodium iodide (110 mg, 0.48 mmol) was added to a solution of ***(1)*** (200 mg, 0.48 mmol) in THF (20 mL) and stirred under reflux for 2 h. After cooling of the reaction mixture in a dry ice acetone bath, liquid ammonia (20 drops) was added in one portion to the mixture at –78°C, and the mixture was left overnight at room temperature (RT). After evaporation *in vacuo*, the residue was purified by chromatography using ethyl acetate/methanol (95/5) to obtain 180 mg (98%) of ***(2)***.

^{1}H NMR ($CDCl_3$): δ (ppm) 10.08 (s large, 1H, N-H), 8.68 (d, 2.2 Hz, 1H), 8.10 (d, 2.2 Hz, 1H); 4.37 (s large, 1H); 4.21 (s, 1H); 3.53 (s, 2H); 2.88 (m, 1H), 2.04 (dd, 1H); 1.97 (m, 1H); 1.86 (m, 1H); 1.67 (m, 1H); 1.43 (s, 9H). MS (ES) m/z 381/383 [M+H$^+$];^{13}C NMR ($MeOD_3$): δ (ppm) 28.53 (3C), 29.60, 30.30, 40.54, 45.43, 45.92, 57.38, 63.21, 81.30, 130.81, 132.70, 140.39, 142.90, 144.04, 156.62, 174.13.

N-Chloro-piperidine *(5)*

Piperidine ***(4)*** (8 mL, 6.82 g, 79.8 mmol) was added to a stirred mixture of N-chlorosuccinimide (NCS) (11.9 g, 87.8 mmol) in dry diethyl ether (380 mL) under N_2. The reaction mixture was stirred 4 h at RT, then washed with distilled water and the organic extracts were concentrated on a rotary evaporator to obtain ***(5)*** as a colourless oil (8,7 g, 90%).

^{1}H NMR ($CDCl_3$): δ (ppm) 1.44 (br s, 2H); 1.70 (m, 4H); 3.12 (br, 4H).

2,4-Dihydroxy-5-chlorobenzaldehyde *(6)*

N-chloro-piperidine ***(5)*** (8.48 g, 70.88 mmol) was added drop-wise to a stirred solution of ***(3)*** (8.90 g, 64.4mmol) in 200 mL of 1/1 mixture of H_2SO_4/H_2O. The mixture was stirred at RT under N_2 atmosphere for 24 h and then filtered. The filtrate was washed five times with distilled water. The solid was dried under high vacuum overnight to give the desired product ***(6)*** as a purple powder (9.65 g, 85%).

^{1}H NMR ($CDCl_3$): δ (ppm) 6.21 (s, 1H); 6.63 (s, 1H); 7.53 (s, 1H); 9.71 (s, 1H); 11.27 (s, 1H) MS (ES) m/z 170.96/172.96 $[M-H]^-$.

6-aminocaproate ethyl ester hydrochloride *(9)*

A solution of 6-amino caprylic acid ***(8)*** (3 g, 22.9 mmol) in 15 mL of ethyl alcohol was cooled to 0°C and thionyl chloride (1.8 g, 40.5 mmol) was added drop-wise. The mixture was stirred first at 0°C for 1h, then at 60°C for 1h. The solvent was concentrated under reduced pressure to obtain ***(9)*** as a white solid (3.3g, 90%).

^{1}H NMR ($CDCl_3$): δ (ppm) 1.24 (t, J= 6.8 Hz, 3H); 1.44 (m, 2H); 1.58 (m, 2H); 1.77 (m, 2H); 2.32 (t, J= 7.17 Hz, 2H); 3.02 (br s, 2H); 4.12 (q, J= 7.17; 14.34 Hz, 2H); 8.27 (br s, NH)

N-(5'-Carboxypentyl)-6-chloro-7-hydroxy-coumarin-3-carboxamide *(11)*

6-Chloro-7-hydroxy-coumarin-3-carboxylic acid ***(7)*** was synthesized according to Zhao *et al.* [1]. To a solution of ***(7)*** (200 mg, 0.833 mmol) in DMF (3 mL), N-hydroxysuccinimide (95 mg, 0.833 mmol) was added. After dissolving, the reaction mixture was cooled at 0°C for 1 h and DCC (88 mg, 0.916 mmol) was added. The reaction mixture was stirred first for 30 min at 0°C, then for 2 h at RT. To this mixture, a solution of 6-aminocaproate ethyl ester hydrochloride ***(9)*** (200 mg, 1.25 mmol) and triethylamine (350 µL, 2.5 mmol) in DMF (1 mL) was added. The resulting mixture was stirred at RT overnight before being extracted with ethyl

acetate. The residue was purified by chromatography using ethyl acetate as eluent to obtain 220 mg (70%) of ***(10)***.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.26 (t, J = 7.08 Hz, 3H); 1.57-1.70 (m, 6H); 2.32 (t, J = 7.34 Hz, 2H); 3.5 (m, 2H); 4.13 (q, J= 7.1; 14.2 Hz, 2H); 7.07 (s, 1H); 7.68 (s, 1H); 8.78 (s, 1H). MS (ES) m/z 380/382 $[M-H]^-$.

A solution of ethyl ester ***(10)*** (220 mg, 0.5789 mmol) in dioxane (15 mL) was stirred for 1 h with 2 N hydrochloric acid (20 mL) followed by the addition of 5 drops of 12 N hydrochloric acid. The reaction mixture was stirred overnight at RT. The solvent was concentrated under reduced pressure. The remaining solution was lyophilised to yield ***(11)*** (155 mg, 70%).

EPB-coumarin *(14)*

To a solution of ***(11)*** (10 mg, 0.0283 mmol) in dimethyl formamide (3 mL), N-hydroxy-succinimide (3.5 mg, 0.0283 mmol) was added. After dissolution, the reaction mixture was cooled at 0°C for 1 h and DCC (6.7 mg, 0.0311 mmol) was added. The reaction mixture was stirred for 30 min at 0°C followed by 2 h at RT. To this mixture, a solution of ***(2)*** (20 mg, 0.0526 mmol) and diisopropylamine (90 µL, 0.526 mmol) in dimethyl formamide (2 mL) was added. The resulting mixture was stirred at RT overnight and purified by HPLC. The elution was performed at a flow rate of 4 mL/min with a linear gradient of acetonitrile in an aqueous solution of TFA (0.1%) from 0 to 100% (v/v) over 30 min. The remaining solution was freeze dried to obtain ***(13),*** the BOC-protected derivative of compound ***(14)***. To this BOC derivative dissolved in 2 mL anhydrous methylene chloride, 2 mL of trifluoroacetic acid was added drop-wise. The mixture was stirred at RT for 2 h. The solvent was evaporated under reduced pressure and the compound was finally triturated in an anhydrous HCl/ether solution to obtain quantitatively the hydrochloride salt of compound ***(14)***. The mixture was purified by HPLC. The elution was performed at a flow rate of 4 mL/min with a linear gradient of acetonitrile in an aqueous solution of TFA (0.1%) from 0 to 100% (v/v) over 30 min. The yield was 35% (6 mg).

^{1}H NMR (CD_3OD): δ (ppm) 8.68 (s, 1H); 8.31 (s, 1H); 8.12 (s, 1H); 7.82 (s, 1H); 6.85 (s, 1H); 4.53 (br s, 1H); 4.33 (s, 1H); 4.07 (s, 2H); 3.42 (m, 2H); 2.45 (dd, 1H); 2.34 (t, 2H); 1.90- 2.12 (m, 6H); 1.61- 1.78 (m, 4H); 1.45-1.53 (m, 2H). The compound was further characterized by high resolution MS; $C_{29}H_{32}Cl_2N_5O_6$ $[M+H]^+$ m/z: found 616.1713/ 618.1688, calcd 616.1724/ 618.1703.

EPB-Cy5 ***(18)***

20 µL (0.41 µmol) of Epi derivative ***(2)*** solution (1 mg of ***(2)***, 120 µL of DMF and 5 µL of DIEA) and 30 µL (0.378 µmol) of ***(17)*** solution (1 mg of ***(17)***, 100 µL of DMF) was stirred for 4 h at RT. The mixture was purified by HPLC. The elution was performed at a flow rate of 4 mL/min with a linear gradient of acetonitrile in an aqueous solution of TFA (0.1%) from 0 to 100% (v/v) over 30 min. The remaining solution was freeze dried to give the BOC-protected derivative of ***(18)***. To this BOC derivative dissolved in 2 mL anhydrous methylene chloride, 500 µL of trifluoroacetic acid was added drop-wise. The mixture was stirred at RT for 2 h and purified by HPLC. The elution was performed at a flow rate of 4 mL/min with a linear gradient of acetonitrile in an aqueous solution of TFA (0.1%) from 0 to 100% (v/v) over 30 min. The yield was 76%; HRMS ESI negative external standard H_3PO_4 0.01%: for $C_{46}H_{54}ClN_6O_8S_2$ $[M-H]^-$ m/z: found 917.3114/ 919.3103, calcd 917.3139/ 919.3132; $[M-2H]^{2-}$ m/z: found 458.1511/ 459.1526, calcd 458.1533/ 459.1530; HPLC: t_R = 14 min (99% purity).

EPB-Cy3 ***(20)*** **and EPB-Atto610** ***(20)***

Compounds ***(20)*** and ***(22)*** used a similar procedure to compound ***(18)*** for there syntheses by coupling precursors ***(19)*** and ***(21)***, respectively, to EPB derivative ***(2)*** before final deprotection. Purification by HPLC yielded these compounds with (65%) and (50%) yield, respectively. HRMS ESI negative external standard H_3PO_4 0.01% ***(20)***: for $C_{44}H_{51}ClN_6O_8S_2$: $[M-H]^-$ m/z: found 891.2963/ 893.2955 calcd 891.2982/ 893.2974; $[M-2H]^{2-}$ m/z: found m/z 445.1413/ 446.1409, calcd 458.1455/ 459.1451; HPLC: t_R= 12.5 min (99% purity) and MS (ES) of ***(22)*** $C_{38}H_{48}ClN_6O_2$: $[M-2H]^{2-}$, m/z 327.2475/ 328.2478; HPLC: t_R = 17 min (99% purity).

Fluorescence quantum yields

Fluorescence spectra were recorded in phosphate buffered saline, pH 7.4 at RT using a FluoroLog®-3 spectrofluorimeter (Jobin Yvon). The following fluorescence standards were used to determine fluorescence quantum yields (Φ_f): for EBP-Cou and EBP-Cl-Cou we used quinine sulfate in 0.1 M sulfuric acid (Φ_f = 0.53) [4], for EBP-Atto610, EBP-Cy3 and EBP-Cy5 we used Atto 610-NHS (Φ_f = 0.7), rhodamine 6G in

MeOH (Φ_f = 0.95) [5], Cy5-NHS (Φ_f = 0.27) [6], respectively. The results are shown in Table 1.

Table 1. Optical properties of the fluorescent EPB ligands.

Ligand[a]	ε ($mM^{-1}cm^{-1}$)[b]	QY[c]	λ_{abs} (nm)[d]	l_{em} *(nm)*[e]
EPB-Cou	28	0.80±0.04	406	451
EPB-Cl-Cou	28	0.79±0.04	410	454
EPB-Atto610	150	0.7	615	634
EPB-Cy3	150	0.10±0.02	550	570
EPB-Cy5	250	0.26±0.02	648	670

[a] Abbreviations: see Scheme 1; [b] ε = absorption coefficient; [c] QY = quantum yield; [d] wavelength of absorbance maximum; [e] wavelength of fluorescence maximum.

Functional characterization of fluorescent EPBs

Radioligand binding assays

Binding of [^{3}H](-)-cytisine to rat brain membranes

Binding conditions were modified from the procedures described by Pabreza *et al*.[7]. Samples that contained 200 to 400 μg of protein, 6 nM [^{3}H](-)-cytisine (30.4 Ci/mmol; New England Nuclear) and various concentrations of ligands (0.1 nM to 1 μM) were incubated in a final volume of 200 μl for 75 min at 4°C in triplicate. The suspensions were filtered through GF-C (Whatman) and counted. Non-specific binding was determined in the presence of 10 μM (-)-nicotine. α4β2 is the predominant nAChR in rat brain.

Binding of [^{125}I]-α-bungarotoxin to nAChR containing membranes

[^{125}I]-α-Bungarotoxin (α-BgTx) (Amersham) binding was determined for membranes prepared from transfected HEK cells to express α7-5HT_3 chimeric receptors[8] and from *Torpedo marmorata* containing $\alpha_2\beta\gamma\delta$ nAChR [9].

Binding of α-BgTx to α7-5HT_3 chimeric receptor

The protection constants *K*p of the ligands, which reflect the protection by the probe against the association of the neurotoxin to its specific binding site, were determined from the decrease in the initial binding of [^{125}I]-α-BgTx to α7-5HT$_3$ chimeric receptors (150 pM α-BgTx binding sites in 150 µl), ligand binding domain of the chicken α7 fused to the complementary domain of the mouse 5HT$_3$ receptor. After preincubation of HEK membranes containing α7-5HT$_3$ receptors with ligands in PBS pH 7.2 (supplemented with protease inhibitor, Roche), [^{125}I]α-BgTx (2 nM) was incubated for 6 min at 25 °C, filtered through GF-C (Whatman) pre-coated with dehydrated milk 1% in PBS, and counted. Competing ligands were tested in triplicate at a range of concentrations of 1 nM to 10 µM. Non-specific binding was determined in the presence of 100 µM (-)-nicotine.

***Binding of* *a*-BgTx to *a*$_2$*bgd* nAChR from Torpedo marmorata**

Because of the slow association kinetics of the toxin, protection constants (*K*p) values were determined from the decrease in the initial binding of [^{125}I]-α-BgTx to *Torpedo marmorata* receptors (8 nM α-BgTx binding sites in 150 µl). After preincubation of *Torpedo* membranes with ligands in PBS, pH 7.2 supplemented with Triton X-100 (0.1 %), [^{125}I]-α-BgTx (2 nM) was incubated for 6 min at 25°C, filtered through Millipore HAWP, and counted. Competing ligands were tested in triplicate at nM range concentrations. Non-specific binding was determined in the presence of 150 µM carbamoycholine.

Electrophysiology

Channel activity of neuronal nAChRs expressed in Xenopus Oocytes

cDNA of rat nAChR subunits was injected into oocytes and receptor expression was tested using standard methods as previously described[10]. Oocytes were held at –100 mV using a two electrode voltage clamp (Geneclamp, Axon Instruments, Forster City, Ca, USA). Drugs were dissolved in the superfusion medium just prior to use and applied using electromagnetic valves controlled by a computer. Currents were digitized and stored on a Macintosh using Matlab (Mathworks Inc.). To determine dose-response curves, peak currents were measured and compared to acetylcholine

evoked currents. Values were statistically analyzed using t-Test with two tails and independent standard deviations. Dose-response curves were also fitted using a single empirical Hill equation.

Channel activity of nAChR expressed in HEK cells

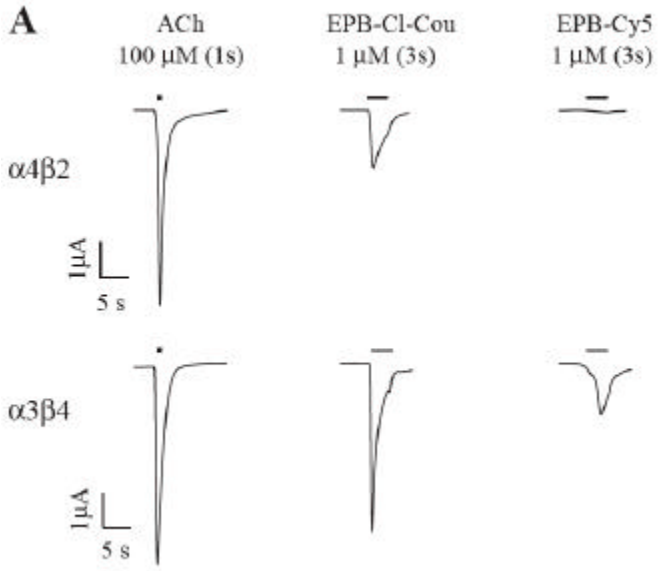
Electrophysiological experiments have been performed on α7-5HT_3 receptors or human foetal muscle-type nAChR, carrying the point mutation δS268K, which shifts concentration-response values for ACh to about 10-fold lower concentration values. This mutation was introduced by PCR using as primers (MWG, Germany) 5' CGGTGCTCCTGGCTCAG**AAG**GTCTTCCTGCTGC and 5' GCAGCAGGAAGAC**CTT**CTGAGCCAGGAGCACCG; the introduced mutations are underlined and the codon for Lys is in bold. cDNA coding for subunits of human foetal muscle-type nAChR or chimerical α7-5HT_3 receptors was co-transfected with cDNA coding for GFP into HEK293-cells using the Effectene Transfection Kit (Qiagen); and experiments were performed 24-48 h later. Recordings were performed in the whole-cell configuration at a holding potential of -60 mV at 18°C on cells that showed green fluorescence. Borosilicate patch pipettes were pulled with a P-87 micropipette puller (Sutter Instruments) and filled with a solution containing 140 mM NaCl, 10 mM EGTA and 10 mM HEPES (adjusted to pH 7.4 with NaOH), showed a resistance of 5-10 MΩ. Transmembrane currents were recorded with an EPC9 patch-clamp amplifier (HEKA). Data were filtered at 2.9 kHz, sampled at 400 Hz and analyzed with the software Pulse (HEKA). Cells were continuously washed with the following solution 147 NaCl mM, 12 mM glucose, 10 mM HEPES, 2 mM KCl, 1 mM $MgCl_2$ (adjusted to pH 7.4 with NaOH).

Solutions containing different concentrations of either epibatidine and its fluorescent derivatives or 100 µM ACh as a reference were applied to the cells using a solution changer (RSC200, Bio-Logic) until the maximal response was reached. Peak currents were baseline corrected, normalized to the current elicited by 100 µM ACh, and fitted using IGOR (Wavemetrics) by a Hill equation yielding the EC_{50} and normalized maximal current values.

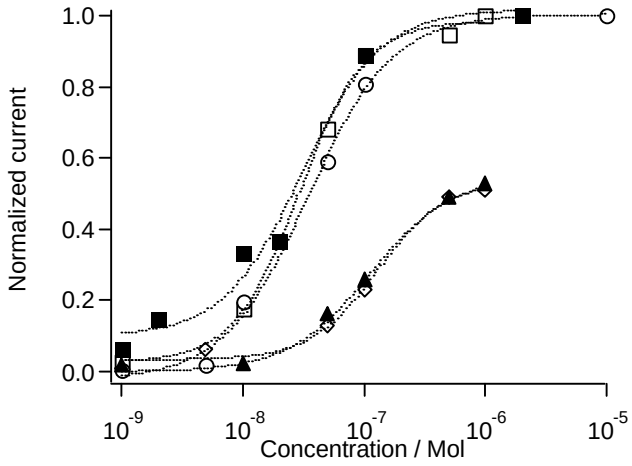
Activation of AchRs by fluorescent EPBs

The ability of the fluorescent EPBs to activate the nAChR was investigated by whole-cell electrophysiology where the rat neuronal α3β4 and α4β2 nAChRs were

expressed in oocytes (Supplementary Fig. 1), and the fetal human muscle nAChR was expressed in HEK cells (Supplementary Fig. 2). Evaluation of the data is presented in Table 2 of the main text.



Supplementary Figure 1. Rat neuronal α3β4 and α4β2 nAChR subtypes expressed in oocytes were challenged with 1 µM EPB-Cl-Cou or 1 µM EPB-Cy5; the extent of activation, normalized to currents evoked by 100 µM ACh, is reported in Table 2 of the main text.



Supplementary Figure 2. Fetal human muscle nAChR with the mutation δS268K expressed in HEK293 cells were challenged with the indicated concentrations of EPB-Cou (○), EPB-Cl-Cou (□), EBP-Atto610 (?), EPB-Cy3 (σ) and EPB-Cy5 (ν). Responses are normalized to currents evoked by 100 µM ACh and fitted by Hill-

equations (dotted lines) from which EC_{50} values are evaluated and listed in Table 2 of the main text.

Microscopic imaging of the binding of fluorescent ligands to living cells

Experiments were performed using an Axiovert 200 inverted wide-field microscope (Zeiss, Germany). An Ar^+-laser at 488 nm for selection of GFP-fluorescent cells or a helium-neon laser (both Coherent, USA) at 633 nm for Cy5 excitation were directed into the microscope equipped with a dichroic mirror (Chroma, USA) and a 63x objective (C-Apochromat, water, 1.2 NA; Zeiss). Fluorescence signals were detected by an intensified CCD camera (I-PentaMAX 512 EFT, Roper Scientific, USA) for 50 ms per image with acquisition rates of 1-12 Hz. Between image recording laser illumination was interrupted by a shutter (LS3T2, Vincent Associates, USA) to reduce photobleaching. Buffer solutions were exchanged using a continuous perfusion system (VC-77SP, Warner Instruments, USA). Movies were recorded using Winview32 software (Roper Scientific).

Experiments were carried out in patch-clamp buffer (see above) with HEK293 cells, growing on 0.17 mm glass slides (Menzel, Germany) and expressing wild-type human adult nAChR. The accumulation and depletion kinetics was fitted by single exponential curves using IGOR. The rate constant of the increasing fluorescence signal was described by a linear combination of the binding rate constant k_{on}, the dissociation rate constant κ_{off} and the rate of photobleaching k_{bleach} of the fluorophores:

$k_{acc} = k_{on} + k_{off} + k_{bleach}$. Correspondingly, the apparent depletion rate constant k_{depl} of the decreasing fluorescence signal was described as $k_{depl} = k_{off} + k_{bleach}$ [11].

Single-molecule tracking

Experiments were carried out as described above at an image acquisition rate of 12 Hz. First, HEK293 cells expressing wild-type adult muscle-type nAChR were perfused with 5 nM Epi-Cy5 for 10 s and then excess of ligand was washed off during image acquisition. Intensity profiles of bright spots were fitted with a 2D-Gaussian function:

$$I = I_0 \cdot \exp\left[\frac{-1}{2(1-cor^2)} \cdot \left(\frac{(x-x_0)^2}{x_{half}} + \frac{(y-y_0)^2}{y_{half}} - \frac{2\cdot(x-x_0)(y-y_0)}{x_{half}\cdot y_{half}}\right)\right]$$

I_0 is the maximum intensity at the mean position coordinates x_0 and y_0, with the standard deviations x_{half}, y_{half}, and a factor *cor* to account for the asymmetry of the 2D-intensity profile. The signal-to-noise ratio and the standard deviations have to fulfil the limit values >1.5 and = 0.17 μm, respectively, for each image in order to validate spots as single molecules [12], in addition to single step photobleaching.

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Supplementary Information - Movie 1: Reversible labelling of nAChR with EPB-Cy5.

Quick-Time movie (100 frames in real time) showing one HEK293-cell expressing adult muscle-type nAChR during 2 cycles of EPB-Cy5 labelling and subsequent washing with pure buffer solution. Kinetics of binding of EPB-Cy5 molecules to nAChR can be measured as accumulation and depletion of fluorescence on the cell membrane.

Supplementary Information - Movie 2: Single-molecule imaging of nAChR labelled with EPB-Cy5.

Quick-Time movie (100 frames in real time) showing the fluorescence signal of one HEK293 cell expressing adult muscle-type nAChR, labelled with 5 nM EPB-Cy5 and excited at 633 nm.