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Long-term exposure to the new nicotinic antagonist 1,2-bis*N*-cytisinylethane upregulates nicotinic receptor subtypes of SH-SY5Y human neuroblastoma cells

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1 Nicotinic drug treatment can affect the expression of neuronal nicotinic acetylcholine receptors (nAChR) both *in vivo* and *in vitro* through molecular mechanisms not fully understood. The present study investigated the effect of the novel cytisine dimer 1,2-bis*N*-cytisinylethane (CC4) on nAChR natively expressed by SH-SY5Y neuroblastoma cells in culture.

2 CC4 lacked the agonist properties of cytisine and was a potent antagonist ($IC_{50} = 220$ nM) on nAChRs. Chronic treatment of SH-SY5Y cells with 1 mM CC4 for 48 h increased the expression of ^{3}H-epibatidine (^{3}H-Epi; 3–4-fold) or ^{125}I-α-bungarotoxin (^{125}I-αBgtx; 1.2-fold) sensitive receptors present on the cell membrane and in the intracellular pool. Comparable data were obtained with nicotine or cytisine, but not with carbamylcholine, *d*-tubocurarine, di-hydro-β-erythroidine or hexametonium.

3 Immunoprecipitation and immunopurification studies showed that the increase in ^{3}H-Epi-binding receptors was due to the enhanced expression of α3β2 and α3β2β4 subtypes without changes in subunit mRNA transcription or receptor half-life. The upregulation was not dependent on agonist/antagonist properties of the drugs, and did not concern muscarinic or serotonin receptors.

4 Whole-cell patch clamp analysis of CC4-treated cells demonstrated larger nicotine-evoked inward currents with augmented sensitivity to the blockers α-conotoxin MII or methyllycaconitine.

5 In conclusion, chronic treatment with CC4 increased the number of nAChRs containing β2 and α7 subunits on the plasma membrane, where they were functionally active. In the case of β2-containing receptors, we propose that CC4, by binding to intracellular receptors, triggered a conformational reorganisation of intracellular subunits that stimulated preferential assembly and membrane-directed trafficking of β2-containing receptor subtypes.
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Abbreviations: Abs, polyclonal antibodies; ACh, acetylcholine; αBgtx, α-bungarotoxin; CC4, 1,2-bis*N*-cytisinylethane; CYT, cytoplasmic peptide; αCntxMII, α-conotoxin MII; Epi, epibatidine; K_d, dissociation constant; K_i, inhibitory constant; MLA, methyllycaconitine; nAChR, neuronal nicotinic acetylcholine receptor

Introduction

Experimental studies on the effects of chronic exposure to nicotine (or nicotinic drugs) on neuronal nicotinic receptor (nAChR) subtypes may help to clarify the tolerance, dependence and withdrawal syndrome associated with nicotine addiction. Furthermore, because of the substantial decrease in nAChRs during ageing, Alzheimer's and Parkinson's diseases (reviewed in (Gotti & Clementi, 2004)), and of the epidemiology and *in vitro* studies suggesting that nicotine may protect against Parkinson's disease (Quik, 2004), investigating the consequences of protracted use of nicotinic drugs might aid our understanding of the pathophysiological mechanisms of certain neurodegenerative conditions.

Post-mortem brain tissue from human smokers (Benwell *et al*., 1988) or from animals chronically exposed to nicotine contains a much higher density of nicotine-binding sites (Marks *et al*., 1985, 1992; Schwartz & Kellar, 1985). This increase varies among different brain regions and mainly involves the α4β2 subtype that is abundantly expressed in the mammalian brain (Fenster *et al*., 1999; Harkness & Millar, 2002; Nelson *et al*., 2003; Gotti & Clementi, 2004; Nguyen *et al*., 2004). The central nervous system also expresses subtypes containing the α3, α5, α6, β3 and β4 subunits, and the α7-containing subtype (the predominant α-bungarotoxin (αBgtx) binding receptor in the mammalian brain). The effects of chronic nicotine treatment on the expression of these subtypes is still a matter of debate (Gentry & Lukas, 2002; Hogg *et al*., 2003; Gotti & Clementi, 2004; Lai *et al*., 2005).

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Even more importantly, it remains debatable if the function (rather than just the number) of nAChRs is enhanced by chronic exposure to nicotine, especially in view of the strong ability of nicotine to desensitise nAChRs (Paradiso & Steinbach, 2003). Following chronic application of nicotine, oocyte-expressed $\alpha4\beta2$ (Peng *et al*., 1994; Fenster *et al*., 1999) and striatal $\alpha6$ receptors (Lai *et al*., 2005) show marked reduction in ACh-evoked currents and function, whereas the opposite occurs for $\alpha4\beta2$ receptors transfected in mammalian cells (Buisson & Bertrand, 2001) or natively expressed by cerebral cortical neurons (Nguyen *et al*., 2004), and for $\alpha7$ receptors of cultured brain neurons (Kawai & Berg, 2001). The biological complexity of studying upregulated receptors has recently been increased by the observation that the extent of this phenomenon depends not only on the presence of $\alpha4$ and $\beta2$ subunits, but also on their relative ratio (Nelson *et al*., 2003; Lopez-Hernandez *et al*., 2004) that is influenced by chronic treatment with nicotine (Nelson *et al*., 2003).

It is noteworthy that chronic application of certain nicotinic antagonists can also enhance nAChR function (Buisson & Bertrand, 2001; Kawai & Berg, 2001; Nguyen *et al*., 2004), though this is not reported in other studies (Peng *et al*., 1997; Wang *et al*., 1998). This issue is of potential importance in understanding the consequences of the strong block of brain nAChR function caused by the β-amyloid peptide present in Alzheimer's disease plaques (Dougherty *et al*., 2003). In fact, interaction between nAChRs and β-amyloid peptide is suggested to be 'a pivotal mechanism involved in the pathophysiology of Alzheimer's disease' (Wang *et al*., 2000).

In the present study, we focused our attention on the effects of chronic nicotinic treatment on $\alpha3$-containing receptors that are natively expressed in nervous tissue (Gotti & Clementi, 2004).

We investigated this aspect using a series of new nicotinic drugs that have recently been synthesised (Carbonnelle *et al*., 2003) starting from the parent compound cytisine, which has been reported to have partial agonist activity on $\beta2$-containing receptors (Covernton *et al*., 1994; Papke & Heinemann, 1994; Chavez-Noriega *et al*., 1997; Houlihan *et al*., 2001; Slater *et al*., 2003), and both partial (Chavez-Noriega *et al*., 1997; Wenger *et al*., 1997; Houlihan *et al*., 2001; Slater *et al*., 2003) and full agonist activity on $\beta4$-containing receptors (Covernton *et al*., 1994; Wong *et al*., 1995; Fischer *et al*., 2005).

These derivatives have a common cytisine skeleton whose amine moiety has been replaced by residues that increase lipophilicity. In particular, we studied one of them, namely 1,2-bis*N*-cytisinylethane (CC4; Carbonnelle *et al*., 2003) consisting of two cytisine molecules joined by a polymethylene chain. Thus, CC4 is a dimer of cytisine that resembles nicotinic blockers (Canu Boido & Sparatore, 1999; Boido *et al*., 2003) such as hexamethonium, decamethonium, *d*-tubocurarine, alkane- and azaalkanediguanidium, possessing two ammonium (or similar cationic) groups joined by a chain, the length of which can change the receptor subtype selectivity (Paton & Zaimis, 1949; Villarroya *et al*., 1996).

We then used SH-SY5Y neuroblastoma cells as a model because they natively express both $\alpha3$ and $\alpha7$ AChRs, resemble human fetal sympathetic neurons and may thus have conserved certain endogenous regulatory mechanisms controlling receptor expression perhaps lacking in transfected heterologous cells. Thereafter, we studied the differential nAChR expression on the plasma membrane and within the cell cytoplasm before and after exposure to CC4 and other cholinergic drugs, identified the upregulated receptor subtypes, and determined whether the membrane-bound receptors were functional.

Methods

$(\pm)^3$H-Epibatidine (Epi) with a specific activity of 56–66 Ci/mmol, was purchased from NEN; ^{125}I-Epi with specific activity of 2200 Ci/mmol was from Perkin-Elmer; ^{125}I-α-bungarotoxin (^{125}I-αBgtx) with specific activity of 200–214 C/mmol was from Amersham; GE Healthcare, Little Chalfont, Buckinghamshire, U.K.; nonradioactive Epi was from RBI; α-conotoxin MII (αCnTxMII) was a generous gift of Dr M.J. McIntosh (University of Utah, Salt Like City, Utah, U.S.A.), the nonradioactive αBgtx and the drugs nicotine, acetylcholine (ACh), di-hydro-β-erythroidine, *N*-methyl-scopolamine, mecamylamine and hexamethonium were from Sigma. Carbamylcholine was from two different sources, namely Sigma (St Louis, MO, U.S.A.) and Fluka (Buchs, SG, Switzerland).

Cytisine derivatives synthesis

Cytisine was extracted from the seeds of *Laburnum anagyroides*, CC1, CC2, CC3, CC4, CC5, CC6 and CC7 were synthesised as previously described (Carbonnelle *et al*., 2003).

Cell culture

SH-SY5Y cells were grown in RPMI 1640 medium (Sigma), supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin–streptomycin at 37°C in a 5% CO_2 humidified incubator. The drugs were sterile-filtered and the pH of the nicotine solution was adjusted with 2 N NaOH before being added to the media.

Binding assays and pharmacological experiments

Binding to cell membranes The cells were collected, rinsed thrice with phosphate buffer saline (PBS) containing 2 mM phenylmethylsulfonylfluoride (PMSF), homogenised in an excess of buffer A (50 mM Tris–HCl, pH 7, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 2 mM PMFS) using a Potter homogeniser, and then washed twice by centrifugation for 60 min at 30,000 × *g* before final suspension in the same buffer containing 20 μg ml^{-1} of leupeptin, bestatin, pepstatin A and aprotinin protease inhibitors.

The saturation membrane binding experiments were performed by incubating cell membranes overnight with 0.01–5 nM ^{3}H-Epibatidine (^{3}H-Epi) at 4°C or 0.01–10 nM ^{125}I-αBgtx at 20°C. To prevent binding of ^{3}H-Epi to the αBgtx-binding receptors, the membranes were first preincubated with 2 μM αBgtx. In the case of ^{125}I-αBgtx, 2 mg ml^{-1} of bovine serum albumine (BSA) was added to the suspension buffer. Specific radioligand binding was defined as total minus nonspecific binding determined in the presence of 250 nM cold Epi or 1 μM cold αBgtx.

To calculate the inhibitory constant (K_i) for each drug, aliquots of the membranes of control or drug-treated cells were incubated with increasing drug concentrations (1 nM to 250 μM for nicotine and cytisine; 0.03 nM to 1.4 mM for CC4; 0.1 nM to 20 mM for carbamylcholine) for 30 min at room temperature,

followed by overnight incubation with a final concentration of 0.15 nM ^{3}H-Epi or 0.75 nM ^{125}I-αBgtx. These ligand concentrations were used for the competition binding experiments because they were within the range of their K_d values for the two classes of nAChRs.

To determine the number of receptors present after treatment with either different concentrations of nicotinic drugs (0.01–1 mM), or a constant 1 mM concentration applied for different times (6–48 h), membrane homogenates were incubated with a saturating concentration of ^{3}H-Epi (2–5 nM) in the presence (nonspecific binding) or absence (total binding) of 250 nM cold Epi, or a concentration of 8 nM ^{125}I-αBgtx in the presence (nonspecific binding) or absence (total binding) of 1 μM cold αBgtx. The same binding protocol was used to determine the number of receptors present after treatment with 100 μM CC4 in the presence or absence of 1.5 mM *d*-tubocurarine. In order to study the effect of a 48 h treatment with 1 mM CC4 on receptor turnover, cells were washed and incubated with fresh medium containing 35 μM cycloheximide (with or without 1 mM CC4), and processed (as described above) at various times (8, 24, 30, 48 and 55 h).

^{3}H-*N*-methyl-scopolamine (specific activity 84 Ci/mmol; Amhersham) was bound to the membrane homogenates by incubating aliquots of membranes diluted in Tris–HCl (50 mM, pH 7.4) with a mixture of protease inhibitors and concentrations of unlabelled ligand ranging from 1 pM to 5 nM for 2 h at room temperature. Nonspecific binding was obtained in parallel from the data in the presence of 1 μM unlabelled ligand. The samples were processed as described above, and their radioactivity was counted in a β-counter.

Binding to whole cells The cells were plated on 12-well plates at a density of 3×10^5 per well. The control cells or cells treated for 48 h with 1 mM CC4 were washed several times with KMB buffer (NaCl 125 mM, KCl 5 mM, D-glucose 10 mM, HEPES 20 mM, $CaCl_2$ 2.5 mM, L-ascorbic acid 5 mM, $MgSO_4$ 1 mM, KH_2PO_4 1 mM, pH 7.4), and binding was performed by adding 2 nM ^{3}H-Epi or 8 nM ^{125}I-αBgTx in the absence (total binding) or the presence (nonspecific binding) of 250 nM unlabelled Epi or 1 μM αBgTx. The contribution of surface ^{3}H-Epi receptors to the total population was determined in cells plated on 12 wells as previously described (Whiteaker *et al*., 1998). The cells (untreated or treated with 1 mM drug concentration for 48 h) were extensively washed with KMB buffer. Binding was carried out by adding 2 nM ^{3}H-Epi in KMB buffer alone (total binding), in the presence of 250 nM cold Epi (nonspecific binding), or 1 (or 5) mM carbamylcholine (a concentration sufficient to block all surface binding). The wells were incubated for 3 h at room temperature and then washed with PBS to remove the unbound ligand. Cells were then dissolved with 500 μl 1 N NaOH, and samples were counted in a β or γ counter.

Binding to 2% Triton X-100 extracts, and immunoimmobilisation of receptors by subunit-specific antibodies The binding to aliquots of Triton X-100 extract, and immunoimmobilisation of receptors was performed as previously described (Moretti *et al*., 2004).

To immunoimmobilise the β2- or β4-containing receptors, the 2% Triton X-100 extract from control or CC4-treated cells was diluted (1 : 2) with buffer A containing 2 mg ml^{-1} BSA and 0.05% Tween 20, and added to affinity-purified human anti-β2 or anti-β4 Abs bound to microwells (Moretti *et al*., 2004). The immobilised receptors were incubated overnight at 4°C with 200 μl of ^{125}I-Epi diluted in buffer A at concentrations of 0.005–1 nM. Specifically labelled ligand binding was defined as total binding minus the binding in the presence of 250 nM unlabelled Epi.

Binding data analysis The experimental data obtained from saturation and competition binding experiments using membrane receptors or immunoimmobilised subtypes were analysed with a nonlinear least-square method using the LIGAND program as described by Balestra *et al*. (2000). The calculated binding parameters were obtained by simultaneously fitting three-four independent experiments.

Cell viability assay

Cell viability assays of SH-SY5Y cells treated with different concentrations of CC4 were carried out with the CellTiter 96 Aqueous One Solution Proliferation Assay kit (Promega) as described in the manufacturer's manual. Briefly, the cells were plated onto flat-bottomed 96-well plates at a density of 2×10^4 cells/well, and cultured for 48 h with different concentrations of CC4. After adding 20 μl of one solution reagent MTS to each well containing 100 μl of fresh culture medium, the plate was incubated for 4 h at 37°C in a humidified, 5% CO_2 atmosphere. Absorbance at 490 nm was recorded using a plate reader.

Evaluation of CC4 stability in the incubation conditions

A 400 ng ml^{-1} solution of CC4 dissolved in cell culture medium was prepared. One aliquot was kept at 4°C for 48 h and another one was incubated at 37°C for 48 h. Both samples were diluted 10 times with trifluoro acetic acid (TCA) in water and injected (1 μl-50 pmol) into the HPLC-ESI-MS system. The analysis was performed with an Agilent (Palo Alto, CA, U.S.A.) 1100 HPLC coupled with an Agilent 1100 MSD trap mass spectophotometer. The column used was a Zorbax RP C 18 (5 μm, 0.5 × 150 mM) and the eluents were A: 0.1%TFA in water and B 0.1%TFA in acetonitrile; the flow was set to 15 μl/min. All mass spectra were acquired in positive ion mode with a capillary potential of 3700 V.

Antibody production and characterisation

The sequences of polyclonal antibodies (Abs) against the human sequence of α2 (CHPLRLKLSPSYHWLESNVDA EEREV), α3 (TRPTSNEGNAQKPRPLYGAELSNLNC), α4 (SPSDQLPPQQPLEAEKASPHPSPGPc), α5 (cgDRYFTQK EETESGSGPKSSRNTLEA), α6 (PRGLARRPAKGKLASH GEPRHLKEC), β2 (RQREREGAGALFFREAPGADSCTy), β3 (LEKAADSIRYISRHVKKEHFc) and β4 (GPDSSPARA FPPSKSCVTKPEATATSPPyg) peptides were chosen for all the subunits of the heteromeric receptors in the cytoplasmic peptide (CYT) loop between M3 and M4, which is the most divergent subunit region. The antibodies raised against the peptides were purified on an affinity column made by coupling the corresponding peptide to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions. The specificity and immunoprecipitation capacity of most antibodies were tested using human transfected cell lines

obtained from Eli-Lilly. To this end, the anti-α2 α3, α4, α5, α6, β2, and β4 Ab specificity and immunoprecipitation capacity were tested on cell extracts obtained from HEK cells transfected with α2β4, α4β2, α3β4, α2β2, α3α5β4, α4α6β4 human subunits (a generous gift from Dr E. Sher, Eli-Lilly and Co., Ltd, U.K.). The anti-α2, α3, α4, β2 and β4 Abs only recognised the Epi receptors present in the cell transfected with the corresponding subunit, and no specific immunoprecipitation was determined using the Abs directed against the other subunits. The immunoprecipitation efficiency ranged from 90 to 100% of the ^{3}H-Epi labelled receptors (mean ± s.e.m. of three independent experiments). The anti-α5 and anti-α6 Abs did not recognise any receptors in the extracts obtained from α4β2, α3β4, α2β2 cell lines; they only recognised receptors in the α3α5β2 (anti-α5) cells and α4α6β4 (anti-α6) cells. The immunoprecipitation efficiency was 50% for the anti-α5 Abs, and 30% for the α6 Abs. We believe that this lower immunoprecipitation capacity was due to the fact that, in these trimeric cell lines, only a fraction of the receptors was actually trimeric.

Immunoprecipitation of ^{3}H-Epi-labelled receptors by antisubunit-specific antibodies

The 2% Triton X-100 membrane extracts from transfected, control treated with 1 mM CC4 cells or the flowthrough samples of the affinity columns, were preincubated with 2 μM αBgtx, labelled with 2 nM ^{3}H-Epi, and then incubated overnight with a saturating concentration of affinity purified Abs (20–30 μg). The immunoprecipitate was recovered by incubating the samples with beads containing bound anti-rabbit goat IgG (Technogenetics, Milan, Italy). The level of Ab immunoprecipitation was expressed either as the percent of ^{3}H-Epi-labelled receptors immunoprecipitated by the antibodies (taking as 100% the amount present in the Triton X-100 extract solution before immunoprecipitation), or as the absolute amount (fmol) of immunoprecipitated receptors per mg of protein.

Receptor subtype immunopurification

Cell membrane homogenates were solubilised by adding 2% Triton X-100 and processed as previously described (Balestra *et al*., 2000). The extracts were incubated thrice with 5 ml of Sepharose-4B with bound human anti-β4 Abs to remove the β4-containing receptors; bound receptors were eluted by competition with 100 μM of the corresponding β4 peptide used for Ab production. The subunit content of the purified β4 receptors was determined by immunoprecipitation using the purified subtypes eluted with the β4 peptide, and that of the flowthrough samples labelled with 2 nM ^{3}H-Epi and subunit-specific Abs.

Gel electrophoresis and Western blotting

The extracts were obtained as described above for control and treated cells were processed as previously described (Moretti *et al*., 2004). The scanned films were quantified using NIH Image.

Sucrose gradient centrifugation

Linear 5–20% sucrose gradients in phosphate buffered saline plus 1 mM PMFS and 0.1% TritonX-100 were prepared using a Buckler (Fort Lee, NJ, U.S.A.) gradient maker, and stored for 4 h at 4°C before use. The volume of each gradient was 12 ml. Of 2% Triton X-100 extracts, 500 μl was obtained from Torpedo electric organ (0.5–1 g, labelled with 8 nM ^{125}I αBgtx) and 500 μl (approximately 300–500 μg of protein) of 2% Triton X-100 extracts obtained from control and CC4-treated cells, were loaded onto the gradients and centrifuged for 14 h at 40,000 r.p.m. Fractions (0.5 ml) were collected from the gradient top and counted with a γ counter (in the case of the Torpedo gradients) or added to the affinity-purified human anti-β2 or anti-β4 Abs bound to microwells. ^{125}I-Epi (0.1 nM) was added to each well and incubated overnight at 4°C. After incubation, the wells were washed seven times with ice-cold buffer containing 0.05% Tween 20, and the bound radioactivity recovered by incubation with 200 μl of 2 N NaOH for 2 h. The bound radioactivity was then determined by liquid scintillation counting in a γ counter.

Northern blot

Total cell RNAs were isolated from control and drug-treated cells using RNAfast-II (Molecular Systems, San Diego, CA, U.S.A.) according to the manufacturer's instructions. Of total RNA, 10 μg were loaded onto a 2.2 M formaldehyde/1% agarose gel, transferred to the membrane with a Turboblotter (Schleicher & Schuell), and UV-cross-linked. The membranes were incubated overnight in a 0.125 M Na_2HPO_4, 1 mM EDTA, 0.25 M NaCl, 7% sodium dodecyl sulphate (SDS) 10% polyethelene glycol (PEG) and 1% BSA buffer containing salmon sperm at 65°C.

Hybridisation was performed using ^{32}P-labelled cDNAs corresponding to the region encoding the cytoplasmic domain of human α3, β2 and β4 subunit at 10^6 CPM ml^{-1} for 2 h at 65°C.

The membranes were washed at 65°C in a stringency of saline-sodium citrate (SSC) 2× +0.1% SDS, SSC 1× +0.1% SDS and SSC 0.1× +0.1% SDS and exposed to films. rRNA 18S cDNA was used to normalise the α3, β2 and β4 signals. The scanned images were quantified using NIH Image.

Electrophysiological experiments

SY5Y cell cultures and patch-clamp recording For chronic drug treatment, the culture medium was replaced after 24 h with the same medium containing 1 mM or 10 μM CC4. The cells were then exposed for 48 h to CC4 and extensively washed (for 45 min) in control saline solution before recording (Di Angelantonio *et al*., 2003b). In brief, cell-containing culture dishes were superfused with control solution containing (mM): NaCl 135, KCl 3.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 15, HEPES 10 (pH adjusted to 7.4 with NaOH, osmolarity 285 mOsm). This solution was applied for about 15 min to naïve cells (30–45 min to chronically treated cells) to ensure full washout of the culture medium. The patch pipettes were filled with (mM) CsCl 130, HEPES 20, $MgCl_2$ 1, Mg_2ATP_3 3, BAPTA 10 (240 mOsm); pH = 7.2. When 5-HT was used as the agonist, the pipette solution was (mM): KCl

140, HEPES 10, $CaCl_2$ 0.5, $MgCl_2$ 2, Mg_2ATP_3 2, GTP 0.2, EGTA 10 (240 mOsm); pH = 7.2. The cells were voltage-clamped at −70 mV (unless otherwise indicated) in the whole-cell configuration (series resistance was compensated by 60%). After the whole-cell configuration was obtained, an equilibration period of 5 min was used to establish adequate solution exchange between the patch pipette and the cell interior.

Drugs and application method Agonists (nicotine or serotonin; 5-HT) were delivered by pressure application (10–20 psi) from glass micropipettes positioned 15–25 μm away from the recorded cell. Puffer applications were used to minimise the strong receptor desensitisation known to develop very rapidly (< 100 ms) especially with nAChRs (Di Angelantonio *et al*., 2003a). Antagonists such as αCntxMII and methyllycaconitine (MLA) were diluted with extracellular solution to the final concentration and applied *via* a rapid superfusion system (Di Angelantonio *et al*., 2003b). The time for solution exchange was about 30 ms. We measured agonist-induced current peak amplitude (from baseline) and decay (from its peak). The latter was quantified on the basis of its time constant (τ), which was best fitted monoexponentially for control cells and biexponentially for the cells treated with 1 mM CC4. The dose–response curves were fitted using a logistic equation. Nicotine-evoked currents in the presence of the antagonist were expressed as a fraction of the current amplitude obtained in its absence. The data were then plotted using the logistic equation to express inhibitory potency in terms of IC_{50} value. In the case of MLA, we used a single concentration known to be selective for the α7-subtype (Jones *et al*., 1999) to avoid loss of nAChR subtype selectivity. The data are presented as mean values ± s.e.m. (n = number of cells). The normal distribution of the raw data was first assessed with SigmaStat, and data meeting this requirement were further analysed using the unpaired Student's t-test. Statistical significance of nonparametric data was assessed with the Mann–Whitney rank-sum test. Multiple comparisons with the control group were made using the Dunn's procedure. A value of $P \leqslant 0.05$ was accepted as indicative of a statistically significant difference.

Results

Effect of chronic treatment with cytisine and cytisine derivatives on ^{3}H-Epibatine receptor expression

Treatment for 48 h of SH-SY5Y cells with different concentrations (10, 50, 200 and 1000 μM) of cytisine and cytisine derivatives (Carbonnelle *et al*., 2003) affected the level of ^{3}H-Epi sensitive receptors. Preliminary experiments showed that CC1, CC2, CC3, CC6 or CC7 evoked a dose-dependent upregulation, which peaked at 200 μM concentration (corresponding to 1.8–2.2-fold increase in receptor number) and then declined. While CC5 (10–1000 μM) did not induce any upregulation, cytisine or CC4 induced the highest level of upregulation (3.5–4-fold at 1000 μM).

Since the strongest upregulation was obtained with CC4, which is also a dimer of cytisine, we decided to investigate in detail the effect of this compound on receptor upregulation.

Upregulation of ^{3}H-Epi sensitive receptors

Initial experiments explored the CC4 concentration and time dependence for changes in receptor expression of SH-SY5Y cell homogenates. As shown in Figure 1a, the highest level of upregulation (five-fold) was obtained using 2.5 mM CC4.

Parallel cell viability assays showed that with CC4 concentrations up to 1 mM there was no deleterious effect. Higher concentrations were, however, toxic (see supplementary Figure 1). For this reason, further studies relied on 1 mM CC4 that yielded a nearly four-fold increase in ^{3}H-Epi binding. The upregulation effect started after 6 h and was strongly developed at 48 h, the time point selected for subsequent experiments (Figure 1b).

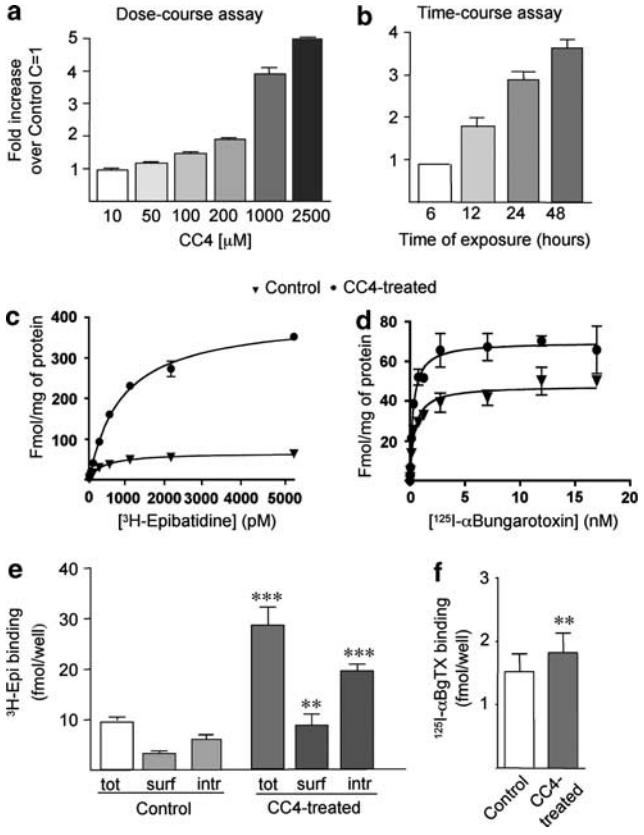

Figure 1 CC4-induced upregulation of nAChRs in SH-SY5Y cells. Dose (a) and time dependence (b) of CC4-induced upregulation of ^{3}H-Epi receptors in SH-SY5Y cells. ^{3}H-Epi labelling of nAChRs was measured on membrane homogenates preincubated with 1 μM αBgtx. The data are expressed as fractional increase (mean ± s.e.m.) over control (n = 3–14 experiments) with the control value being 117.6 ± 18.8 fmol of ^{3}H-Epi receptors/mg of proteins. Saturation curve of specific ^{3}H-Epi (c) and ^{125}I-αBgtx (d) binding to membrane homogenates of control and CC4-treated cells. The binding curves are from a representative experiment in which ligand concentrations were tested in triplicate. (e) Binding to surface and intracellular populations of ^{3}H-Epi receptors. The cells were grown in the presence or absence of 1 mM CC4 for 48 h. The total and intracellular populations of ^{3}H-Epi receptors were determined using 2 nM ^{3}H-Epi (in the presence or absence of 250 nM unlabelled Epi) in the absence or presence of carbamylcholine (5 mM to displace cell surface ^{3}H-Epi binding) and are expressed as fmol/well. Paired t-test with $^{***}P < 0.0001$; $^{**}P < 0.001$. (f) Binding to surface αBgtx receptors (for details see d). The specific binding of ^{125}I-αBgtx to surface receptors expressed as fmol/well was measured using 8 nM ^{125}I-αBgTx (in the presence or absence of 1 μM unlabelled αBgTx). Paired t-test $^{**}P < 0.001$.

In order to differentiate between increased receptor binding affinity and increased number of binding sites, we performed saturation binding analyses which showed the maximum level of ^{3}H-Epi sensitive receptors in the CC4-treated cells to be 417.9 ± 80.8 fmol mg^{-1} of protein, a value significantly larger ($n=7$, $P<0.005$) than that (117.6 ± 18.8 fmol mg^{-1} of protein) of control cells. Conversely, the apparent dissociation constant (K_d) of ^{3}H-Epi binding to control cells was 115 pM (coefficient of variation, (CV) 17%), a value not significantly different from the one of CC4-treated cells (78.5 pM, CV 29%).

We also analysed the saturation binding curves of control and CC4-treated cells (Figure 1c) to find out if there were two classes of binding site with different affinities. However, we could not obtain a statistically better fit by assuming the existence of two sites (see also the results with immunoprecipitation and binding experiments).

SH-SY5Y cells express heteromeric nAChRs on the cell membrane and in their cytoplasm (Peng *et al*., 1997). In order to discriminate whether the receptor increase was restricted to a particular receptor pool, we performed competition binding experiments on intact cells using 250 nM Epi (which binds both intracellular and surface receptors) and 5 mM carbamylcholine which, as a quaternary amine, is a cell impermeable ligand. On intact cells, carbamylcholine inhibited total binding to a maximum of $36.8 \pm 2.6\%$; ($n=11$), whereas at the same concentration it displaced (K_i 1.6 μM) almost the whole fraction of ^{3}H-Epi binding in cell homogenates ($90 \pm 3\%$; $n=5$), suggesting that $>50\%$ of nAChRs were intracellular (see supplementary Figure 2).

Using the carbamylcholine displacement method, we found (mean $\pm$ s.e., $n=10$) 8.7 ± 0.6 fmol of ^{3}H-Epi receptors/well in control cells (3.1 ± 0.4 fmol on the cell surface and 5.6 ± 0.5 in the intracellular pool; Figure 1e). After CC4 treatment, there was an increase in the total number of receptors (24.9 ± 1.1 fmol; $n=8$), of which 6.6 ± 0.8 fmol on the cell surface and 18.4 ± 0.9 fmol in the intracellular pool.

We also checked ^{3}H-Epi receptor binding upregulation induced by other nicotinic drugs at 1 mM concentration (see Table 1): nicotine and cytisine strongly upregulated ($P<0.0001$) membrane-bound and intracellular receptors, mecamylamine only slightly increased their expression ($P<0.05$), whereas carbamylcholine, *d*-tubocurarine, di-hydro-βerythroidine, or hexamethonium did not change receptor expression. Note that drug ability to upregulate nAChRs (Table 1) was unrelated to K_i value on ^{3}H-Epi receptors, because, despite similar nAChR enhancement, nicotine and cytisine had much smaller K_i values (0.37 μM and 0.26 μM, respectively) than CC4 (K_i 30 μM).

Table 1 Effects of nicotinic agonist and antagonist chronic treatments on the expression of ^{3}H-epibatidine binding receptors

	Fractional changes over control	*Surface*	*Intracellular*
Control	1	0.34 ± 0.03	0.66 ± 0.03
Nicotine	3.58 ± 0.45***	0.32 ± 0.04	0.68 ± 0.04
Cytisine	2.78 ± 0.27***	0.33 ± 0.03	0.67 ± 0.03
CC4	2.97 ± 0.31***	0.32 ± 0.03	0.68 ± 0.03
Carbamylcholine	0.84 ± 0.08		
d-tubocurarine	1.08 ± 0.13		
Dihydro-β-erytroidine	0.97 ± 0.09		
Hexamethonium	1.08 ± 0.1		
Mecamilamine	1.38 ± 0.2*		

Control value was 8.69 ± 0.7 fmol. The results were obtained from 3 to 10 separate experiments always performed in triplicate. Statistical analysis used the Student's paired *t*-test. ***$P<0.001$, *$P<0.05$.

Although CC4 and *d*-tubocurarine have similar K_i values (30 and 32 μM, respectively), a large excess of *d*-tubocurarine (1.5 mM; co-incubated with CC4) had no significant effect on the upregulation induced by CC4 (100 μM), as the increase over control cells was 1.21 ± 0.17-fold for cells treated with 100 μM CC4 alone vs 1.21 ± 0.19 fold for cells treated with 100 μM CC4 plus 1.5 mM *d*-tubocurarine ($n=5$).

Upregulation of ^{125}I-αBgtx receptors

As in the case of ^{3}H-Epi sensitive receptors, we first determined the dose-dependent effect of CC4 on αBgtx receptors in SH-SY5Y homogenates ($B_{max} = 68.4 \pm 18$ fmol mg^{-1} of protein) and found that the highest upregulation was obtained with 1 mM CC4 ($B_{max} = 82.9 \pm 18$ fmol mg^{-1} of protein; $n=7$), although the increase was more limited (1.2-fold) than the one of ^{3}H-Epi receptors ($P<0.05$). Saturation binding experiments with homogenates of control and CC4-treated cells showed the increase to be due to enhanced B_{max} values with no change in K_d values (Figure 1d). A statistically significant ($P<0.05$) increase was also obtained when binding was performed with intact cells (the rise in plasma membrane receptor was from 1.51 ± 0.29 to 1.82 ± 0.30 fmol/well; $n=10$; Figure 1f).

Specificity of the effects of CC4 on nicotinic receptors

In order to exclude the possibility that the effects of CC4 were due to drug hydrolysis to cytisine or other breakdown products during cell incubation, we used HPLC to analyse the culture medium of cells exposed for 48 h to 1 mM CC4. We found that that the extracted ion current peak integration of cytisine and CC4 showed a very low cytisine/CC4 ratio (approximately 2%, $n=3$) and that there was no significant difference between the nonincubated and 48 h-incubated samples, indicating that the upregulation of nAChRs was actually due to CC4.

To establish whether the effect of CC4 was nAChR-specific, we investigated expression of SH-SY5Y native muscarinic receptors with saturation binding experiments using the antagonist ^{3}H-methylscopolamine. In control cell homogenates ($n=4$), the apparent K_d value was 117 pM (CV 17%) with $B_{max} = 493 \pm 66$ fmol mg^{-1} of protein, against an apparent K_d value of 86 pM (29%) and $B_{max} = 426 \pm 83.6$ fmol mg^{-1} of protein for the CC4-treated cells: these differences were not statistically significant.

CC4 upregulated receptors are not due to increased post-translation of receptor subunits or changes in receptor half-life

We tested whether the CC4-evoked upregulation of ^{3}H-Epi sensitive receptors was caused by increased transcription of ^{3}H-Epi receptor subunits by making a quantitative Northern blot analysis of mRNA levels for the α3, β2 and β4 subunits that make up the main ^{3}H-Epi sensitive receptors of SH-SY5Y

cells (Balestra *et al*., 2000; Ridley *et al*., 2001). Average values from three independent experiments showed CC4 treated to control ratio of 0.90 ± 0.1 for $\alpha 3$ mRNA, 1.1 ± 0.1 for $\beta 2$ mRNA, and 0.98 ± 0.1 for $\beta 4$ mRNA. These results indicate that CC4 did not upregulate the steady-state amounts of mRNA for the $\alpha 3$, $\beta 2$ and $\beta 4$ subunits, and that upregulation of ^{3}H-Epi sensitive receptors did not depend on trascriptional events.

It has previously been reported that protein synthesis is important for the maintenance of both surface and intracellular nAChRs (Free *et al*., 2005). In particular, the upregulation of the $\alpha 4\beta 2$ subtype in transfected cells is due to a nicotine-induced decrease in its turnover (Peng *et al*., 1994). In order to investigate whether the CC4-induced upregulation of ^{3}H-Epi sensitive receptors might have a similar origin, in control and CC4-treated cells we blocked protein synthesis with cycloheximide (35 μM), and monitored ^{3}H-Epi receptor half-life ($t_{1/2}$) in the presence or absence of CC4 (48 h application). In the absence of CC4, the receptor $t_{1/2}$ value in the control and CC4-treated cells was almost identical (24 and 25 h, respectively, $n = 3$).

While the protein synthesis inhibitor puromycin decreases nAChR expression by bovine chromaffin cells (Schroeder *et al*., 2003; Free *et al*., 2005), the protein synthesis inhibitor cycloheximide applied for 2 days to neuroblastoma cells increases their nAChR expression (Schroeder *et al*., 2003). Cycloheximide induces modest (20%) loss of cells in culture (Cardenas *et al*., 1995; Schroeder *et al*., 2003). While our experiments do not rule out the possibility of direct effects of cycloheximide on nAChRs, the very similar $t_{1/2}$ values of receptors in control and CC4-treated cells after 24 h (despite the very different number of receptors) suggest that the rate of receptor disappearance in the two experimental conditions was analogous and did not strongly influence the total receptor number measured in the current study.

Identification of CC4 up-regulated Epi receptor subtypes

Immunoprecipitation and binding experiments After incubating Triton X-100 (2%) extracts of control or CC4-treated cells with 2 nM ^{3}H-Epi, labelled receptors were immunoprecipitated with saturating concentrations of subunit-specific Abs. As shown in Figure 2a, nearly all receptors contained the $\alpha 3$ subunit, the large majority contained the $\beta 4$ subunit, and a smaller fraction contained $\beta 2$ and/or $\alpha 5$ subunits. There were no receptors containing the $\alpha 4$, $\alpha 2$, or $\alpha 6$ subunits. CC4 treatment led to a large increase in the number of receptors (expressed as fmol of immunoprecipitated receptors/mg of protein, $n = 4$) containing the $\alpha 3$ (from 69.9 ± 15.4 to 185.3 ± 11.6, $P < 0.0001$), $\alpha 5$ (from 22.0 ± 3.5 to 47.8 ± 8.6, $P < 0.0001$), $\beta 2$ (from 41.6 ± 1.1 to 156.7 ± 3.1, $P < 0.0001$) and $\beta 4$ (from 75.3 ± 8.6 to 138.1 ± 2.8, $P < 0.001$) subunits.

We also determined the affinity of ^{125}I-Epi for the receptors present in the extracts obtained from control and CC4-treated cells by performing saturation binding experiments on receptors immunoimmobilised on anti-$\beta 2$ and anti-$\beta 4$ Abs bound to microplates. In control cells, the $\beta 2$-containing receptors had a K_d of 41 pM (CV 27%) and the $\beta 4$-containing receptors had an affinity of 130 pM (CV 21%; $n = 3$). In CC4-treated cells, the $\beta 2$-containing receptors had a K_d of 16.5 pM (CV 33%) and the $\beta 4$-containing receptors had an affinity of 45 pM (CV 41%; $n = 3$).

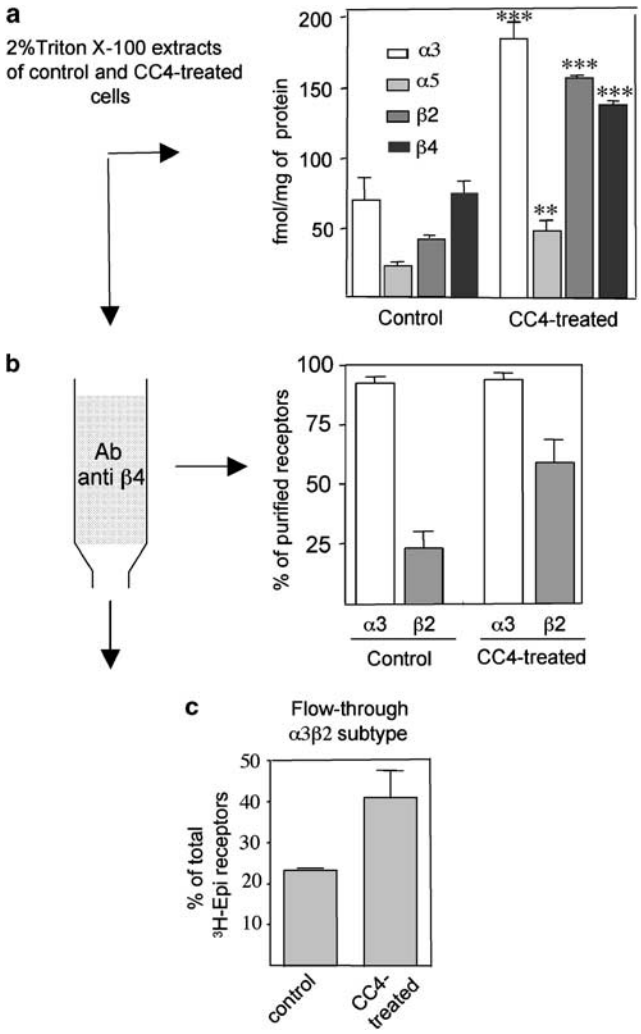

Figure 2 Immunoprecipitation of ^{3}H-Epi-labelled receptors and receptor subtype immunopurification from control and CC4-treated SH-SY5Y cell extracts. (a) An aliquot of the extracts was labelled with 2 nM ^{3}H-Epi and incubated overnight with a saturating concentration of affinity-purified IgG. Immunoprecipitation was carried out as described in Methods, and the results are expressed as mean $\pm$ s.e.m. of fmol ^{3}H-Epi labelled immunoprecipitated receptors/mg of proteins ($n = 4$). The increase in immunoprecipitation for the $\alpha 3$, $\alpha 5$, $\beta 2$ and $\beta 4$ subunits in CC4-treated cells were statistically significative for all four subunits ($P < 0.001$). (b) The remaining extracts were incubated three times with 5 ml of Sepharose-4B with bound human anti-$\beta 4$ Abs in order to immunodeplete the $\beta 4$ containing-subtypes. The bound receptors were eluted by competition with the corresponding $\beta 4$ peptide, labelled with 2 nM ^{3}H-Epi, and then immunoprecipitated by the indicated subunit-specific Abs. Immunoprecipitation was carried out as described in Methods using saturating (20–30 μg) concentrations of antisubunit Abs. The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG, and the results are expressed as the percentage of ^{3}H-Epi binding of purified $\beta 4$-containing receptors present in the solution before immunoprecipitation. Each data point is the mean value $\pm$ s.e.m. of three determinations performed in triplicate. (c) Binding and immunoprecipitation analyses of the flow through of the anti-$\beta 4$-affinity column revealed the presence of ^{3}H-Epi receptors containing the $\alpha 3$ and $\beta 2$ subunits. This population, respectively, represented $23.4 \pm 0.3\%$ and $41.5 \pm 3.9\%$ of the total number of ^{3}H-Epi receptors present in the starting 2% Triton X-100 extracts of control and CC4-treated cells, and was defined as the ($\alpha 3\beta 2$) population. The results are the mean value $\pm$ s.e.m. of three determinations.

We then used immunopurification studies to identify the subtypes of the receptors upregulated by CC4, and investigated their subunit composition. To this end, β4 subunit-containing receptors were first immunodepleted from 2% Triton X-100 extracts of control and treated cells by incubating extracts with Sepharose beads coated with anti-β4 Ab. Immunoprecipitation analysis of receptors (bound by anti-β4 Abs and recovered by competition with the β4 peptide) showed that nearly all receptors contained the α3 and β4 subunits, and that a fraction also contained the β2 subunit (Figure 2b). This indicates that, in addition to the subtype containing α3 and β4 subunits, there was also a subtype containing the α3, β2 and β4 subunits corresponding to $23\pm8\%$ of purified receptors in control cells, and rising to $59\pm9\%$ in CC4-treated cells ($n=3$, $P<0.05$) (Figure 2b).

To identify other subtypes not bound by the β4-affinity column, immunoprecipitation analysis (combined with binding studies of flow-through β4-affinity column) was carried out. We detected immunoreactivity for α3 and β2 subunits, thus indicating the presence of the $\alpha3\beta2$ subtype ($23.4\pm0.3\%$ of ^{3}H-Epi sensitive receptors in the extracts from control cells growing to $41.5\pm3.9\%$ in the CC4-treated cells, $n=3$, $P<0.05$, Figure 2c). Saturation binding experiments performed on these flow-through receptors (containing only the $\alpha3\beta2$ subtype) immunoimmobilised on anti-β2 Abs gave ^{125}I-Epi K_d values of 15 pM (CV 21%) and 13 pM (CV 15%) for control and CC4-treated cells.

Taken together, the immunoprecipitation studies of purified β4-containing receptors plus flow-through experiments indicated that CC4 treatment greatly increased the expression of subtypes containing the β2 subunit ($\alpha3\beta2$ and $\alpha3\beta2\beta4$).

Furthermore, binding experiments performed on immuno-immobilised subtypes confirmed that receptor populations with different affinities for ^{125}I-Epi were present in control and CC4-treated cells. In particular, the $\alpha3\beta2$ subtype present in the flow-through of the β4-affinity column of control or CC4-treated cells had an affinity (13–15 pM) very similar to that obtained from the total extract of CC4-treated (16.5 pM) cells, though higher than the one found in control cells (41 pM). This difference was probably due to the $\alpha3\beta2\beta4$ receptor subtype (immuno-immobilised by anti-β2 Abs) present in a variable ratio with the $\alpha3\beta2$ subtype in control and CC4-treated cells.

The presence of $\alpha3\beta2\beta4$ subunits immunoimmobilised by anti-β4 Abs in control and CC4-treated cells was also likely to be responsible for the different affinity between the β4-containing receptors of control (130 pM) and CC4-treated (45 pM) cells.

Western blotting We also checked the level of the ^{3}H-Epi receptor subunits by separating extracts from control or CC4-treated cells on 9% polyacrilamide SDS gel and analysing them with Western blotting with anti-α3, β2 and β4 Abs. As shown in Figure 3a and b, there was only a modest increase in the level of immunoreactivity for the α3, β2 and β4 subunits in the 2% Triton X-100 extracts from treated cells, clearly less than the one observed with immunoprecipitation experiments (Figure 3b).

Sucrose gradient analysis of ^{3}H-Epi receptors present in control and CC4-treated cells Sucrose density-gradient centrifugation was used to ascertain whether the β2- and β4-containing receptors Epi receptors were incorporated into correctly assembled pentameric subtypes. For this purpose, the size of detergent β2- and β4-containing receptors was measured in control and CC4-treated cells. An identical amount of protein obtained from control and treated cell extracts was loaded onto a 5–20% sucrose gradient and centrifuged. The fractions were collected and stratified on microplates with bound anti-β2 or anti-β4 Abs and labelled with 0.1 nM ^{125}I-Epi. We found that ^{125}I-Epi receptors sedimented in the 11S region both in control and CC4-treated cells as a single species slightly larger than the Torpedo monomer (9 S) but lower than the Torpedo dimer (13 S). No evidence of partially assembled receptors was found. As shown in Figure 3c, CC4-treated cells had approximately 3–4-fold more assembled receptors containing the β2 subunit than control cells, but only twice the number of β4-containing receptors, in accordance with immunoprecipitation data.

These results clearly confirmed that Epi sensitive receptors measured by binding and immunoprecipitation studies were correctly assembled receptors and that there was an increase in both β2- and β4-containing receptors of CC4-treated cells. From our data, it is likely that the increase in β4-containing receptors was mainly due to the large increase in the $\alpha3\beta2\beta4$ subtype.

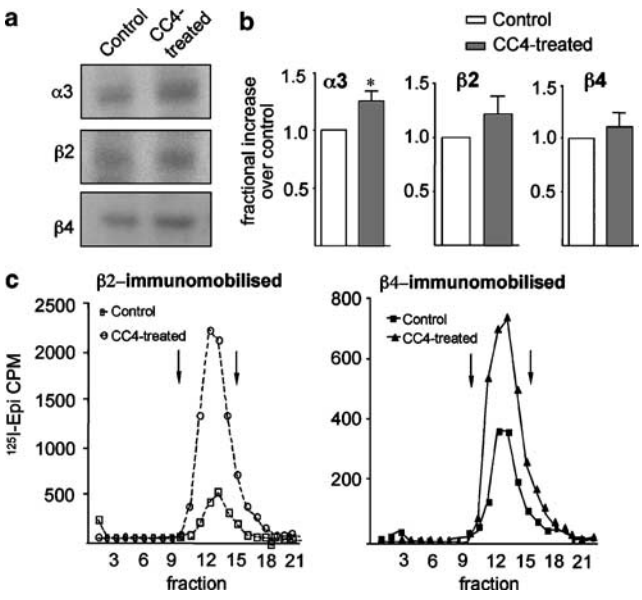

Figure 3 Western blot analysis and quantification of extracts from control and CC4-treated cells (a, b) and sucrose gradient analysis of the β2 * and β4 *-containing ^{3}H-receptors present in the extracts of control and CC4-treated cells (c). (a) Of extracts 20 μg were separated on 9% acrylamide SDS gel, electrotransferred to nitrocellulose, and probed with 10 μg ml^{-1} of anti-α3, β2 and β4 Abs. The bound Abs were revealed by means of ^{125}I labelled protein A. (b) The films were quantitatively analysed using NIH image; the CC4 values, expressed as fractional increase over control, were 1.25 ± 0.08 for α3, 1.21 ± 0.01 for β2 and 1.10 ± 0.14 for β4 (mean $\pm$ s.e.). *$P<0.05$ $n=5$. (c) 500 μl of 2% Triton X-100 extracts were loaded onto a 5–20% (wt/vol) sucrose gradient in PBS pH 7.5, 0.1% Triton X-100 and 1 mM PMFS, and centrifuged for 14 h at 40,000 r.p.m. in a Beckman rotor at 4°C. The fractions were collected, added to anti-β2 or β4 Abs bound to microwells, left for 24 h, and then assayed for ^{125}I-Epi binding as described in Methods. As a standard, ^{125}I-αBgtx labelled Torpedo AChRs were subjected to sucrose gradient centrifugation in parallel, the fractions were collected, and the radioactivity determined by γ counting. The arrows indicate the position of the Torpedo monomer and dimer in each gradient.

Functional and pharmacological characterisation of nicotine-induced currents in the presence of acutely applied CC4

First, we characterised the principal pharmacological properties of CC4 on nicotine-induced currents of control SH-SY5Y cells. A 50 ms pressure application of nicotine (1 mM) induced an inward current whose peak amplitude was reduced by 35% after 15 s preapplied CC4 (100 nM; *via* the fast superfusion system; Figure 4a). This agonist application was selected to produce a maximal response amplitude (also see Figure 4c) with a brief delivery pulse in order to minimise nAChR desensitisation (Di Angelantonio *et al*., 2003a). The CC4-induced block of nicotine currents had relatively rapid onset followed by fast recovery after washout (49 ± 6% after 60 s). It is worth noting that CC4 (up to 1 mM) *per se* did not change the baseline current or input resistance of the cells, thus indicating that it had no agonist activity. Using the same 50 ms puffer pulse duration of 1 mM nicotine and different concentrations of CC4, we quantified the reduction in the nicotine-evoked current. Figure 4b shows a plot of the fractional reduction in current amplitude against log concentrations of CC4 (10 nM–10 μM; $n = 13$); from these data the calculated IC_{50} value for CC4 was 220 nM. Nicotine-induced currents were fully abolished by 10 μM CC4. Figure 4c shows that increasing the duration (5–50 ms) of nicotine pulses progressively increased current amplitude, with apparent saturation being reached with a 50 ms pulse. When the same protocol was repeated in the presence of 500 nM CC4 (15 s pre-pplication), all responses were similarly reduced. Taking the average responses at approximately the midpoint of the curve (10 ms), 500 nM CC4 led to 37 ± 18% depression in comparison with control amplitudes ($n = 9$, $P < 0.05$ for all nicotine doses).

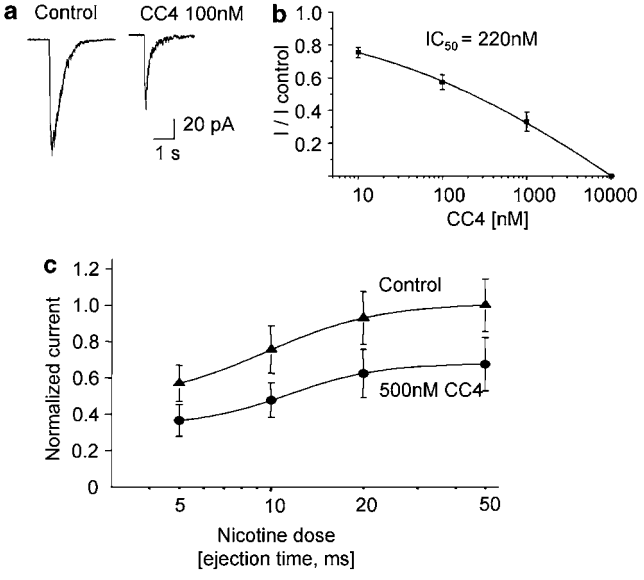

Figure 4 Inhibitory effect of the acute application of CC4 on nicotine-evoked currents in SH-SY5Y cells. (a) Typical nicotine-evoked current (1 mM, 50 ms) under control conditions and in the presence of 100 nM CC4. (b) Plot of nicotine current amplitude (as a fraction of control) vs the log concentration of CC4 ($n = 13$). (c) Nicotine pulse duration–response curve under control conditions and in the presence of 500 nM ($n = 9$, $P < 0.05$ for all nicotine data points).

Larger pulse duration of nicotine application could not restore the full-response amplitude in the presence of CC4 (Figure 4c). This observation cannot, however, be taken to identify the pharmacological nature of the CC4 antagonism because membrane currents were obtained with focal application of the agonist under nonequilibrium conditions. Overall, these data indicate that CC4 was an effective antagonist of SH-SY5Y nAChRs.

Chronic CC4 treatment changes nicotine-evoked currents

To find out if the upregulated receptors present on the cell membrane following chronic exposure to CC4 were functional, we applied 1 mM CC4 for 48 h, washed it out and tested cells for their responses to nicotine pulses (1 mM; 50 ms). Figure 5a compares the inward current induced by nicotine in a control and a CC4-treated SH-SY5Y cell. The treated cell showed a much larger (about 150%) current amplitude and area. Moreover, under control conditions, the deactivation of the nicotine-induced currents was best fitted with a monoexponential function (τ value = 430 ms ± 52; $n = 14$), whereas after treatment with CC4 it was best fitted with a bi-exponential function (τ_1 value = 75 ms ± 10, τ_2 value = 589 ms ± 150; $n = 13$).

The histograms in Figure 5b show raw data of peak amplitude of the nicotine-evoked currents in cells treated with two different concentrations of CC4. After exposure to 1 mM CC4, the amplitude of the nicotine response was significantly enhanced ($P < 0.05$). Similar results were obtained when the area rather than the peak of the nicotine-evoked currents was measured (Figure 5b). After normalising the amplitude values, the peak currents of cells treated with 1 mM CC4 grew to 157 ± 7% of control ($n = 71$; $P < 0.05$). No effect was detected when the cells were treated with 10 μM ($n = 62$), a concentration, however, sufficient to completely block nicotine-evoked current in acute conditions.

We also studied whether chronic treatment with CC4 (1 mM) altered the ability of acutely applied CC4 to antagonise nAChRs. As shown in Figure 5c, 500 nM CC4 uniformly depressed the nicotine-evoked currents in CC4-treated cells with the same pattern of antagonism as that observed in naïve cells (cf. Figure 4c).

The effect of chronic CC4 treatment was selective for nAChRs because the natively expressed receptors for 5-HT were unaffected. In fact, 5-HT (1 mM) puffer-applied (500 ms) to cells elicited a maximal outward current (mediated by 5-HT_{2B} receptors; (Schmuck *et al*., 1994) that was not changed after chronic CC4 (1 mM) treatment (Figure 6a). The histograms in Figure 6b summarise the results obtained from 17 cells to show that chronic CC4 application did not bring about a broad enhancement in membrane receptor function.

nAChR subtype pharmacology after chronic CC4 application

One possibility to account for the different shape of the nicotine-induced current in control and chronically treated cells (see Figure 5a) might have been a change in the relative contributions by different subtypes (Peng *et al*., 1997) to the whole current. In particular, treatment with 1 mM CC4 might have upregulated α7-containing receptors often responsible for the fast activating and inactivating component of the nicotine-

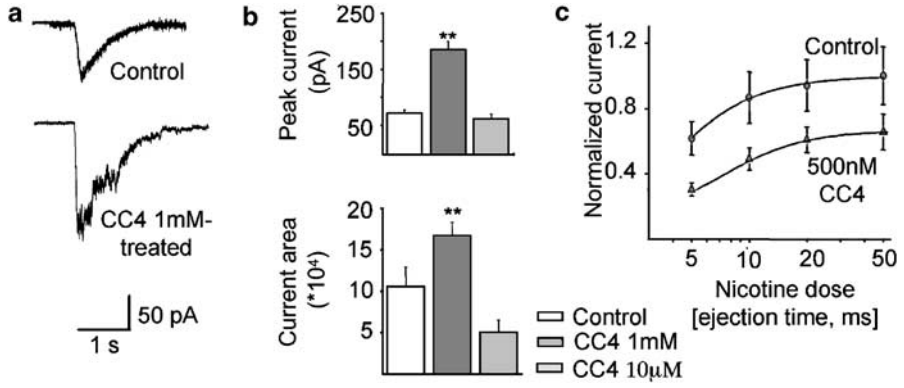

Figure 5 Effects of the chronic application of CC4 on nicotine-evoked currents. (a) Representative nicotine-evoked current (1 mM, 50 ms) in a control cell and a cell treated with 1 mM CC4. (b) Top: mean peak amplitude of nicotine-evoked currents in control cells ($n=68$) and cells treated with CC4 1 mM ($n=71$) or 10 μM ($n=62$). Treatment with 1 mM CC4 significantly increased the mean current amplitude ($P<0.05$). Bottom: mean area of nicotine-evoked currents in control cells ($n=68$) and cells chronically treated with 1 mM ($n=71$) or 10 μM ($n=62$) CC4. Treatment with 1 mM CC4 significantly increased the mean current area ($P<0.05$). (c) The nicotine pulse–response curve was shifted downwards by 500 nM CC4 in the cells treated with 1 mM CC4 ($n=9$, $P<0.05$ for all nicotine doses).

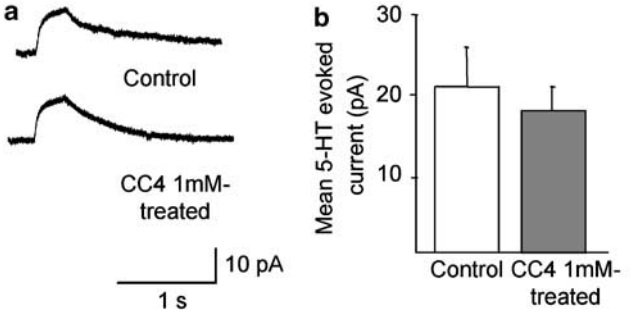

Figure 6 CC4 chronic treatment does not affect 5-HT-evoked current in SH-SY5Y. (a) Example of 5-HT-evoked current (1 mM, 500 ms) in control cells and in cells treated with 1 mM CC4. (b) Mean peak amplitude of the 5-HT-evoked currents in control cells ($n=16$) and cells chronically treated with 1 mM CC4 ($n=17$).

induced current (Jones *et al*., 1999). In order to investigate this issue, we used MLA, a drug selective for the α7-containing subtype at low nanomolar concentration (Jones *et al*., 1999). The histograms in Figure 7a show the fractional reduction in the amplitude of the peak current, evoked by the same nicotine pulse (1 mM, 50 ms) in the presence of 10 nM MLA in control and CC4-treated cells. The inhibitory effect of MLA was significantly ($P<0.05$) higher in the treated cells ($77\pm1\%$; $n=7$) than in control ($88\pm3\%$; $n=6$). An example of the blocking effect of MLA on nicotine-evoked currents in CC4-treated cells is shown in Figure 7b, in which the toxin attenuated the fast, early current component in a manner consistent with a response mediated by α7 receptors (Jones *et al*., 1999). This is in line with the results of the binding studies of surface receptors (Figure 1f) and suggests a contribution by functional α7 receptors in cells chronically treated with 1 mM CC4.

As α3-containing receptors are highly expressed in SH-SY5Y cells (Peng *et al*., 1997; Wang *et al*., 1998), we tested nicotine-evoked currents in the presence or absence of αCntxMII, a toxin highly selective for α3- and α6-containing receptors (reviewed in Gotti *et al*., 2005) although the latter subunit is not expressed in SH-SY5Y cells (see immunoprecipitation results) (Peng *et al*., 1997; Wang *et al*., 1998; Ridley *et al*., 2001).

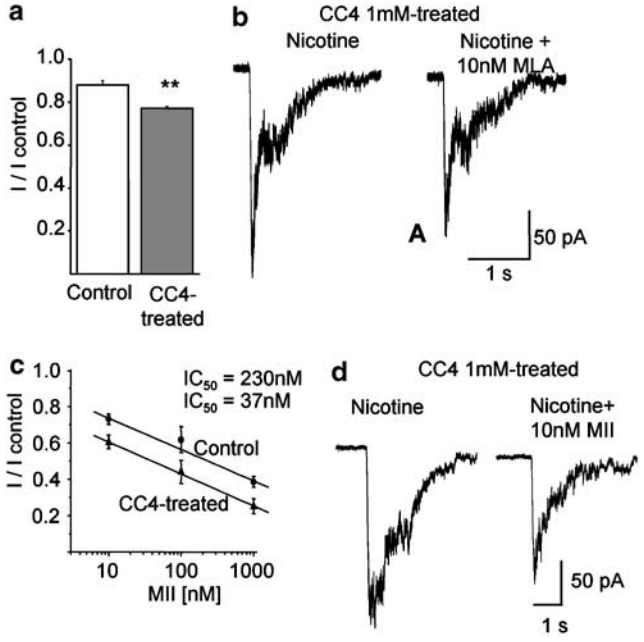

Figure 7 MLA (a, b) and αCnTxMII sensitivity (c, d) of upregulated nAChRs. After washout of CC4 (1 mM), typical currents induced by nicotine were recorded from control and CC4-treated cells in the presence or absence of 10 nM MLA. (a) Fractional reduction in current amplitude (induced by the same nicotine pulse; 1 mM, 50 ms) in control ($n=6$) or CC4-treated cells ($n=7$). The inhibitory effect was significantly greater in CC4-treated cells ($P<0.05$). (b) Representative nicotine-evoked current in a CC4-treated cell before and after application of 10 nM MLA. After washout of CC4 (1 mM), typical currents induced by the same nicotine pulse were recorded from control and CC4-treated cells in the presence or absence of increasing concentrations of αCnTxMII. (c) The graph shows the fractional reduction in amplitude of nicotine-evoked currents in control cells ($n=6$) or CC4-treated cells ($n=8$) with increasing log concentration of αCnTxMII. The IC_{50} value decreased from 230 nM in control cells to 37 nM in treated cells. (d) Representative nicotine-evoked current in a CC4-treated cell before and after application of 10 nM αCnTxMII.

Figure 7c shows the plot of the fractional reduction in current amplitude against different log concentrations of the αCntxMII when the same nicotine pulse (1 mM; 50 ms) was applied to

control and CC4-treated cells. The calculated IC_{50} value (37 nM) was much lower than the one (230 nM) in control. Figure 7d shows an example of αCnTxMII depression of nicotine-evoked currents on a CC4-treated SY5Y cell. The toxin inhibited the slow component of the current, which is consistent with its identification as an α3-containing receptor-mediated response. This result suggests that, after chronic treatment with CC4, functional α3-containing receptors made a larger contribution to the whole-membrane current evoked by nicotine.

Discussion

The present study is the first report of the long-term action of the novel nicotinic drug CC4 on native nAChRs of SH-SY5Y cells. This compound, that had potent nicotinic receptor blocking properties unlike the agonist parent drug cytisine, induced a large upregulation of nAChRs involving $\alpha3\beta2\beta4$, and $\alpha3\beta2$ receptors, and, to a lesser degree, homomeric α7 receptors. We also report that the upregulated nAChRs were functional as demonstrated with patch clamp studies. It is suggested that higher receptor numbers were achieved by increased assembly of pre-existing receptor subunits, perhaps *via* an intracellular site of action of CC4.

Identity of nAChR subtypes of SH-SY5Y cells

Using subunit-specific Abs, extensively tested in transfected human cell lines for their immunoprecipitation specificity and efficiency, we could identify various subtypes of nAChRs. In agreement with previous data (Peng *et al*., 1997; Wang *et al*., 1998; Balestra *et al*., 2000), we found that SH-SY5Y cells expressed α3, α5, α7, β2 and β4 subunits. Control biochemical experiments indicated that heteromeric nAChRs comprised (with or without α5 subunit) $\alpha3\beta4$ (46%), $\alpha3\beta2\beta4$ (30%) and $\alpha3\beta2$ (24%). Electrophysiological data with very low concentrations of the $\alpha3\beta2$ selective αCnTxMII (Cartier *et al*., 1996) and the selective α7 blocker MLA (Gotti *et al*., 2005) confirmed that these receptors were functional and differentially contributed to the kinetics of the nicotine-activated currents. Since either antagonist only partially blocked nicotine-evoked currents, it is plausible that the residual responses were mediated by the other biochemically detected subtypes and for which we lacked selective antagonists.

Plastic changes in receptor activity due to chronic application of nicotinic agents: correlating biochemical and electrophysiological data

Even if long-term exposure of nAChRs to nicotine leads to an increase in receptor number (reviewed by Hogg *et al*., 2003), the identity of these new receptors remains incompletely understood in terms of subunit composition, stoichiometry, post-translational modifications and function. Several studies suggest that receptor upregulation is actually due to the agonist-evoked persistent desensitisation block (reviewed in Hogg *et al*., 2003). It is, however, difficult to ascertain if, under those conditions, all nAChRs are fully desensitized and thus inactivated as a prerequisite to generate upregulation. Hence, it seemed interesting to employ a more direct approach to the link between nAChR block and upregulation by studying whether chronic application of a chemically-stable antagonist could actually change receptor structure and function. In the present study, we investigated how the new cytisine derivative CC4 (a dimer of cytisine) with rapid, strong antagonist effect on nAChRs of SH-SY5Y cells could modulate nicotinic receptors and what mechanisms were underlying its long-term effects.

In analogy with previous studies that had to rely on high concentrations of nicotinic agents largely in excess of the receptor saturating doses (Peng *et al*., 1997; Molinari *et al*., 1998; Wang *et al*., 1998), the CC4 concentration necessary to upregulate receptors was approximately 1000 times higher than the one necessary to block nicotine-evoked currents. Perhaps upregulation implied drug binding to yet unidentified accessory receptor sites with low affinity. While the reason for this discrepancy remains unclear, the observed action was not an indiscriminate upregulation of membrane receptors, because CC4 differentially enhanced native ^{3}H-Epi and ^{125}I-αBgtx sensitive receptors without affecting the expression of muscarinic receptors or the functional responses of 5-HT receptors.

It is noteworthy that, after chronic application of CC4, together with a large rise in the number of intracellular nAChR, an extensive number of them was also detected (with binding and electrophysiological techniques) at membrane level, including heteromeric and homomeric subtypes. The increased number of receptors in the intracellular pool might have therefore provided the substrate for the increased level of surface receptors. By using high-resolution patch clamp experiments on native receptors, we could conclude that increased surface nAChRs were functional at single cell level.

Heteromeric nAChR subtypes upregulated by chronic application of CC4

Chronic CC4 treatment preferentially increased the number of receptors containing the β2 subunits, especially the $\alpha3\beta2$ subtype that has a higher affinity for Epi. Increased expression of subtypes containing the β2 subunit was confirmed by electrophysiological data indicating enhanced blocking potency of αCnTxMII (from 230 nM IC_{50} in control to 32 nM IC_{50} after CC4). αCnTxMII has an apparent affinity (K_i) for transfected human $\alpha3\beta2$ and $\alpha3\beta4$ receptors of 32 and 1500 nM, respectively (C Gotti *et al*., unpublished results) and its IC_{50} on the oocyte-expressed rat $\alpha3\beta2$ subtype is 0.5–2.2 nM, whereas it is 200 times less potent on the $\alpha3\beta4$ subtype (Cartier *et al*., 1996). Although αCntxMII has high affinity and selectivity towards $\alpha6\beta2$-containing receptors (reviewed in Gotti & Clementi, 2004), lack of α6-containing receptors in SH-SY5Y cells suggests that the stronger blocking effect of this toxin observed in the present study should be attributed to its binding to $\alpha3\beta2$ subunits contributing to the overall nicotine-evoked currents.

Mechanisms underlying nAChR upregulation evoked by CC4

Following chronic exposure to CC4, the increased number of total ^{3}H-Epi sensitive receptors might have been due to enhanced synthesis, assembly or stability of an internal precursor, increased efficiency of their insertion into the plasma membrane, or their stabilisation on the cell membrane. Our results show that treatment with CC4 did not affect the level of various subunit mRNAs, had no apparent effect on the half-life of total ^{3}H-Epi sensitive receptors (estimated from

the result of cycloheximide application), and only slightly affected subunit neosynthesis.

Immunoprecipitation and sucrose gradient analysis, however, showed a much higher level of assembled receptors containing the $\beta2$ subunit in CC4-treated cells.

Processes mediating heteromeric receptor upregulation

As far as heteromeric nAChRs are concerned, up-regulation of $\beta2$-containing receptors after chronic nicotine treatment has been previously reported (Peng *et al*., 1997; Sallette *et al*., 2004). Chronic nicotine exposure upregulates $\alpha3\beta2$ to much higher levels than $\alpha3\beta4$ mainly because it promotes maturation of $\alpha3\beta2$ receptors (even in the absence of protein synthesis (Wang *et al*., 1998). By constructing $\beta2/\beta4$ subunit chimeras, Sallette *et al*. (2004) have recently identified two segments (amino acids 74–89 and 106–116) in the extracellular domain of the $\beta2$ subunit which are critical for the upregulation of $\beta2$-containing subtypes. The amino acids in these segments form a compact microdomain that contributes to the subunit interface with the ACh binding site. Sallette *et al*. (2004) suggest that, by binding to immature oligomers, nicotine elicits a conformational reorganisation of the microdomain, strengthens the interaction between adjacent subunits, and facilitates the maturation process toward high-affinity receptors. A more recent study has clearly shown that nicotine enhances a critical step in the intracellular maturation of the $\alpha4\beta2$ receptors (Sallette *et al*., 2005).

Since our present study as well as others (reviewed in Hogg *et al*., 2003) have observed that both agonist and antagonist nicotinic ligands can induce receptor upregulation, this process does not seem to be related to the same conformation change present in channel opening.

In our study, nicotine, cytisine, cytisine derivatives and mecamylamine upregulated ^{3}H-Epi sensitive receptors, whereas the agonist carbamylcholine and other antagonists like *d*-tubocurarine, di-hydro-β-erythroidine, and hexamethonium did not. Although these results are consistent with those previously reported by Peng *et al*. (1997) concerning nicotine, *d*-tubocurarine and di-hydro-β-erythroidine, the lack of chronic effect by carbamylcholine (obtained from two different manufacturers) remains a discrepancy perhaps due to the much longer incubation time (4 days in Peng's *et al*., work).

One factor which might have influenced the drug ability to upregulate receptors is cell permeability. Nicotine, cytisine, mecamylamine and CC4 are lipophylic compounds at least at the concentrations used for upregulation, whereas carbamylcholine, *d*-tubocurarine and hexamethonium are cell-impermeable, charged compounds (Whiteaker *et al*., 1998). Nevertheless, the cell permeability issue does not explain the lack of effect of the lipophylic di-hydro-β-erythroidine. These results suggest that upregulating drugs could act perhaps after their compartmentalisation within the intracellular milieu and binding to immature receptors to trigger a conformation favouring the maturation of $\beta2$-containing receptors. However, the binding characteristics of the upregulation sites indicated low affinity for ligands and a certain pharmacological specificity (e.g. insensitivity to di-hydro-β-erythroidine).

Processes involved in $\alpha7$ receptor upregulation

One interesting finding of this study is that the CC4 treatment also increased αBgtx-labelled receptors, which have characteristics typical of MLA-sensitive $\alpha7$ homomeric receptors (Jones *et al*., 1999). While previous reports indicate that nicotine can upregulate $\alpha7$ homomeric receptors (Peng *et al*., 1997; Molinari *et al*., 1998; Ridley *et al*., 2001), in the present study the extent of this phenomenon was comparatively smaller than for heteromeric nAChRs. The mechanism related to the $\alpha7$ receptor enhancement appears different from the one of heteromeric receptors in so far it depends on receptor neosynthesis (Kawai & Berg, 2001). Furthermore, it is debated if antagonists can upregulate $\alpha7$ receptors (Peng *et al*., 1997; Molinari *et al*., 1998; Ridley *et al*., 2001). The present study observed that the MLA-sensitive component of the nicotine-evoked current mainly corresponded to the fast response peak, indicating relatively rapid kinetics typical of the $\alpha7$ receptor mediated activity (Jones *et al*., 1999). On CC4-treated cells the fraction of the MLA-antagonised current was significantly increased, suggesting upregulation of $\alpha7$ receptors expressed on the cell surface.

Implications for future studies

The present report indicates that, unlike standard nAChR blockers, high concentrations of the novel nicotinic antagonist CC4 could differentially upregulate functional nAChRs. These results imply that nAChR upregulation was not an unselective homeostatic process triggered by sustained receptor block with any nicotinic inhibitor. Since certain nicotinic antagonists are proposed to help the treatment of nicotine addiction and require long-term use (Damaj *et al*., 2004), it is interesting that the possibility of enhancing nAChRs is dependent on the type of antagonist, requires high concentrations and is expressed differentially for various subunits. It is important to note that, in the present study, this conclusion was reached by comparing different receptor subtypes natively expressed by a homogeneous cell population.

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