S.I.S.S.A. I.S.A.S.

SCUOLA INTERNAZIONALE SUPERIORE DI STUDI AVANZATI
INTERNATIONAL SCHOOL FOR ADVANCED STUDIES
Via Beirut 2-4, 34014 Trieste (Italy) tel.040-37871 - fax: 040-3787.249

Studies of neuronal nicotinic receptors of autonomic neurons: expression and function

Thesis submitted for the degree of
"Doctor Philosophiae" in Biophysics

Neurobiology sector

Candidate
Cosetta Matteoni

Supervisor
Prof. Andrea Nistri

TRIESTE

NOTE

The present thesis is based on a series of studies which have been published in the following papers:

Silvia Di Angelantonio, **Cosetta Matteoni**, Elsa Fabbretti, Andrea Nistri (2003) "Molecular biology and electrophysiology of neuronal nicotinic receptors of rat chromaffin cells" European Journal of Neuroscience, Vol. 17, pp. 1–9, 2003

Loredana Riganti, **Cosetta Matteoni**, Silvia Di Angelantonio, Andrea Nistri, Annalisa Gaimarri, Fabio Sparatore, Caterina Canu-Boido, Francesco Clementi, Cecilia Gotti (2005) "Long-term exposure to the new nicotinic antagonist 1,2-bisN-cytisinylethane upregulates nicotinic receptor subtypes of SH-SY5Y human neuroblastoma cells" British Journal of Pharmacology 146, 1096–1109

Elena Sokolova, **Cosetta Matteoni**, Andrea Nistri (2005) "Desensitization of neuronal nicotinic receptors of human neuroblastoma SH-SY5Y cells during short or long exposure to nicotine" British Journal of Pharmacology 146(8):1087-95.

The candidate declares that she personally performed the experiments reported in paper #1 with some collaboration with S. Di Angelantonio for electrophysiological results and E. Fabbretti for molecular biology.
The candidate performed all the electrophysiological experiments reported in paper #2, while data on cellular and molecular biology were obtained in collaboration with the group of C. Gotti (Milan).
The work related to paper #3 was performed in collaboration with E. Sokolova who performed the part related to receptor desensitization.

ABSTRACT

Neuronal nicotinic receptors (nAChRs) are important membrane proteins to signal intercellular communication in health and disease. Against a large body of data related to brain or muscle nAChRs, there is comparatively less information concerning such receptors on autonomic sensory neurons. The present investigation was focused on characterizing the expression and function of nAChRs in chromaffin cells of the rat adrenal medulla, on exploring how novel synthetic compounds could modulate acutely or chronically nAChRs by using SH-SY5Y cells which are tumor-derived cells of chromaffin cell lineage, and how long-term application of nicotinic agents could alter the function of such receptors.

Reverse transcription-polymerase chain reaction analysis indicated the presence of α2, α3, α4, α5, α7, β2 and β4 transcripts (α6 and β3 could not be detected) in rat chromaffin cells. Immunocytochemistry and western blot analysis did not confirm the expression of the α7 subunit. Inward currents elicited by nicotine pulses were insensitive to α-bungarotoxin and low doses of methyllycaconitine, demonstrating lack of functional α7 receptors. Partial block of nicotine currents was observed with either AuIB α-conotoxin (selective against α3β4 receptors) or MII α-conotoxin (selective against α3β2 receptors). Antagonism by dihydro-β-erythroidine (selective at low doses against α4β2 receptors) summated nonlinearly with AuIB and MII inhibition, confirming heterogeneity of neuronal nicotinic acetylcholine receptor populations. These results suggest that the most frequently encountered receptors of rat chromaffin cells should comprise α3β4, α3β2 with the addition of α5 subunits, and much less commonly α2β4, without excluding other subunit combinations.

Using SH-SY5Y cells, more stable in culture and therefore suitable for chronic treatment, we investigated the effect of the novel cytisine dimer 1,2-bisN-cytisinylethane (CC4). On nAChRs CC4 lacked the agonist properties of cytisine and was actually a potent antagonist (IC_{50} = 220 nM). Chronic treatment of SH-

SY5Y cells with 1 mM CC4 for 48 h significantly increased nicotine-evoked currents with augmented sensitivity to the blockers α-conotoxin MII or methyllycaconitine, indicating a relative increase of functional nicotinic receptors comprising β2 and α7 subunits on the cell membrane.

Chronically treating SH-SY5Y cells with nicotine showed that, despite desensitization, they preserved a degree of responsiveness to nicotine pulses, and that they rapidly recovered on washout to generate larger responses without changes in kinetics or pharmacology.

In summary, these data have demonstrated the subunit expression and function of nAChRs on peripheral autonomic cells, and have identified new properties like their sustained ability to preserve function even in the presence of chronic agonist application and their compensatory up-regulation. The present study, thus, sheds new light on the plasticity of nAChRs and outlines new strategies for their pharmacological modulation. Because of the role of nAChRs in the control of chromaffin cell function in regulating blood pressure, it is suggested that changes in nAChR activity might influence the mechanisms responsible for changes in blood pressure in health and disease.

INTRODUCTION

Structure of neuronal nicotinic receptors

Nicotinic acetylcholine receptors (nAChRs) are cationic channel which belong to the superfamily of ligand gated ion channel that includes γ-aminobutyric acid ($GABA_A$), glycine and serotonin 3 ($5\text{-}HT_3$) receptors.

Historically nAChRs expressed by the vertebrate neuromuscular junction and *Torpedo marmorata* or electrical eel electroplaque organs were the first ones to be characterized. Receptors contained in these tissues have been used as a model of nicotinic receptors because of their strong expression which enabled purification studies. In fact, during 70s, a glycoprotein of 290 kDa has been purified from these tissues; it comprises different subunits (named α, β, γ, δ) according to their increasing molecular weight (reviewed by Changeux, 1990).

On the basis of their amino-acidic sequence, nicotinic subunits can be classified into two groups: α and non-α subunits. α Subunits contain disulfide bridges between contiguous cysteine residues (residues 192 and 193 in α1 subunit); non-α subunits do not contain cysteine residues.

Eleven α subunits (α1-10) and seven non-α subunits (β1-β4, γ, δ, ε) have been identified in vertebrate species. α1, β1, γ, δ, ε subunits are typical of the skeletal muscle and fish electrical organs, with $(\alpha1)_2\beta1\gamma\delta$ and $(\alpha1)_2\beta1\gamma\varepsilon$ pentameric structure in fetal or adult form, respectively. Muscle receptors are potently blocked by α-bungarotoxin (α-Bgtx).

Neuronal nAChRs (Fig. 1) can be homomeric or heteromeric. Homomeric receptors bind α-Bgtx and contain five identical subunits (α7, α8 or α9). Between the subunits capable of forming homomers, only the α7 subunit is widely distributed in the mammalian brain. Heteromeric nAChRs are α-Bgtx-insensitive and are generally composed by two α and three β subunits (Fig. 1 B) so that many different

subunit combinations are possible. All α subunit (except α5) can form functional channels when coexpressed with β2 or β4, and more than two different subunits can be present in a single receptor. Heteromeric receptors (α-Bgtx sensitive) containing only α subunits also exist (α7α8 and α9α10). The α8 subunit has been found in avian tissue, but it has not been detected in mammals (for reviews see Paterson and Nordberg, 2001, Gotti et al., 2006, Dani and Bertrand, 2007).

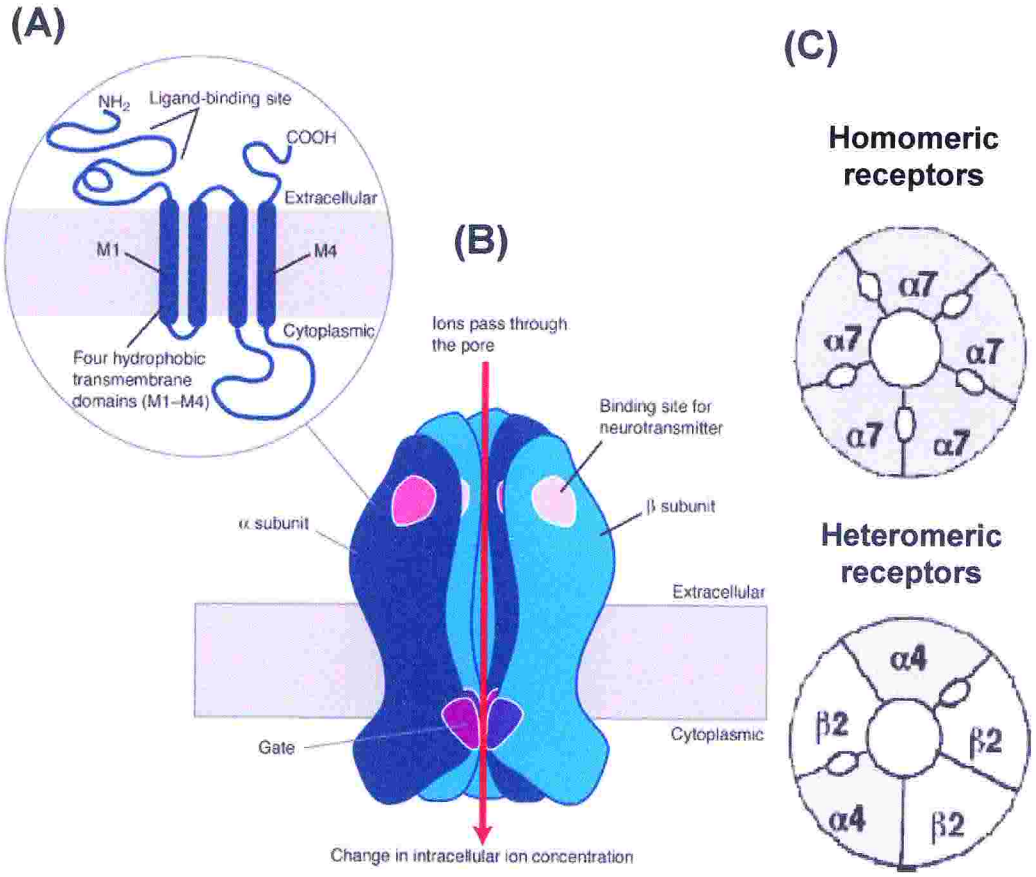

Fig. 1. (A-C) Organization and structure of nAChRs. (A) Schematic representation of the putative transmembrane topology of nAChR subunits. (B) Pentameric arrangement of nAChR subunits in an assembled heteromeric receptor. (C) Subunit arrangement in the homomeric α7 and heteromeric α4β2 subtypes, and localization of the ACh binding site (Gotti and Clementi, 2004).

All nicotinic receptors are composed by five membrane-spanning subunits around a central ion channel (Fig. 1). Each subunit comprises a long hydrophilic extracellular N-terminal domain, a compact hydrophobic domain split into three segments of about 19-27 amino acids termed M1-M3, a short highly variable hydrophilic intracellular loop, a fourth hydrophobic trans-membrane domain, termed M4, and a short C-terminal domain (Fig. 1 A).

The N-terminal domain probably contains phosphorylation sites and plays a role in ligand binding. The M2 trans-membrane domains of each subunit line the ion channel. While the precise subunit composition of nAChRs in the brain *in situ* has yet to be elucidated, it is clear that at least eight functional neuronal nAChR subtypes can be assembled when subunit cDNAs are transfected in oocytes (Luetje and Patrick, 1991, Sequela et al., 1993): several of these receptors have physiological and pharmacological properties similar to the native receptors found in the CNS (Mulle et al. 1992). The combination of the neuronal nAChR subunits present in a given pentamer dictates the pharmacological and functional properties not only via interactions at the acetylcholine binding site, but also through separate sites distinct from the classical binding site.

Acetylcholine binding site

Muscle receptors have two acetylcholine (ACh) binding sites that interact in a positive, co-operative manner: both sites must be occupied by ACh (or other nicotinic agonists) to induce channel activation. The amino-acids which contribute to the ACh binding site in muscle and *Torpedo* organ are at the interface between α and β subunits and involve cysteine-residues 192 and 193 (Kao and Karlin, 1986; Galzi and Changeux, 1995). Similarly, in heteromeric neuronal nicotinic receptors, two ACh binding sites are thought to exist at the interface between α and β subunits (Fig. 1C) (Alkondon and Albuquerque, 1993). Conversely, homomeric receptors

contain five identical ACh binding sites, due to the identical nature of the α subunits making up the receptor protein (Fig. 1C).

Agonists of neuronal nicotinic receptors

Other classical nAChR agonists are nicotine (Nic), dimethylphenylpiperazinium (DMPP) and cytisine (Cyt) (reviewed by Gotti et al., 1997). Although the majority of the available agonists demonstrate limited selectivity, there is a rank order of potencies that depends on the different α/β subunit composition of oocyte-expressed receptors and can be used for subtype characterization (Papke, 1993). Some agonists can also act as functional antagonists under certain experimental conditions: for example, cytisine seems to competitively inhibit the ACh response of β2 containing receptors (whereas it activates β4 containing receptors) (Papke, 1993), but these findings differ from other studies with an α4β2 transfected cell line in which cytisine is as active as nicotine in stimulating ACh release (Marks et al., 1993). Similar unusual agonist/antagonists profile as been observed with nicotine, which can function as an antagonist at the α9 homo-oligomer subtype (Elgoyhen et al., 1994).

EC_{50} values for different nAChR subtypes and principal agonists are shown in Table 1 and their chemical structure in Fig. 2.

Table 1. Potency (EC_{50}) of the principal nicotinic agonists on different human nicotinic subtypes (Chavez-Noriega et al., 1997)

Agonist	α4β2	α3β2	α3β4	α7
ACh	68.05 μM	442.90 μM	203.14 μM	179.57 μM
Nic	5.47 μM	132.44 μM	80.30 μM	113.34 μM
Cyt	2.61 μM	67.09 μM	72.18 μM	71.42 μM

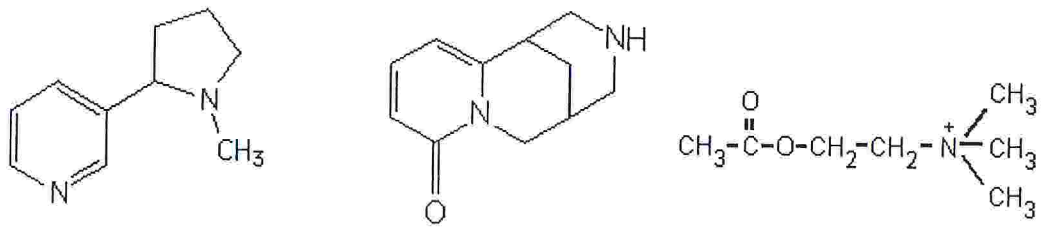

Fig. 2. Chemical structure of the principal nicotinic agonists.

Antagonists of neuronal nicotinic receptors

Neurotoxins, some of which are large peptides, have been used to distinguish neuronal nicotinic subunit combinations *in vitro*. These distinct agonist/antagonist profiles observed in oocytes suggest that it may be possible to develop novel agents selectively targeting different nAChR subtypes *in vivo*.

Neuronal bungarotoxin (n-Bgtx) completely blocks ACh-induced currents in oocytes injected with α3β2, but it has no effect on α2β2 and α4β2 functions (Luetje et al., 1990).

The competitive antagonists dihydro-β-erythroidine (DHβE) and erysodine are alkaloids isolated from erythrina seeds; they display nanomolar affinity for neuronal nAChRs. [^{3}H]DHβE binding has a regional distribution similar to that seen with [^{3}H]-Nic and can be displaced by nicotine and the nicotinic agonists lobeline and cytisine, but not by the nAChR antagonists, mecamylamine, pempidine and hexamethonium. In addition, ACh gated-currents in *Xenopus* oocytes containing α4β2 subunits are 16- and 27- fold more sensitive to antagonism by DHβE than are ACh-gated currents in oocytes expressing α3β2 or α2β2 subunit combinations (Luetje, 1990). These findings suggest that DHβE may exhibit a certain degree of subtype selectivity. DHβE has been suggested to be selective for α4β2 receptors

when used at sub-micromolar concentrations (Chavez-Noriega at al., 1997; Zoli et al., 1998).

Methyllicaconitine (MLA), isolated from the *Delphinium brownii* plant, is a potent (Ki = 4 nM) competitive inhibitor of [^{125}I]α-Bgtx binding in rat brain. MLA has 200-fold greater selectivity for α7 compared with (α1)2β1γδ receptors, and 50- to 100-fold greater selectivity compared with α3β4, α4β2 and α3β2 subtypes when expressed in oocytes. MLA is, thus, the only commercially-available antagonist that differentiates clearly between α-Bgtx sensitive sites on neuronal and muscle nAChRs. MLA is 1,000-fold more selective for rat α7 than α4β2 subtypes (Dowskin et al., 2001).

Chlorisondamine and mecamylamine are non-competitive nicotinic antagonists that do not inhibit [^{3}H]-ACh or [^{3}H]-Nic binding to rat brain (El-Bizri and Clarke, 1994, Schwartz et al., 1982). These compounds are believed to act by blocking the nAChR channel.

AuIB and MII, two toxins isolated from the venom of the *Conus* sea snail, are believed to be selective (at nM concentrations) and very potent antagonists against α3β4 and α3β2 receptors respectively (Fig. 3) (Cartier et al., 1996, Kaiser et al., 1998, Luo et al., 1998).

Table 2 shows the differential sensitivity of principal nicotinic subtypes to some nicotinic antagonist.

Table 2. Sensitivity (IC_{50}) of principal nicotinic subtypes to various partially selective agonists. (Cartier et al., 1996; Chavez-Noriega et al., 1997; Dwoskin and Crooks, 2001; Luo et al., 1998)

Antagonist	α4β2	α3β2	α3β4	α7
DHβE	0.11 μM	1.62 μM	13.77 μM	19.69 μM
MLA	~2.5 nM	~2.5 nM	~2.5 nM	25 pM
MII	0.4 μM	0.5 nM	1.1 μM	0.1 μM
AuIB	>100 μM	~750 nM	~0.75 nM	7.5 nM
d-tubocurarine	3.16 μM	2.41 μM	2.24 μM	3.10 μM

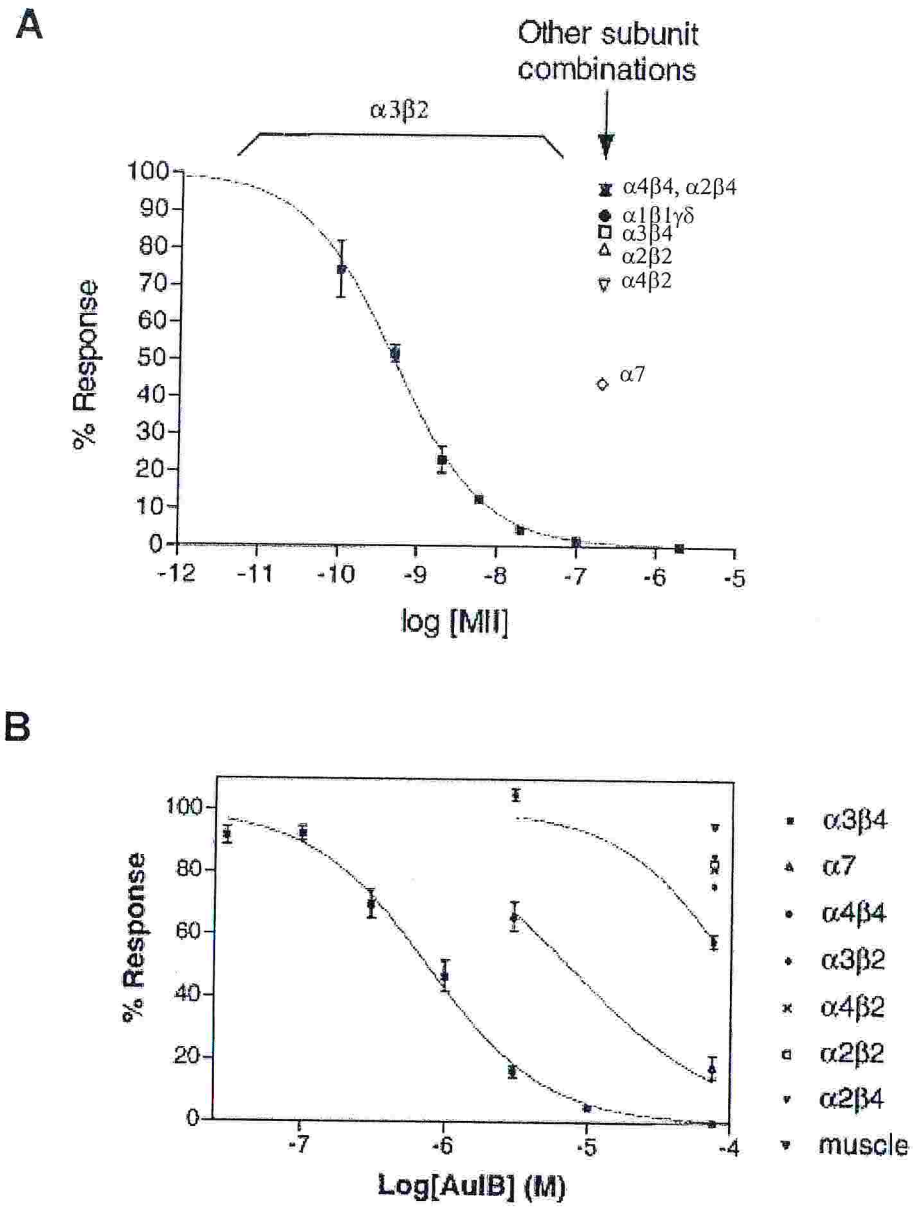

Fig. 3. Selectivity of a-conotoxin MII (A) and AuIB (B). (A) Oocytes expressing various nAChR subunit combinations were voltage clamped and the response to ACh measured. The IC_{50} for α-conotoxin MII block of the α3β2 receptor is 0.5 nM. Oocytes expressing other nAChR subunit combinations were perfused with 200 nM α-conotoxin MII (400 X the IC_{50} on α3β2 receptors). The mean % response to ACh was as follows: α4β4 96, α2β4 96, α1β1γδ 89, α3β4 85, α2β2 80, α4β2 70, α7 44. (Cartier et al., 1996) (B) α-conotoxin AuIB preferentially blocks α3β4 versus other nAChR subunit combinations. The dose–response curve shows that α-conotoxin AuIB blocks α3β4 receptors with an IC_{50} of 0.75 mM. By comparison, AuIB is about 10-fold (α7) and >100-fold less potent on other nAChR subtypes. (Luo et al., 1998)

Treatment of SH-SY5Y cells with different concentrations of cytisine and cytisine derivatives (Carbonnelle *et al.*, 2003) affects the level of ^{3}H-Epi sensitive receptors. Cytisine and 1,2-bisN-cytisinylethane (CC4; Fig. 4)) induce the highest level of up-regulation (3.5-4 fold at 1000 μM). CC4 is a potent nicotinic receptor antagonist as demonstrated in the present thesis.

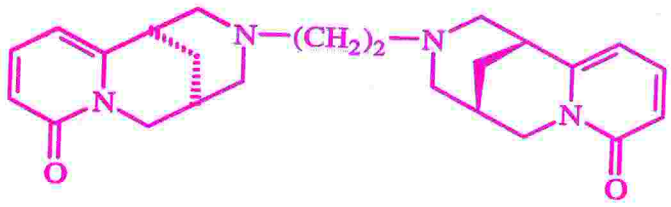

Fig. 4. CC4 chemical structure.

Allosteric modulators

The function of neuronal nAChRs (like the activity of most ionotropic neuroreceptors in the vertebrate brain) is subject to modulation by a variety of chemically different substances that can modify the properties of nAChRs via interactions with sites other that the classical ACh binding sites. This process is called allosteric modulation (reviewed by Paterson and Nordberg, 2000). Allosteric effectors that potentiate nAChR function are positive effectors (for example, the cholinesterase inhibitors physostigmine, galantamine and tacrine and the muscle relaxant benzoquinonium); those that inhibit are negative effectors (including chlorpromazine, phencyclidine, local anesthetics, ethanol and barbiturates).

Other compounds act as nicotinic receptor modulators by binding specific sites on the receptor protein, for example steroids and L-type Ca^{2+} channel antagonists.

Different steroids produce positive or negative modulation. A small number of amino-acid residues at the C-terminal end of the α4 subunit mediate 17-β-estradiol

potentiation (Paradiso et al., 2001). This example illustrates the importance of the subunit-specific structural domains to enable allosteric modulation .

Phosphorylation by protein kinase A, protein kinase C or tyrosine kinase of certain residues within the cytoplasmic loop results in desensitization of the receptor channel. A variety of pharmacological substances indirectly enhances nAChR desensitization via phosphorylation. This generally occurs through changes in intracellular Ca^{2+} concentration and activation of Ca^{2+} sensitive protein kinases. For example, the neuropeptide CGRP (calcitonin gene related peptide) and substance P both enhance nicotinic receptor desensitization through activation of phosphorylating enzymes (Paterson and Norberg, 2000; Di Angelantonio et al., 2003).

Activation and desensitization of nAChRs

Although different subtypes display a range of different functional and pharmacological properties, they share basic features. They occupy three main functional states in response to agonists: closed at rest, open channel, and closed because desensitized. Upon binding ACh, the nAChR ion channel is stabilized in the open conformation for several milliseconds; thereafter, it closes to a non-conducting state. Prolonged exposure to low concentrations of nicotine, as obtained during tobacco use, produces significant desensitization, which stabilizes the receptor in a closed state unresponsive to agonists for milliseconds or seconds (Giniatullin et al., 2005). When open, nAChRs have high permeability to cations that cause membrane depolarization and may produce further intracellular signals (especially in the case of Ca^{2+}).

Although sodium and potassium carry most of the nAChR current, calcium can also make a significant contribution (Castro and Albuquerque, 1995, Decker and Dani, 1990, Vernino et al., 1992 Vernino et al., 1994). Unlike voltage-gated calcium channels and NMDA receptors, that require membrane depolarization to pass

current freely, nicotinic receptor channels open and readily pass current at negative potentials because these provide a strong driving force for cation entry into the cell owing to the fact that the reversal potential is near 0 mV. Thus, calcium influx mediated by nAChRs has different voltage dependence from one of other calcium permeable ion channels, and a different spatial distribution depending on the location of nAChRs on the cell surface.

Desensitization is a general characteristic of ligand-gated ion channels, whereby a decrease or loss of biological response occurs following prolonged or repetitive receptor activation. While desensitization was first investigated at the skeletal neuromuscular junction, even brain nicotinic acetylcholine receptors can readily desensitize (Giniatullin et al., 2005). Usually at a cholinergic synapse, vesicular release produces a high ACh concentration in the synaptic cleft for only a few milliseconds before diffusion and acetylcholinesterase remove the neurotransmitter. In response to these high ACh concentrations, the high-affinity $\alpha4\beta2$ nAChRs desensitize with slower kinetics than the rapidly desensitizing $\alpha7$ nAChRs. Because of their high affinity for agonist, the $\alpha4\beta2$ nAChRs display significant, slowly-developing desensitization for agonist concentration below 0.1 μM.

On the other hand, the $\alpha7$ receptors are not effectively desensitized by agonist concentrations below approximately 1 μM ACh (Wooltorton et al., 2003). A comparable condition is observed if agonists, such as nicotine or nicotinic drugs, are given systematically for a prolonged time period, for example during smoking, or during treatment of Alzheimer's disease (AD) with cholinesterase inhibitors, or as a result of poisoning by an anticholinesterase agent.

Although the role of nAChR desensitization in normal cholinergic transmission remains unclear, its potential to control cholinergic activity and induce adaptive changes is considerable so that desensitization appears to be an important component of nAChR function. In the short period of seconds to minutes, nAChR desensitization underlies the brief skeletal muscle paralysis caused by agents such as

succinylcholine during general anaesthesia (Tuba et al., 2002). Over a longer time-frame, nAChR desensitization might be important to determine the therapeutic efficacy of various nicotinic drugs currently used to treat the cholinergic dysfunction associated with neurodegenerative disease (Jonnala and Buccafusco, 2001; Bertrand et al., 2002). Furthermore, nAChR desensitization might even lead to chronic modulation of nAChRs in the brain of tobacco smokers (Dani et al., 2001, Wooltorton et al., 2003).

Because of purified nAChRs reconstituted in lipid bilayers can undergo desensitization, this process must be intrinsic to the receptor protein, although it can be modified by a variety of other cellular agents and exogenous signals (Wang and Sun, 2005).

In the mammalian nervous system, nAChRs can be classified as either α7-containing receptors that desensitize rapidly (milliseconds) or non α7- containing receptors that desensitize more slowly (seconds) and are made up of various combinations of α and β subunits. Among non-α7 receptors, the two most common subtypes are α3β4 (mainly expressed by autonomic neurons and moderately susceptible to desensitization) and α4β2 receptors (widely found in the brain and very prone to desensitization) (Giniatullin et al., 2005). When a medium to high (μM to mM) concentration of agonist is applied, nAChRs can desensitize with subsequent recovery after agonist removal. This recovery process develops usually in the range of tens of milliseconds, although different subtypes of nAChRs have differential susceptibility to desensitization recovery (Giniatullin et al., 2005).

Very low agonist concentrations (nM range) can induce desensitization even without apparent receptor activation, a process that has been identified as "high affinity desensitization" (HAD) (Giniatullin et al., 2005). HAD is a slow process (taking second to minutes), and, during chronic exposure to an agonist, it is more likely to be generated than classical desensitization. HAD is also receptor-subtype specific, affecting preferentially α4β2 rather than α7 or α3β4 nAChRs. Together with

desensitization, long (hours to days) exposure to an agonist can produce sustained changes in receptor sensitivity owing to up-regulation of nAChRs.

Desensitization is qualitatively (not quantitatively) similar throughout the nicotinic receptor family and its essential behaviour can be captured in a general cyclical model (Katz and Thesleff, 1957). In this scheme, desensitization represents a classic form of allosteric protein behaviour, in which the receptor is distributed (in the absence of ligand) between several discrete conformations and the agonist simply increases the probability of the receptor transitions between states. An update of the model involves the sequential occupation of two agonist-binding sites on the same receptor to produce activation. Accordingly, HAD might represent the transition of the mono-ligand bound receptor (AR) to a desensitized bound state (AD) (Fig. 5) (Giniatullin et al., 2005).

Because homomeric nicotinic receptors undergo desensitization, the α subunits must be sufficient to confer this channel property. Subunit splice variants, different subunit assembly, and different α:β subunit ratio can all dramatically change the desensitization properties (Giniatullin at al., 2005). In terms of agonist concentration effective for desensitization, α7-containing receptors are much less sensitive to agonist than heteromeric nAChRs (Alkondon et al., 2000, Frazier et al., 1998).

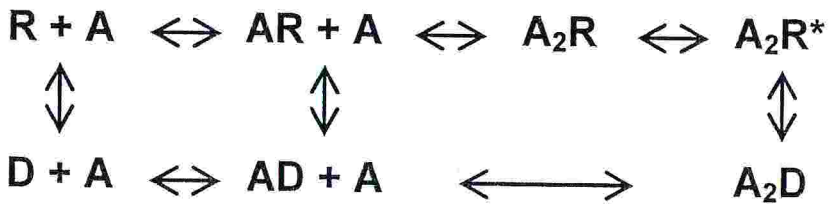

Fig 5. This model suggests binding of two agonist molecules and transition into the desensitized state from both the agonist-bound closed state (AR) and from the open receptor state (A_2R^*). The former has a high affinity for agonist because it can be evoked by low (nanomolar) concentration of agonist, and it could represent the receptor conformation responsible for generating high-affinity desensitization (HAD) because no activation is involved. (Giniatullin et al., 2005).

A good deal of information about desensitization of heteromeric nAChRs is available (reviewed by Quick and Lester, 2002) through a combination of electrophysiological and binding assays. Results indicate that both α and β subunits contribute to the agonist affinity to activate and desensitize, whereas the type of β subunits is important for the onset rate of desensitization (receptor containing β2 subunits desensitize faster than those containing β4 subunits). In experiments in which the β subunit is kept constant, receptor containing α3 desensitize faster than those containing α4. Thus, overall heteromeric nAChRs can be ordered in terms of their desensitization kinetics (from fastest to slowest): α3β2 > α4β2 > α3β4 > α4β4. The contribution of α2 subunits has been less studied, perhaps due to their restricted CNS distribution. The inclusion of an additional α5 subunit can change desensitization properties of receptors: α5α3β4 receptors desensitize approximately threefold faster that α3β4 receptors (Gerzanich et al., 1998; Quick et al., 1999); α5 is often found associated with α3β4 receptors (Conroy and Berg, 1995; Sheffield et al., 2000). Likewise, the presence of α5 increases the time course of desensitization of α4β2 receptors (Ramirez-Latorre et al., 1996). Subunit splice variants or different α:β subunit ratio can also produce changes in desensitization properties. For instance, α7 receptors of autonomic ganglion neurons desensitize more slowly that those of central neurons, probably because of cell-dependent expression of an α7 subunit isoform (Cuevas and Berg, 1998; Severance et al., 2004). Data from recombinant α4β2 receptors show that, even with the same subunit composition, desensitization is largely enhanced when the ratio of β and α subunits increases (Lopez-Hernandez et al., 2004).

Other factors regulating the kinetics of receptor activation and desensitization are temperature, membrane potential, post-translational modifications, ligands, ions and interactions with the cytoskeleton. One of these factors is the endogenous peptide substance P, which powerfully facilitates desensitization by binding to an allosteric

site distinct from the ACh-recognition sites (Di Angelantonio et al., 2003). Other modulators are intracellular messengers (e.g. Ca^{2+}) that primarily target the recovery from, rather than the onset of, desensitization (Quick and Lester, 2002; Giniatullin et al., 2005). Fenster et al. (1997) have shown that Ca^{2+} promoted recovery, whereas Khiroug et al. (1998) have directly demonstrated that elevated levels of intracellular Ca^{2+} following nAChR stimulation inhibit recovery from desensitization. This difference may be explained by distinct receptor subtypes, cell systems, or the relative stimulation of second messengers by Ca^{2+}. Indeed, if nicotine-induced intracellular transients in chromaffin cells are limited by using the fast chelator BAPTA, recovery from desensitization is always fast, even in the presence of PKC inhibition, which would otherwise markedly delay the recovery phase. Additional data showing that inhibition of the Ca^{2+}-dependent phosphatase calcineurin and stimulation of PKC activity by phorbol ester both promote recovery from desensitization have lead to hypothesis that Ca^{2+} regulates desensitization by altering the balance of kinase/phosphatase activity, as increased phosphorylation (PKA- or PKC- dependent) promotes recovery (Quick and Lester, 2002).

Alteration in the time course of recovery from desensitization implies that protein phosphorylation acts to regulate the rate constants that govern transition between activatable and desensitized receptor conformations. Longer-term receptor inactivation, possibly in concert with phosphorylation, may have an important role in neuronal nAChR function and dysfunction, although is still unclear what the exact relationship is between desensitization and inactivation.

Up-regulation of nAChRs

The effects of desensitization on receptors include alterations in affinity and density. The generally accepted view is that overstimulation induced by agonists leads to a reduction in the number of receptors. However, desensitization by long-term exposure to nicotine triggers an increase in the number of nAChRs in different

species including humans (Wonnacott, 1990). The desensitization and up-regulation of nAChRs are presumably involved in nicotine tolerance and dependence.

Up-regulation possibly relies on the fact that brain nAChRs undergo rapid desensitization and consequent inactivation after prolonged exposure to this agonist (Fenster et al., 1999): inactivation, which occurs in the presence of even low concentration of nicotine, is then counteracted by an increase in the number of nicotinic receptor molecules expressed by neurons. The molecular mechanisms underlying this increased expression of cell surface receptors have been investigated *in vivo* and *in vitro*.

A larger number of nicotinic receptors has been documented in the brain of tobacco smokers (Benwell et al., 1988) and it is strongly suspected to play a role in the molecular mechanisms underlying withdrawal symptoms (Dani and Heinemann, 1996). An increase in nicotinic binding site density has also been observed in the brain of rodents after chronic nicotinic treatment *in vivo* (Wonnacott et al., 1990; Flores et al., 1992; Rowell and Li, 1997). High affinity binding of [^{3}H]-nicotine (and [^{3}H]-cytisine) has been correlated with nicotinic AChRs comprised of α4 and β2 subunits (Flores et al., 1992; Zoli et al., 1998). Up-regulation of the α4β2 nAChR subtype has also been demonstrated in stably transfected cell lines (Peng et al., 1994; Bencheriff et al., 1995; Zhang et al., 1995; Gopalakrishnan et al., 1997; Whiteaker et al., 1998; Buisson and Bertrand, 2001). When the function of nicotinic receptors after chronic treatment has been investigated, an increase has also been observed in $^{86}RB^+$ efflux (Gopalakrishan et al., 1997) and electrophysiological responses (Buisson and Bertrand, 2001).

Both *in vivo* and in cell lines, up-regulation of high affinity [^{3}H]-nicotine binding sites by nicotine reflects an increase in the number of binding sites (B_{max}) with no change in affinity (k_D; Wonnacott, 1990; Gopalakrishnan et al., 1997) and is not accompanied by an increase in subunit mRNA, which clearly suggests that post-transcriptional mechanisms are responsible for this phenomenon (Marks et al., 1992; Peng et al., 1994; Bencherif et al., 1995; Zhang et al., 1995). Recent investigations

of the maturation and trafficking of the α4β2 nAChRs demonstrate that up-regulation is initiated in the endoplasmic reticulum soon after protein translation. The data so far accumulated provide evidence that nicotine elicits up-regulation by promoting maturation of nAChR precursors that would otherwise be degraded (Corringer et al., 2006).

Other subtypes of neuronal nicotinic receptors are also up-regulated by chronic nicotine treatment. [^{125}I]-α-Bgtx labels a nicotinic site that is correlated with the α7 subunit in rodent brain (Séguéla et al., 1993; Barrantes et al., 1995; Orr-Urtreger et al., 1997). Chronic nicotine treatment *in vivo* up-regulates the [^{125}I]- α-Bgtx binding sites in some brain regions, although this response is less robust then that observed for [^{3}H]-nicotine binding site and requires higher nicotine concentrations that may not be relevant to human smokers (Wonnacott, 1990; Pauly et al., 1991). As in the case of the up-regulation of α4β2 nAChRs, no change in α7 mRNA levels has been observed (Marks et al., 1992). Up-regulation of [^{125}I]- α-Bgtx binding sites has been reported also *in vitro*: primary hippocampal cultures chronically treated with nicotine show a small, yet significant increase in the number of surface [^{125}I]- α-Bgtx binding sites (Barrantes et al., 1995), a result replicated in SH-SY5Y cells (Peng et al., 1997; Ke et al., 1998; Ridley et al., 2001), in cell lines transfected with the α7 subunit (Quik et al., 1996; Molinari et al., 1998), and in rat cortical neurons (Kawai and Berg, 2001). Whenever investigated, the electrophysiological responses were also increased (Molinari et al., 1998; Kawai and Berg, 2001).

Up-regulation of α7-receptors can be also evoked by persistent depolarization induced by high K^+ (De Koninck and Cooper, 1995); in contrast to nicotine evoked up-regulation, this response is accompanied by an increased level of α7 mRNA. K^+ evoked up-regulation is proposed to result from Ca^{2+} influx through L-type Ca^{2+} channels and activation of a Ca^{2+}-calmodulin dependent kinase pathway.

More recently, up-regulation of α3-containing receptors has been measured by studying epibatidine binding in various protocols, mostly utilizing SH-SY5Y cells

(Peng et al., 1997; Ke et al., 1998; Wang et al., 1998; Warpman et al., 1998; Svensson et al., 2000; Ridley et al., 2001). Nevertheless, up to now, there are no electrophysiological studies addressing this issue at single cell level.

A differential process of up-regulation seems to involve α3-containing receptors: chronic nicotine treatment increases the amount of α3β2(α5) receptors, but it has no effect on α3β4(α5) receptors (Wang et al., 1998; Ridley et al., 2001). No change in mRNA synthesis for α3 nAChR subunits is present (Peng et al., 1997; Ke et al., 1998; Wang et al., 1998).

Nevertheless, two recent studies demonstrate a different behaviour for the α6β3-nicotinic receptors: Visanji et al. (2005) show that chronic nicotine treatment enhances the response to systemic nicotine and increases the levels of α6 and β3 nAChR subunit mRNA in the substantia nigra pars compacta of the rat. Conversely, Mugnaini et al. (2006) demonstrate that α6β2- nicotinic receptors, unique within the nicotinic acetylcholine receptor family, are down-regulated following chronic nicotine treatment in rat dopaminergic mesostriatal pathway. These findings further demonstrate the complexity of the neuronal network response to nicotine (or other nicotinic drugs) exposure.

Whereas all other nicotinic agonists up-regulate all nAChRs (Peng et al., 1997; Gopalakrishnan et al., 1998; Molinari et al., 1998; Wang et al., 1998; Warpman et al., 1998; Witheaker et al., 1998; Ridley et al., 2001), the ability of nicotinic antagonists to either elicit or prevent up-regulation is less consistent between studies, and between receptor subtypes (see Table 3).

In SY5Y cells, Peng et al. (1997) report that millimolar concentrations of d-tubocurarine, DHβE and mecamylamine cannot up-regulate either α3 or α7 subtypes and do not prevent the nicotine-induced up-regulation of α3, but partially block the up-regulation of α7 nAChRs in response to nicotine.

Table. 3. Ability of different antagonists to induce up regulation (reference and cell type).

	DHβE	MLA	d-tubocurarine	mecamylamine	hexametonium
α4β2	**No change** (Whiteaker et al., 1998, M10 cells) **Up-regulated** (Gopalakrishnan et al., 1997, HEK cells) **Up-regulated** (Buisson and Bertrand, 2001, HEK cells)	**No change** (Whiteaker et al., 1998, M10 cells) **Up-regulated** (Gopalakrishnan et al., 1997, HEK cells) **Up-regulated** (Buisson and Bertrand, 2001, HEK cells)	**No change** (Whiteaker et al., , M10 cells) **Up-regulated** (Gopalakrishnan et al., 1997, HEK cells)	**No change** (Whiteaker et al., 1998, M10 cells) **No change** (Gopalakrishnan et al., 1997, HEK cells)	**No change** (Whiteaker et al., 1998, M10 cells)
α7	**No change** (Peng e al., 1997, SY5Y) **Up-regulated** (Quik et al., 1987, chromaffin cells)	**Up-regulated** (Molinari et al., 1998, HEK cells)	**No change** (Peng e al., 1997, SY5Y) **Up-regulated** (Quik et al., 1987, chromaffin cells)	**No change** (Peng e al., 1997, SY5Y) **Up-regulated** (Quik et al., 1987, chromaffin cells)	**No change** (Quik et al., 1987, chromaffin cells)
α3*	**No change** (Peng et al., 1997, SY5Y)	**Up-regulated** (Ridley et al., 2001, SY5Y)	**No change** (Peng et al., 1997, SY5Y) **Up-regulated** (Ridley et al., 2001, SY5Y) **No change** (Svensson, 2000, SY5Y)	**No change** (Peng et al., 1997, SY5Y) **No change** (Svensson, 2000, SY5Y)	

These observations have led to the tentative suggestion that up-regulation of α3 and α7 nAChRs may proceed via different mechanisms, the latter requiring receptor activation by agonists. Whiteaker et al. (1998) and Svensson (2000) also could not find up-regulation of α4β2 receptors in transfected M10 cells chronically treated with antagonists; the same result was observed in SY5Y cells for α3 and α7 containing receptors (Svensson, 2000). This result is, however, not supported by other findings that d-tubocurarine and MLA both up-regulates [^{125}I]- α-Bgtx binding sites (Quik et al., 1987; Molinari et al., 1998; Ridley et al., 2001). Antagonist-induced up-regulation is not additive to that produced by agonists (Ridley et al., 2001).

In summary, at least certain antagonists can induce up-regulation, suggesting that binding to (or near) the agonist binding site, rather that activation, is sufficient to trigger up-regulation, perhaps by stabilizing a particular conformation of the receptor. The non-competitive antagonist chlorisondamine (El-Bizri and Clarke, 1994) does not induce up-regulation, indicating that receptor block is not sufficient to observe this phenomenon. Recently, Kuryatov et al. (2005), expressing α4β2 receptors in permanently transfected tsA201 human embryonic kidney cells lines, have reported that both membrane permeable ligands like nicotine and much less permeable quaternary amine cholinergic ligands can act as pharmacological chaperones within the endoplasmatic reticulum to promote the assembly of AChRs. Agonists are more potent pharmacological chaperones than antagonists, presumably because activated or desensitized conformations assemble more efficiently.

Localization of neuronal nAChRs

nAChRs, that are widely distributed in the brain, seem principally localized to terminal boutons (for reviews see Dani, 2001, Gotti and Clementi, 2004, Gotti et al., 2006). They are present in postsynaptic, presynaptic or non-synaptic areas. However, anatomical and functional evidence suggests that most nAChRs are

preferentially located on the presynaptic boutons regulating neurotransmitter secretion in several parts of the brain (see Wonnacott, et al. 1995; Wonnacott, 1997). Since nAChRs are highly Ca^{2+} permeable and receptor activation can cause a significant calcium influx, different effects can be predicted depending on the sub-cellular receptor localization. For instance, when presynaptic nicotinic receptors are activated, increased calcium in the presynaptic bouton facilitates neurotransmitter release (Dani, 2001). In particular, presynaptic nAChRs have been implicated in promoting the release of ACh, noradrenaline, dopamine (DA), glutamate and GABA.

In addition to controlling and modulating the release of various neurotransmitters, presynaptic nAChRs can play other roles under particular conditions (i.e. denervation and development) such as axon path finding and neuritogenesis (Zheng et al., 1994; Role and Berg, 1996). Although cholinergic systems provide a diffuse innervation to practically all brain areas, a relatively small number of cholinergic neurons innervate each region (Kasa, 1986; Woolf, 1991). Despite this sparse innervation, cholinergic activity drives or modulates a wide variety of behaviours. There is also evidence that high affinity nAChRs are located on postsynaptic membranes in the somatodendritic regions of a few brain areas (Clarke, 1993).

The location of the αBgtx binding sites (corresponding to the human α7 subtype) has been studied in man only at macroscopic cell level. In animals they have been found at both synaptic and non-synaptic sites, indicating that, perhaps in addition to controlling rapid communications between pre- and postsynaptic neurons, these receptors may regulate other cell functions by increasing Ca^{2+} influx (for example, neuroprotection; Gotti and Clementi, 2004).

The predominantly presynaptic localization of nAChRs on nerve terminals containing different neurotransmitters, and the presence on the same boutons of different nAChR subtypes (Gotti and Clementi, 2004) is the basis of the pleiotropic effects of nicotinic drugs as modulator of several pathways.

On the basis of the results of in situ hybridisation studies, it seems that the most important and diffused receptor subtype in human brain is the α4β2, but the α4β4/β2 subtype is also present in regions such as the striatum, hippocampus, habenula and cerebral cortex. Unlike the rodent brain, the primate brain contains a substantial presence of α2β2. There is also a selective, functionally-important distribution of α6β3 subunits in the mesolimbic nuclei (Gotti and Clementi, 2004).

α7 Receptors are present in autonomic ganglia, hippocampus, cerebellum, pituitary and pineal glands, and cortex. The α9/α10 subtypes are mainly expressed in the cochlea.

nAChRs are also largely expressed in autonomic ganglia and in non-neuronal tissues (lymphocytes, macrophages, dendritic cells, adipocytes, keratinocytes, endothelial cells, epithelial cells of the intestine and lung; Gotti and Clementi, 2004; Gahring and Rogers 2006). The fast nicotinic synaptic current recorded from autonomic ganglion neurons is mainly mediated by α3β4/α5 sutypes.

Effects of nicotine on the CNS

Nicotine is a potent modulator of CNS function. It enhances ion fluxes and neurotransmitter release, augments or gates a number of neuronal systems, and elicits a variety of behavioral states. Nicotine administration increases heart rate and blood pressure, and stimulates the secretion of prolactin and adrenocorticotropic hormone, resulting in a subsequent increase in corticosterone secretion (Benowitz et al., 1989). In humans, nicotine increases arousal, visual attention and perception, but it also decreases reaction time, prevents a decline in efficiency over time and improves the ability to suppress inappropriate responses (Wesnes and Warburton, 1983; Jones et al., 1992). Despite these important effects elicited by nicotine, the exact role of brain nAChRs in behavior remains unclear.

Knockout (Ko) mouse experiments have shown that brain nAChRs are not essential for survival or the execution of very basic behaviors: for example, β2 and α4 Ko

mice, which completely lack the most abundant high-affinity nAChRs, can accomplish routine behavior (for a review, see Gotti and Clementi, 2004). However, nAChRs are important for the fine control of more sophisticated and complex behaviors that can be evaluated only with appropriate tests or during particular conditions like ageing.

These findings put nAChRs in a different and perhaps more important perspective in terms of their involvement in brain pathologies and as potential drug targets. Many pathological situations involve a lack of fine control and tuning rather than complete loss of function, and the pharmacological restoration of appropriate tuning may have an important clinical effect.

Nicotine interacts with a variety of presynaptic nAChRs to facilitate the release of a number of neurotransmitters implicated in mediating/modulating behavioral tasks (Wonnacott and Thorne, 1990; McGehee et al., 1995). Thus, it as been proposed that the role of nAChRs in the brain is to modify the excitability of neurons, that is to produce the optimal performance of neurons by adjusting their excitability, an action which is likely to be particularly important for cognitive processes (McGehee et al., 1995). The most obvious behavioral action mediated by nAChRs is addiction to nicotine smoking. The mesolimbic DA pathway is thought to mediate the addictive effects of nicotine and other substances of abuse, as nicotine is known to stimulate release of DA in this pathway (Pich et al., 1997).

nAChRs have also a role in development and neuronal plasticity as suggested by the pattern of their expression especially high during the stage of synaptic formation. *In vitro* experiments indicate that nAChRs (particularly $\alpha 7$) may control the development of neuronal architecture, stabilize synapse formation, and orient and control neurite outgrowth (Lipton and Kater, 1989; Pugh and Berg, 1994; Zheng et al., 1994; Quik, 1995; Role and Berg, 1996).

Nicotinic receptors seem to be important during ageing: there is general consensus that nicotine binding and the nAChR mRNA slightly decrease in brain rat during ageing, with some regional specificity (Cimino et al., 1994). The finding that there is

marked neurodegeneration in β2 Ko mice indicates that β2-containing receptors are involved in neuronal survival during ageing (Zoli, 2000). In fact, β2 subunit presence declines with age in all structures of the human brain (Flynn and Mash, 1986; Nordberg et al., 1992; Court and Clementi, 1995).

These data indicate that the use of nicotinic drugs during fetal development and ageing could have important consequences: the exposure of fetal brain to nicotine may greatly modify brain circuitry, whereas the use of nicotinic drugs to prevent or decrease the brain degeneration during ageing could be a useful tool.

Some of the pathologies resulting from dysfunction of neuronal nicotinic receptors are, for example, Alzheimer's disease, Parkinson's disease, epilepsy, and other mental diseases such as schizophrenia, Tourette's syndrome, anxiety and depression. Further work is necessary to determine what nAChR subtypes are involved in cognitive processes and to elucidate the interactions of these receptors with other neurotransmitter systems critical for cognitive function.

Role of peripheral nAChRs

The autonomic nervous system (ANS) maintains the body homeostasis by regulating cardiovascular, temperature, gastrointestinal, genitourinary, and exocrine functions. Its activity, however, is dependent on the brain and spinal cord and on a range of afferent stimuli from various parts of the body. The excitatory signals in autonomic ganglia are mediated mainly by local release of ACh, stimulating postganglionic nAChRs. The nAChRs, thus, play a central role in signal transmission in autonomic ganglia, and this is the site of action of several drugs (e.g. nicotine or C6). There is a very large potential for nAChR diversity, but only 5 of the 12 nAChR subunit (α3, α5, α7, β2, β4) are known to exist in autonomic neurons (Wang et al., 2002).

In order to study the role of nAChRs in autonomic regulation, it is important to understand nAChRs subunit combinations, distribution and diversity of their physiological and pharmacological properties in the ANS.

The α3 subunit is expressed in all autonomic ganglia, even tough other subunits (α5, α7, β2 and β4) may be coexpressed, to a variable degree, in different tissues and individual ANS neurons. Ko mice lacking α3 subunits show a phenotype similar to human autosomal recessive disease, megacystis-microcolon-intestinal hypoperistalsis syndrome (MMIHS). (Wang et al. 2002). In the small bowel of patients with MMHIS, the mRNA for α3 subunits is not detectable (Richardson et al., 2001), providing further support that mutation in the gene encoding the α3 subunit is responsible for this disease. Follow-up clinical studies report that auto-antibodies against ganglionic α3 nAChRs are involved in a syndrome consisting of idiopathic (or pareneoplastic) autonomic neuropathy, postural tachycardia, as well as in idiopathic gastrointestinal dysmobility (Balestra et al., 2000; Vernino et al., 1998, 2000).

Studies on Ko mice have also demonstrated the functional role of the other nAChR subunits expressed in ANS autonomic ganglia (Wang et al, 2002):

- β4 subunits seem to be the predominant β component in autonomic ganglia. However, their absence can be compensated by up-regulation of β2 subunits forming α3β2 AChRs which maintain near-normal ganglionic transmission. The complex combination of α3β2β4 or α3α5β2β4 may also exist in native nAChRs.
- α5 subunits are normal components of channels in autonomic ganglion neurons, and modulate the interaction between β2 and β4 subunits, or between them and α3 subunits.
- Although α7 subunits are normally expressed in the ANS, little is known about their function. The α7 subunits are apparently involved in the sympathetic baroreflex (Franceschini et al., 2000), suggesting that heteromeric α7-AChRs participate in fast postsynaptic currents in the ANS.

Chromaffin cells

Chromaffin cells are located in the adrenal medulla (the inner part of the adrenal glands). They manufacture, store and secrete a complex mixture of hormones, the most important of which is adrenaline.

Chromaffin cells owe their name to the color produced by their chemical reaction with chromium salts that reacts with oxidized adrenaline to yield a yellowish brown color.

The adrenal medulla is an endocrine gland that affects other tissues and organs by discharging hormones into the blood stream: under conditions of fear or stress, chromaffin cells release adrenaline into the blood vessels to mobilize the body for peak physical responses. Flooding the bloodstream with up to 300 times the normal concentration, adrenaline interacts with receptors on cells in various organs increasing the heart rate, blood pressure, pulmonary ventilation, inhibiting certain "non-essential" processes (for example gastrointestinal secretion) and prompting the release from liver of extra sugar to fuel muscular work. Taken together, these reactions constitute a "fight or flight" response that prepares one to combat an enemy or flee from danger.

Chromaffin cells have the same embryonic origin of the postganglionic sympathetic neurons (Anderson, 1993). Stress leads to activation of both cell types as impulses from the spinal cord reach them (Fig. 6). Chromaffin cells secrete adrenaline, noradrenaline and other substances into the bloodstream, thereby exercising widespread control over tissues and organs; sympathetic neurons discharge noradrenaline locally. Because of the similarities in the activation of chromaffin cells and sympathetic neurons, in their secretion and in their effects, and because they develop from the same stem cells (in the neural crest) during embryogenesis, the adrenal medulla is considered to be a part of the sympathetic nervous system.

When chromaffin cells are grown in culture, they emit axon-like processes, which indicates their close kinship to neurons. Adrenal chromaffin cells are readily collected as a monotypic cell group and they are, therefore, readily accessible to biochemical analysis. As a result, they have served as a laboratory model of neurons and much of what is known about the production and secretion of neurotransmitters was established through studies of the chromaffin cell.

Since chromaffin cells are exposed to acetylcholine released by the splancnic nerve and possess high nAChR density, they have been widely used for pharmacological and physiological studies aiming at characterizing nAChRs (Giniatullin et al.,1999; Khirough et al., 1997; Khirough et al., 1998).

The α3, α5, α7 and β4 subunits have been reported to be expressed in bovine chromaffin cells (Campos-Caro et al., 1997; Criado et al., 1992). In rat chromaffin cells the homomeric α7 receptors does not seem to be expressed: in fact, α-bungarotoxin is not bound by rat chromaffin cell membranes (Di Angelantonio et al., 2000) and α-bungarotoxin fails to inhibit responses mediated by nAChRs (Nooney and Feltz, 1995). Bovine chromaffin cells express α-bungarotoxin sensitive receptors (Campos-Caro et al., 1997; Lopez et al., 1998), but they appear to be irrelevant to secretion mechanisms probably because of their fast desensitization (Tachikawa et al., 2001). Activation of α3β4 and α3β2 containing nAChRs, natively expressed by adrenal chromaffin cells, is essential to mediate release of catecholamines (Tachikawa et al. 2001) into the blood stream with consequent changes in the cardiovascular system.

On chromaffin cells it was observed that nAChR activation mediate a sustained rise in intracellular calcium concentration (Vernino et al., 1994; Amador and Dani, 1995; Khiroug et al., 1997; Khiroug et al., 1998); the fraction current carried by Ca^{2+} has been shown to be about 4 % of the total nicotine-induced current. This Ca^{2+} influx through neuronal nAChRs of rat adrenal chromaffin cells is also insensitive to α-bungarotoxin (Vernino et al., 1994). Using reverse transcription-polymerase chain

reaction (RT-PCR) and *in situ* hybridisation methods on bovine chromaffin cells, Campos-Caro et al. (1997) have reported that the predominant receptor type is α3β4 with variable contribution by α5. More recent work by Mousavi et al. (2001) has suggested that, on rat chromaffin cells, additional subunits (α4, β2) are also present, but whether they make up functional receptors remains unknown.

SH-SY5Y cells

Neuroblastomas are pediatric tumors that are thought to arise from migratory cells of the embryonic neural crest. A number of clonal neuroblastoma cell lines have been stabilized so that they display most features of autonomic neurons including the ability to express ganglionic-type nicotinic receptors (Lukas et al., 1993). SH-SY5Y cells express mRNA for α3, α5, α7, β2 and β4 subunits (Lukas et al., 1993; Peng et al., 1994) and they express on their surface α7-, α3β2- and α3β4-containing receptors (Wang et al., 1998; Ridley et al., 2001). Thus, SH-SY5Y cells possess native nAChRs analogous to those normally found on autonomic ganglion neurons (and chromaffin cells), but they are more stable in culture and, therefore, they are more suitable for studies based on chronic treatments. For such a reason, SH-SY5Y cells have frequently been used as a model to investigate long term alterations in nAChRs in the presence of cholinergic ligands (Peng et al., 1997; Warpman et al., 1998; Ridley et al., 2001).

AIMS OF THE PRESENT STUDY

The aim of the present study is to investigate a number of properties of peripheral nAChRs, in particular:

- To characterize native nAChR subtypes present in rat chromaffin cells with molecular biology, pharmacological tools and electrophysiological experiments.
- To investigate the acute and chronic effects of the new cytisine derivative CC4 on nAChRs of SH-SY5Y cells which are similar to chromaffin cells.
- To examine the function of nAChRs under conditions known to induce their long-term perturbation. Two main issues are to be addressed:
  1. whether sustained agonist application can lead to complete loss of receptor sensitivity and how efficiently such receptors might recover from it;
  2. to understand whether chronic agonist application may lead to compensatory changes in receptor function.

Experimental approach

To meet these aims, a range of different approaches was used.

- To characterize nAChRs expressed by rat chromaffin cells, we started with the subunit identification by molecular biology techniques. We detected various subunit mRNAs by RT-PCR, not all of them corresponding to effective expression measured by immunocytochemistry and western blot. Strong expression of the α3, α4, α5, β2 and β4 subunits was found. At single cell level, co-localization could be demonstrated for α3α5, α3β4 and α5β4 mRNAs by single cell RT-PCR, and for α3α5 and α4β2 by double immuno-staining.
  The identification of the nAChR subunits expressed by rat chromaffin cells left unanswered the issue of how they assembly to form functional receptors and, consequently, what nAChRs subtypes are responsible of the nicotine-evoked currents in these cells. To clarify this issue, we used pharmacological tools able to discriminate between different receptor compositions, although the availability of specific pharmacological compounds was limited. Nanomolar

concentration of MLA (specific for the homomeric α7 receptors) left unchanged nicotinic currents, confirming the absence of α7 receptors.

The results obtained with DHβE (thought to be specific, at low micromolar concentration, for the α4β2 subtype) and the two conotoxins MII and AuIB (specific for the α3β2 and α3β4 containing subtypes, respectively) indicated that the α3β2 and the α3β4 receptors (with a probable contribution by the α5 subunit) are responsible for a large portion of the nicotine-evoked current plus a rather small contribution by the α4β2 subtype.

In order to investigate the long term effects of nicotinic ligands, we used SH-SY5Y cells, a neuroblastoma cell line more stable in culture and, therefore, more suited to chronic treatments than acutely dissociated chromaffin cells. We first characterized the pharmacological properties of the nicotine-evoked currents in such neuroblastoma cells, by fast application of nicotine and related agents.

- We investigated the acute effect of the novel cytosine derivative CC4, showing that this compound, applied alone, lacked any agonist activity. Indeed, CC4 decreased the nicotine-evoked current showing its antagonist activity.
- Chronically treated cells were tested for their response to nicotine, showing that the nicotine-evoked currents were increased after treatment with 1mM CC4 and changed their shape. We then investigated the relative subunit contribution to the total nicotinic current in control cells and in chronically treated cells by using specific antagonists, demonstrating enhanced role of α3- and α7- containing receptors after treatment.
- To investigate the desensitization properties of nicotinic receptors in SH-SY5Y cells, the effect of a sustained application to nicotine was tested on nicotine evoked-currents. These experiments showed that these cells retained a small response to nicotine and completely recovered after wash out. After wash out, we observed functional up-regulation 8-48 hours later. We also investigated the functional properties and the antagonist sensitivity of the up-regulated receptors, demonstrating that they were not significantly different from those of untreated cells.

METHODS

Cell preparation

Rat chromaffin medullary cells were cultured according to the method of Brandt et al. (1976). Adrenal glands were removed from 25-35 day old rats (anaesthetized with slowly raising levels of CO_2), and rinsed in a medium (pH 7.2) containing (mM): NaCl 137, KCl 3, Na_2HPO_4 0.7, HEPES 25, glucose 10 and 350 units/ml of penicillin and streptomycin. Cells were dissociated drawing the adrenal tissue fragments gently up and down inside a Pasteur pipette after incubation with collagenase A and DNAse (0.5 units/ml and 10 μg/ml respectively) at 37°C for 15 min, repeating the treatment until complete dissociation. The suspension was centrifuged at 750 g for 5 min, and the pellet (containing cells) rinsed twice with HEPES-buffered medium. Finally, cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), plated on poly-L-lysine (5 mg/ml)-coated Petri dishes, and cultured at 37°C for 1-2 days under a 5% CO_2 containing atmosphere (Di Angelantonio et al., 2000; Giniatullin et al., 2000; Khiroug et al., 1998).

SH-SY5Y cells were grown in RPMI 1640 (Sigma), supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine and 1 % penicillin-streptomycin at 37°C in a 5 % CO_2 containing atmosphere.

For chronic drug treatment, the culture medium was replaced after 24 h with the same medium containing drugs. The cells were then exposed for a variable period to test drug and extensively washed in control saline solution before use.

Electrophysiology

Cell-containing culture dishes were mounted on the stage of an inverted Nikon Diaphot microscope and superfused (5-10 ml/min) with control saline solution containing (mM) NaCl 135, KCl 3.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 15, HEPES 10 (pH

7.4 adjusted with NaOH, osmolarity 285 mOsm). Patch pipettes pulled from thin glass had 5-6 MΩ resistance when filled with (mM) CsCl 120, HEPES 20, $MgCl_2$ 1, Mg_2ATP_3 3, BAPTA 10 (240 mOsm). The pH of the pipette solution was always adjusted to 7.2 with CsOH.

Cells were voltage-clamped at -70 mV (unless otherwise indicated) in the whole-cell configuration after obtaining GΩ seals (usually not less that 2 GΩ). Series resistance was compensated by 60%. In the whole cell configuration the cell resistance was usually 100-200 MΩ after compensation. After obtaining whole-cell configuration, a 10 min period of stabilization normally elapsed before membrane currents were recorded, filtered at 1 kHz and acquired on the hard disk of a PC by means of pCLAMP software (Molecular Devices, Sunnyvale, California, USA).

Note that SY5Y cells of different origin have been used in two series of experiments: thus, different cell groups showed currents of different amplitude in response to nicotine pulse. To normalize results and avoid variability among different batches of cells, all data are referred to the control current of the same cell group.

Drug application methods and theoretical calculations

Puffer and fast superfusion

In the present study, agonists were delivered by short pressure pulse via a puffer pipette, while antagonists were applied by the rapid solution exchanger.

For pressure application, glass micropipettes (thin glass of 1.5 mm o.d. from Hilgenberg, Malsfeld, Germany) were pulled with a PP830 Narashige puller (Narashige International Limited, London, UK) to obtain a resistance of 4 MΩ when filled with patch electrode solution (tip diameter was about 3 μm). For standard use, the pressure pipette was filled with nicotine diluted in external solution and connected to the Picospritzer II instrument (General Valve Co. Fairfields, New Jersey, USA).The pipette was positioned at about 20 μm from each single cell under

microscopic control. In this position, with a standard puffer pulse, the solution has a rapid and homogeneous distribution over the entire cell (Di Angelantonio and Nistri, 2001).

When using the rapid solution exchanger (BioLogic, Strasbourg, France) we filled a number (up to six) of glass barrels (from Clark Electromedical, Pangbourne, UK; type GC100 F15, 1 mm o.d., 0.58 mm i.d.) with solutions containing antagonists. Applications via fast perfusion system were controlled via a PC which commanded the prompt opening of electromagnetic valves and solution flow. With this arrangement the recorded cell was exposed to the rapid flow from one barrel only, while the others were kept off stream.

Electrophysiological data analysis

Data are presented as mean ± sem (n = number of cells) with statistical significance assessed with Wilcoxon test (for non parametric data) or paired *t*-test (for normally distributed data). A value of $P \leq 0.05$ was accepted as indicative of statistically significant difference.

Dose-response curves were fitted with a logistic equation (Origin software, Microcal Software, Northampton, MA, USA).

RT-PCR

RNA extraction

The total RNA content of chromaffin cell cultures was isolated with TRIzol™ (Gibco) method. Briefly cells were lysed directly in the culture dish by adding 1 ml TRIzol™ reagent, then passed several times through a pipette and incubated 5 minutes at room temperature (RT) to allow the complete dissociation of nucleoprotein complexes. To perform phase separation, after shaking for 15 s and incubating at RT for 2-3 min, samples were centrifuged at 12.000 g for 10 min at 4°C. The aqueous phase, containing RNA, was then collected in a fresh tube to

precipitate RNA by mixing it with isopropyl alcohol (0.5 ml per 1 ml TRIzol™ used). Samples were then incubated at RT for 10 min and centrifuged at 12.000 g for 10 min at 4°C. The pellet contained the precipitate RNA. The supernatant was removed and the pellet was washed with 75% ethanol (1 ml per 1 ml TRIzol™ used), sample was then mixed (by vortexing) and centrifugated at 7.500 g for 5 min at 4°C. Pellet was air-dried for 5-10 min and dissolved in 20 µl RNAse-free water by passing the solution through a pipette tip, and incubating for 10 min at 60°C. RNA was immediately used for RT-PCR or stored at -80°C.

Reverse Transcription reaction and Polymerase Chain Reaction (RT-PCR)

The RT mix consisted of: 50 mM KCl, 10 mM TrisHCl (pH 8.3), 5 mM $MgCl_2$, 1 mM DTT, 1 mM each dNTP, 2.5 mM random hexamers, 0.33 U/µl RNAse inhibitor, 10 U/µl reverse transcriptase (all reagents from Roche), 20 µl RNA, RNAse free water until a final volume of 100 µl.

Reaction was run at 25°C for 10 min, 42°C for 60 min and 95°C for 5 min in a termal cycler. Reaction products were stored at 4°C until running PCR.

The first round of PCR was carried out using the primers listed in Table 5. A standard PCR reaction was initially carried out for α3, α6, β2, β3 and β4 subunits, and β-actin, while a multiplex PCR reaction was performed with the couple of primers reported in Table 5 to amplify α2/α5 and α4/ α7 subunits. Primers used for multiplex reaction were designed to amplify more than one target sequence in order to minimize the possibilities for primer-primer binding. Primers employed for the initial step were designed to span exon-exon boundaries to avoid amplification of genomic DNA. The following solution (in mM unless otherwise stated): 50 KCl, 10 TrisHCl (pH 8.3), 2.5 $MgCl_2$, 0.25 each dNTP, 10 pM each upstream and downstream primers for the nAChR subunits and β-actin primers, 0.05 U/µl DNA Taq polymerase, was added to 10 µl of the RT product up to a final volume of 30 µl.

PCR was held at 94°C for 30 s, and then cycled 25 times (90°C for 15 s, 55°C for 20 s, 72 °C for 30 s each cycle). 2 µl of the multiple PCR products were used as substrate for the second round of specific PCR. A 25 µl reaction was prepared for each specific sequence analyzed.

The specific reaction consisted of (in mM unless otherwise stated): 50 KCl, 10 TrisHCl (pH 8.3), 1.5-3 $MgCl_2$, 0.25 each dNTP, 1 µl each upstream and downstream primers for the nAChR subunit or β-actin, and 0.025 U/µl DNA Taq polymerase in DEPC treated water. The second round (product specific) used the same primers listed in Table 5. Reaction was held at 92°C for 30 s and the cycled 40 times as follows: 92°C for 15 s, annealing temperature different for different primers (55-63°C) for 20 s and 72°C for 30 c. 7 ml samples were analyzed on 2 % agarose gels using ethidium bromide ad U.V. illumination.

Gloves were worn during all experiments with RNA and all tubes and tips were handled to be RNAse free.

Primer design

The first consideration for primer design was the sequence region to amplify. The second consideration was that the upstream and downstream primers should bind the exon regions. On the basis of these two requirements, primers were designed to amplify mRNAs for different nAChR subunits while recognizing their non homologous sequences. This was a difficult task because of the large homology between different subunits; for instance, α3 and α6 subunits share 98 % of their sequence (Le Novère et a., 1996). Candidate primers were examined with the PCRPLAN module of the PC/GENETM software package (kindly provided by Dr. Jerry Yakel, NIH, Research Triangle Park, North Carolina, USA). This program examines primer pairs for a number of parameters, in particular primer self-complementarity, stem-loops formation, complementarity between primers, complementarity to other regions of the template, and compatible calculated

annealing temperature (calculate annealing temperature within 5°C of each other). It is recognized that primers that have self-complementarity (that is, they bind to another identical primer) leading to stem-loops (primer folds over and bind to itself), or that are complementary to the other primer sequence, can remove a large fraction of the primary pool available for PCR. Primers that have a significant complementarity to other regions of the same template (or other unknown DNA sequences) can also generate multiple PCR products.

Candidate PCR primer sequences were also screened against the GenBank DNA sequence database using the BLASTN computer program. Primers that showed possible binding to other known sequences were rejected. Finally, primers were compared with those used by other research group (Klink et al., 2001; Le Novère et al., 1996; Sheffield et al., 2000; Sudweeks and Yakel, 2000).

Table5. List of primer sequences.

Primer	Sequence	Fragment length (bp)
α2/5	GTCTGGCTGAAGCAGGAATGGA AGGGTGACGAAGATCATGGTGAA	670/680
α4/7	ATTGATGTGGATGAGAAGAACCA AGCAGGAAGACGGTGAGAGAAAG	650/644
α2	GATCTGGATCCCAGACATTG CGCCGATGAGTGGGATGACC	572
α3	GGAGAAGTGACTTGGATCC CAAGTGGGCATGGTGTGTG	590
α4	CCAGATGATGACAACCAACG CCACACGGCTATGAATGCTC	356
α5	CGAACGTCTGGTTGAAGC CACCATAATGGATAGGG	549
α6	TCTTAAGTACGATGGGGTGATAAC AACATGGTC TTCACCCACTTG	608
α7	TTGCCAGTATCTCCCTCCAG CTTCTCATTCCTTTTGCCAG	214

β2	GCTGACGGCATGTACGAAG GGAGGTGGGAGGCACAATC	507
β3	CTCATTATCCACCTCCGTTT CTGTATCACTCTCCTTTCCATCC	306
β4	GGTTGCCTGACATCGTGTTG GCCAATGAGCGGTATGTC	541
β-Actin	AAGATCCTGACCGAGCGTGG CAGCACTGTGTTGGCATAGAGG	327

Protein detection

Antiboby staining of cultured chromaffin cells

For single and double staining of cultured chromaffin cells with antibodies against nAChR subunits, cells were fixed for 10 min at room temperature (RT) in 4 % paraformaldehyde in phosphate buffer saline (PBS), washed in PBS and permeabilized in 100 % ethanol for 15 min at -20°C. Cells were permeabilized because some of the antibodies recognized intracellular portions of the epitopes.

After rehydratation with decreasing ethanol concentrations in PBS at RT, cells were incubated overnight at 4°C with polyclonal antibodies against different nAChR subunits at different dilutions (as indicated in the results), diluted in 10 % FCS in PBS plus 0.01 % Tween 20 detergent (PBST). After washes in PBST, cells were incubated 2 h at RT with fluorescent conjugated secondary IgG antibodies, diluted 1:100 in 10 % FCS in PBS. Cells were then washed in PBS twice and mounted in Vectashield (Vector, Burlingame, California, USA). Fluorescence was analyzed with fluorescein and rhodamine filters, under dark field, on a Zeiss Axiophot microscope. As control, some cover-slips were incubated with the secondary antibody only.

For double staining experiments, additional controls were necessary in order to avoid cross-reactivity of the secondary antibodies. Thus, we also tested cells under

the following experimental conditions: absence of one of the primary antibodies, absence of one of the secondary antibodies, lack of primary antibodies.

Western blot of adrenal gland homogenates

Whole extract from rat adrenal glands or kidney were analyzed with Western blot. Fresh tissues were homogenized using a glass potter in 10 volumes of ice-cold lysis buffer containing (in mM): 25 Tris-HCl (pH 7.5), 1 ethylenediaminetetraacetic acid (EDTA), 1 spermidine, 1 iodacetamide, protease inhibitors (Sigma). After adding 1 % Triton X-100, proteins were extracted for 1 h at 4°C under vortexing, and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was recovered, diluted 1:1 (v/v) in Laemmli buffer and loaded onto 8 % sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Proteins were transferred to nitrocellulose membranes (Amersham), blocked at RT for at least 5 h in Tris-buffered saline (TBS) buffer (25 mM Tris pH 7.5, 150 mM NaCl) plus 5 % dry milk and 2 % FCS, and then incubated overnight with antibodies against nAChR subunits in the same blocking buffer at 4°C. After washing with TBS, membranes were incubated with peroxidase-conjugated secondary antibody (Sigma, 1:2000) for 5 h. Bound peroxidase was detected using a chemioluminescence ECL kit (Amersham) and exposed to Hyperfilm ECL (Amersham) for up to 60 min.

RESULTS

1. Functional and molecular characterization of nAChRs in rat chromaffin cells

1.1 RT-PCR analysis

Fig 7 shows an example of RT-PCR analysis for the nAChR subunit transcripts present in chromaffin cells. When the product of the RT reaction was amplified using specific primers for each nAChR subunit, in a PCR reaction, the primers for the α2, α3, α4, α5 and α7 subunits always yielded products of the expected size, whereas the α6 did not (Fig 7A). Fig. 7B shows the results of experiments for the detection of nAChR β subunits and control positive for β-actin. The primers for the β2 and β4 subunits and those for β-actin always yielded products of the expected size, whereas the β3 was never detected. Similar data were obtained by repeating such experiments six times. Despite the use of different primers (Klink et al., 2001; Sheffield et al., 2000; Sudweeks & Yakel, 2000), a signal for α6 or β3 subunits was never detected.

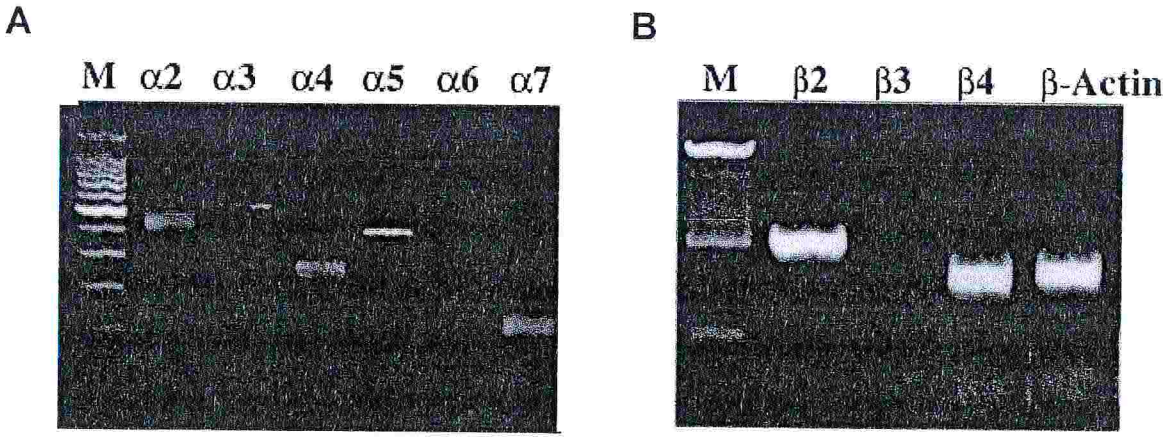

Fig. 7. (A, B) RT-PCR analysis of nAChR subunit cDNA from chromaffin cells. (A) The mRNA content for the different nAChR subunits is amplified by RT-PCR and yields cDNA fragments of the expected size (in bp): α2 (572), α3 (590), α4 (356), α5 (549), α7 (214). (B) Similar approach applied to β subunits is also shown with β2 (507) and α4 (541). As a positive RT-PCR control, β-actin is also amplified (327). No bands relative to α6 and β3 subunits are detected. The marker lane (M) shows bands at 100-bp increments.

Preliminary single-cell RT-PCR experiments confirmed the results obtained with total RNA content extraction from cultured chromaffin cells. On 23 cells (94 ± 4 % of which yielded positive β-actin signal), the relative distribution of the various subunits was (in descending order of percentage distribution ± SEP): 83 ± 8 % β4, 61 ± 10 %, α3, 57 ± 10 % β2, 48 ± 10 %, α5, 30 ± 9 % α2, 26 ± 9 % α4, 13 ± 7 % α7; α6 and β3 was never detected. No false positive data were found. The probability of co-detection of α3α5 ($r = 0.44$), α3β4 ($r = 0.43$) and α5β4 ($r = 0.77$) subunits was statistically significant ($P < 0.03$, $P < 0.05$ and $P < 0.006$, respectively).

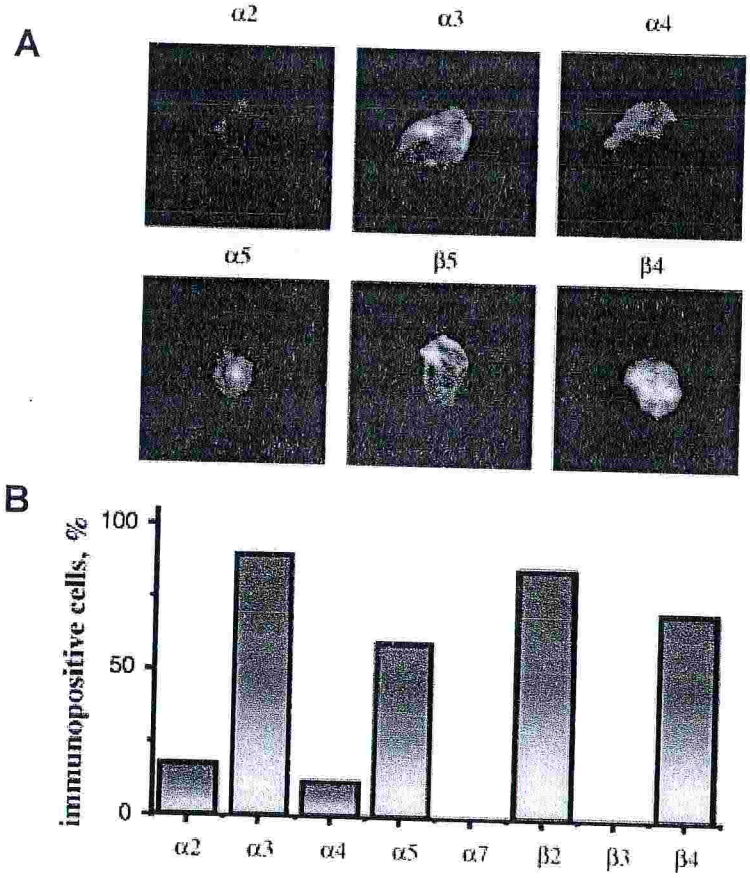

Fig. 8. Immunostaining of nAChRs of chromaffin cells. (A) Expression of α2,α3, α4, α5, β2 and β4 subunits in chromaffin cells is detected by indirect immunofluorescence using specific antibodies. (B) The histograms show the percent of nAChR subunit-specific positive cells over the total number of cells examined. The most frequently observed subunits were (in descending order) α3, β2, β4 and α5.

1.2 Expression of nAChRs detected by immunocytochemistry

An example of immunofluorescence staining from one experiment (repeated in triplicate) obtained with antibodies against α2, α3, α4, α5, β2, or β4 subunits is shown in Fig. 8 A. Fig. 8 B indicates the relative distribution of nicotinic subunits among those chromaffin cells. In particular, expression of the α2 subunit was detected in 8/44 cells, while the α3 subunit was observed in 36/40 cells. In the case of the α4 subunit, staining was found in 4/32 cells only, while immunoreactivity against the α5 subunit was observed on 12/20 cells.

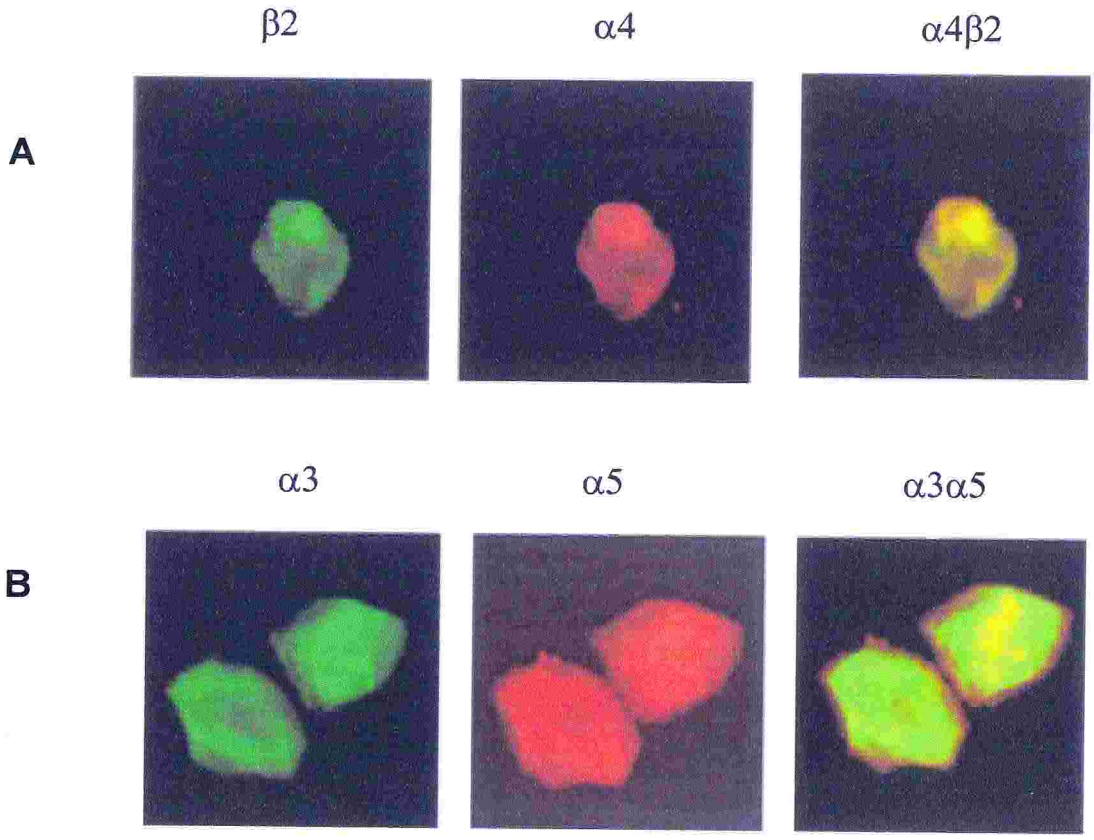

Fig. 9. Simultaneous detection of immunofluorescence staining for α4/β2 and α3/α5 subunits in chromaffin cells. Cells are reacted simultaneously with (A) anti-α4 and anti-β2 or (B) anti-α3 and anti-α5 specific antibodies, showing α4/β2 or α3/α5 co-expression. Colours are generated by excitation of the secondary fluorochrome-conjugated antibodies (green, FITC, and red, CY3, respectively). Overlapping the two independently obtained images produces yellow-like staining (see right panel) to indicate wide topography of expression of either subunit.

As far as β subunits were concerned, the β2 antibody signal was observed in 30/35 cells and the β4 signal in 20/28 cells. No chromaffin cell was immuno-reactive for α7 or β3 subunit. In no instance staining was detected when primary antibodies were omitted.

We then sought to demonstrate co-expression of distinct subunits by the same cell using double immunofluorescence. Double staining experiments were not carried out when the two primary antibodies originated from the same animal species because, in preliminary experiments, omitting one of the two primary antibodies (while retaining both secondary antibodies) consistently yielded artefactual double staining. Thus, we could test co-expression of α3 with α5, and α4 with β2. Testing for α4β2 co-expression seemed interesting on the basis of the pharmacological profile of nicotine-induced responses reported below. Fig. 9 A shows an example of α4 or β2 subunits expressed individually (left, middle panels), or co-expressed (right). Likewise, α3 or α5 subunits were first observed separately (Fig. 9 B, left and middle panels) and subsequently together (Fig. 9 B, right).

1.3 Western blot analysis of rat adrenal gland homogenates

In view of the high homology displayed by the different nAChR subunits, western immunoblots of protein extracts obtained from rat adrenal gland or kidney (used as negative control) were performed to characterize the specificity of the antibodies. After separating adrenal gland homogenates (obtained from 5 experiments) with SDS-PAGE, bands corresponding to the molecular weight of α3, α4, α5, β2 or β4 subunits were demonstrated (Fig. 10). The anti-α3 antibody recognized a single band of about 66 kDa, a value larger than the one (58 kDa) reported previously (Mousavi et al., 2001). To confirm our data, we repeated western blots with protein extracts from SH-SY5Y neuroblastoma cells known to express α3 subunit (Guan et al., 2000; Martin-Ruiz et al., 1999;), and we obtained analogous results as with chromaffin cells (not shown). The anti-α4 antibody recognized a single band at 70 kDa in accordance with Arroyo-Jimenez et al. (1999), while the anti-α5 antibody recognized a band of 45 kDa (similar to the result obtained by Balestra et al, 2000,

for chicken subunit) and the anti-β4 antibody detected a band of 54 kDa (as reported by Zhou et al., 2001).

In the case of β2, the antibody recognized a band at approximately 50 kDa in agreement with data by Jones et al. (2001). In a few cases an additional band (70 kDa) was also present.

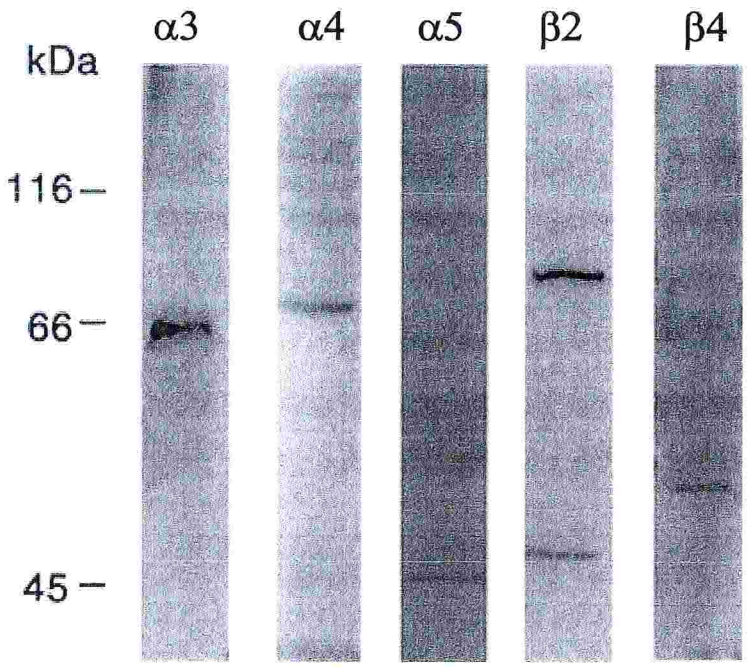

Fig. 10. Western immunoblot data of protein extracts from rat adrenal gland. Gels show protein bands detected after incubation with antibodies against α3, α4, α5, β2 and β4 rat nAChR subunits.

Two distinct antibodies against α2 failed to generate any signal. Likewise, no signal was detected using the anti-β3 antibody, confirming absence of this subunit as demonstrated with PCR and immunofluorescence. When western blot analysis was performed with protein extracts from rat kidneys as negative control, no signal was detected.

1.4 Functional characterization of native rat chromaffin cells using selective blockers

Presence of mRNA, detected with RT-PCR analysis, and expression of subunit proteins, detected with immunostaining and immunoblotting, left unanswered the issue of whether certain receptors were functional and, if they were, what their molecular identity was in view of the complexity of potential subunit assemblies. A useful approach to clarify this question was to employ various pharmacological tools to discriminate between different receptor compositions. Receptor activity was assayed on the basis of membrane currents evoked by brief applications of nicotine (to minimize receptor desensitization; Khiroug et al., 1998). When, under conditions of voltage clamp, nicotine was briefly applied to a chromaffin cell via a puffer pipette, it always generated inward currents, mediated by the activation of nAChRs.

Application of α-bungarotoxin (α-Bgtx) in concentrations up to 5 μM (for 30 min) failed to change nicotine-induced currents (95 ± 10 %, n=8), indicating that no functional α7 homomeric receptors could be detected on chromaffin cells. Fig. 11 A shows that nM concentrations of methyllicaconitine (MLA) left unchanged nicotine evoked currents.

However, larger concentrations of MLA, which do not retain selectivity for α7 receptors (Macallan et al., 1988), reduced nicotine evoked currents (Fig. 11 A) as exemplified in figure 11 B in which nicotine induced currents in control and 1 μM MLA solution are superimposed to demonstrate antagonism (which on average amounted to 44 ± 3 %; n=8, $P<0.05$). Complete current block was obtained with 10 μM MLA.

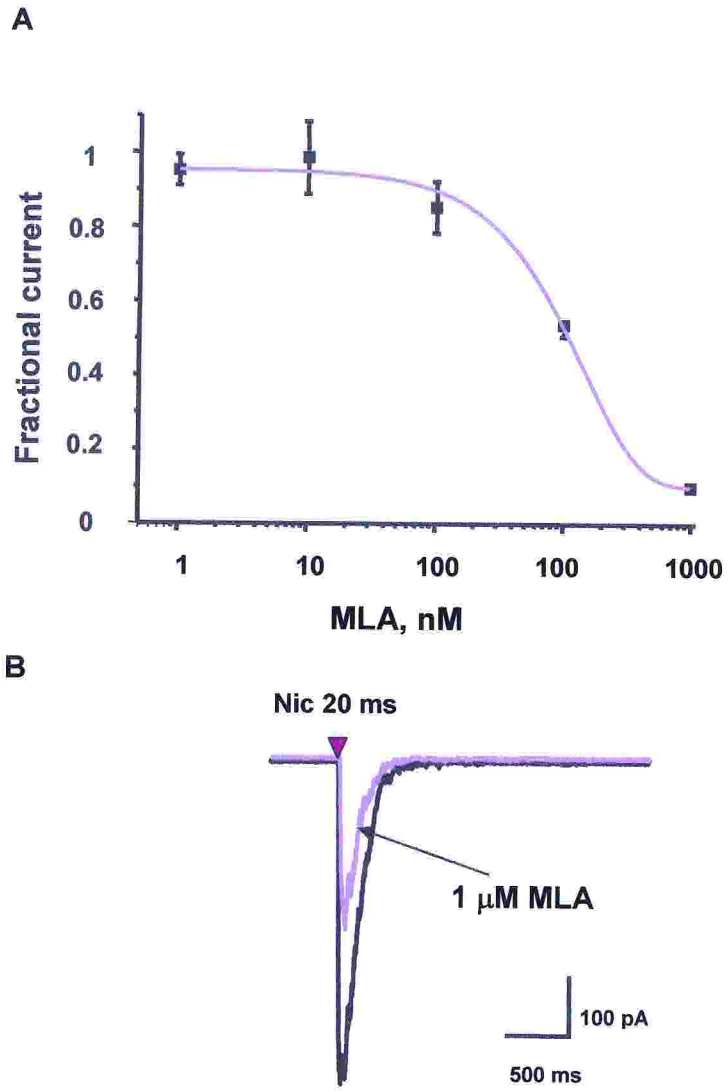

Fig. 11. Effect of the antagonist MLA on nicotine-induced currents. (A) Plot of nicotine current amplitude (as fraction of control) vs. log concentration of MLA. Antagonism is observed only with MLA doses larger than 300 nM. Data are from eight cells. (B) Example of inhibition of nicotine response by large concentration of MLA. Note overall reduction in current without changes in onset or offset.

AuIB and MII, two toxins isolated from the venom of the *Conus* sea snail, are believed to be selective and very potent (nM range of IC_{50}; concentration producing 50% of the maximal inhibition of response amplitude) antagonists against $\alpha3\beta4$ receptors and $\alpha3\beta2$ receptors, respectively (Cartier et al., 1996; Kaiser et al., 1998;

Luo et al., 1998). In the presence of 1 nM MII (see Fig. 12 A), nicotine evoked currents were slightly, yet significantly ($P<0.05$) depressed (by 13 ± 3 %; n=12), indicating presence of α3β2 receptors. However, with 1 μM MII, a residual response to nicotine persisted, indicating antagonism saturation around 50 % of the agonist response.

In order to calculate the IC_{50} value of MII, we therefore took as maximal inhibition the one observed with 1 μM MII and scaled the graph accordingly on the assumption that the residual current was due to a toxin-insensitive component (Fig. 12 B). The calculated IC_{50} value was then 35 nM, that is in line with the one reported for recombinant α3β2 receptors expressed in *Xenopus* oocytes (0.5-8 nM) (Cartier et al., 1996; Harvey et al., 1997; Kaiser et al., 1998). Since the dose-response curve for MII toxin antagonism stretched over four concentration log-units, this observation indicates heterogeneity of chromaffin nAChRs, only a fraction of which was blocked by such a toxin.

Similar observations were obtained with the AuIB toxin, which (as indicated in Fig. 12 A) yielded a shallow inhibition curve, demonstrating it to be a blocker of a limited subgroup of nAChRs. In fact, when the inhibition curve for AuIB was scaled using the same procedure described above for MII antagonism (Fig 12 B), the IC_{50} value (105 nM) was compatible with the one reported for α3β4 receptors (750 nM) expressed in *Xenopus* oocytes (Luo et al., 1998).

In order to evaluate the relative contribution by α3β4 as well as α3β2 receptor complexes to the total membrane current, both conotoxins were applied to the same chromaffin cells (Fig. 13 B). After depressing nicotine evoked responses by 16±3 % (n=9) with 10 nM AuIB, application of 10 nM MII produced further current depression (42±3 %; n= 12), which was larger than the one observed with MII alone. However, on the same group of cells, larger concentrations (100 nM or 1 μM) of co-applied toxins did not intensify the extent of block already found with MII alone (Fig. 13 B).

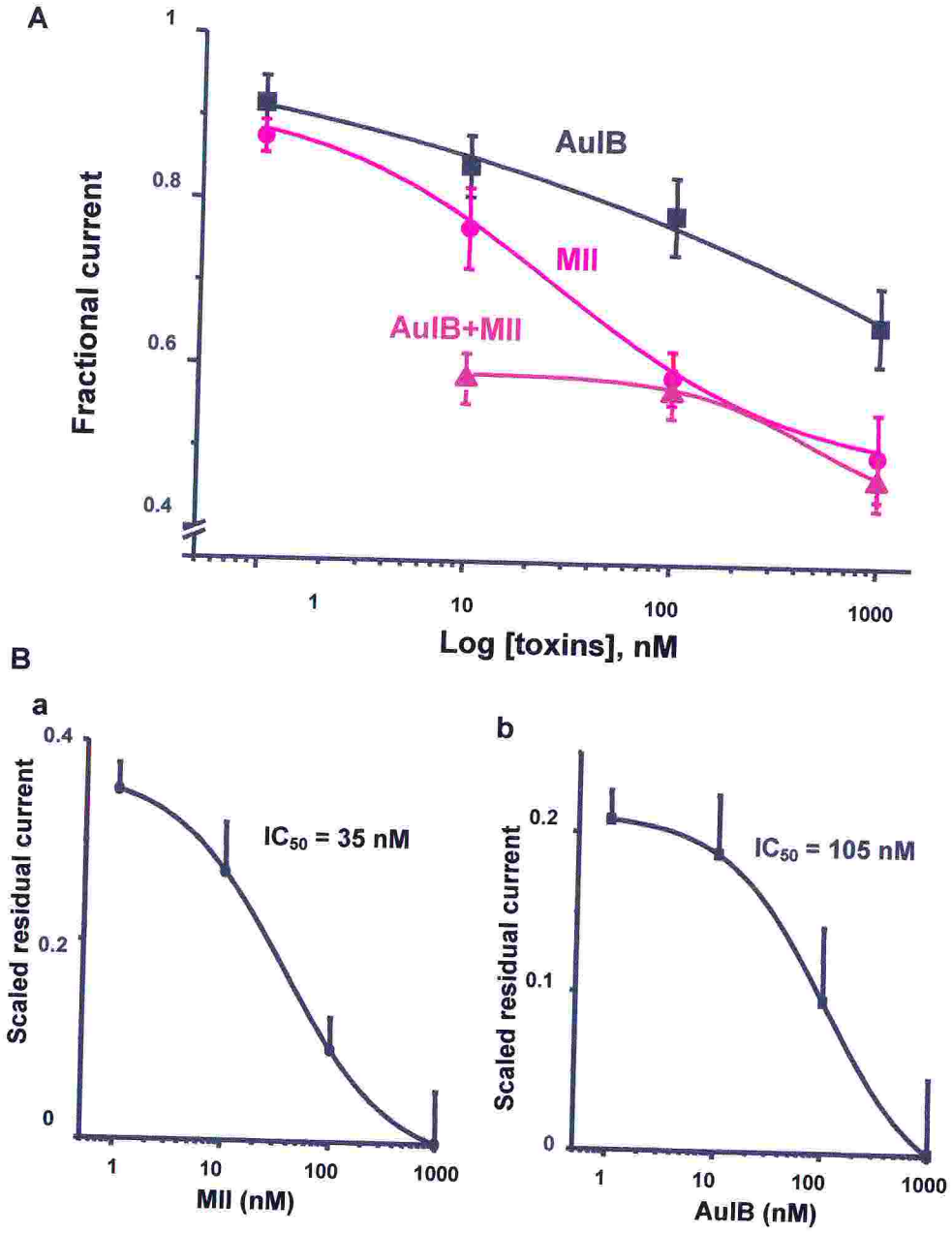

Fig. 12. **(A)** Plot of nicotine current amplitude vs. log concentration of AuIB, MII, or their combination. Note that AuIB or MII produce gradual reduction in response amplitude, leaving a residual current even at the highest concentration tested. With coapplication of both toxins (10 nM), there is antagonism summation which is lost with higher toxin doses. Data are from 9-12 cells. (B) Scaled inhibition curves for (a) MII or (b) AuIB. Note that, assuming complete block of toxin-sensitive receptors, the IC_{50} values are very similar to those reported for selectivity against specific receptor assemblies.

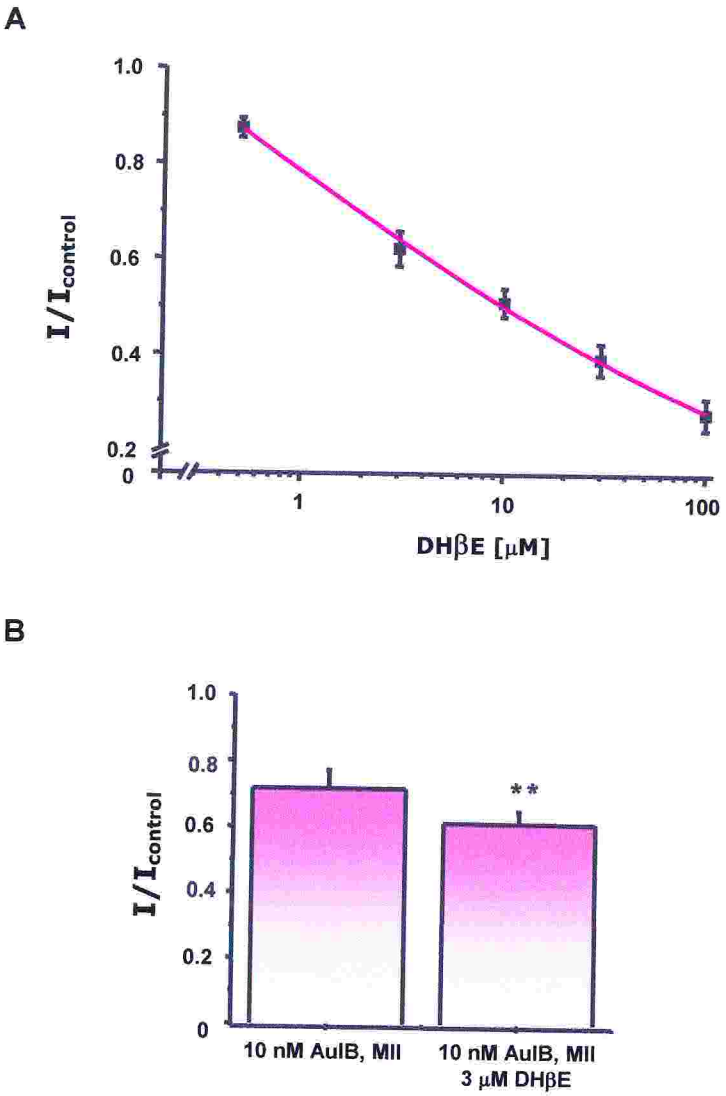

Fig. 13. (A) Plot of nicotine current amplitude vs. log concentration of DHβE. Note gradual decrease in current response with increasing DHβE concentration. Data are from eight cells. (B) Additional effect of DHβE on nicotine evoked currents partially blocked by prior application of 10 nM MII and AuIB.

Dihydro-β-erythroidine (DHβE), suggested to be selective for α4β2 receptors when used at low concentrations (Chavez-Noriega et al., 1997; Zoli et al., 1998), was used to test for the functional presence of α4β2 receptors. The threshold concentration of DHβE for nAChR block was 500 nM (as nicotine evoked responses were blocked by 13 ± 2%, n=8, $P<0.05$) with 10 μM IC_{50} value (Fig. 13 A). On the assumption that DHβE (3 μM) was preferentially selective for α4β2

receptors over α3β4 and α3β2 receptors (Chavez-Noriega et al., 1997), we questioned the relative contribution by α4β2 receptors to the nicotine current left after blocking α3β4 and α3β2 receptors with AuIB and MII. Thus, we tested the effects of combining DHβE with these conotoxins (Fig. 13 B). After obtaining a partial block (35 ± 4 %, n=5) of nicotine evoked responses by 10 nM AuIB together with 10 nM MII, application of 3 μM DHβE further depressed inward currents by 14 ± 6 % (n=5, $P<0.05$), giving an overall residual current of 51 ± 10% with respect to control. When the two conotoxins were used at 100 nM concentration, 30 μM DHβE blocked by 43 ± 6 % the toxin-resistant current (n=4, $P<0.05$), yielding a residual current of 37 ± 10 % of control.

Basic pharmacological properties of nAChRs of SH-SY5Y cells

SH-SY5Y cells posses native nAChRs analogous to those normally found on autonomic ganglion neurons (and chromaffin cells; Ridley et al., 2001; Wang et al., 1998; Wang et al., 2002). Because SH-SY5Y cells are more stable in culture, they are, therefore, suitable for investigating the effects of chronic treatments on nAChRs.

SH-SY5Y cells express functional α7, whereas rat chromaffin cells, as shown earlier, do not (Séguéla et al., 1993). Nevertheless, the presence of α7 mRNA in chromaffin cells suggests the possibility that, under certain conditions, α7 receptors can be functionally expressed. This condition may be species dependent because it has been shown that bovine chromaffin cells normally express α7 homomeric receptors (Garcia-Guzman et al., 1995)

My initial experiments characterized the pharmacological properties of nAChRs in untreated SH-SY5Y cells. Two s-long nicotine pulses, applied by fast perfusion, produced inward currents (as shown in the examples of Fig.14 A, inset) which peaked and decayed before the end of the drug application. The concentration-peak amplitude response curve had 43 μM EC_{50} value and 1.7 Hill coefficient (Fig.14 A). At 1 mM concentration nicotine produced a maximal current amplitude of -169 ± 14 pA (n=6) which fell rapidly to baseline and was followed by a rebound current

on wash, presumably indicating a degree of open channel block (and rapid relief) by

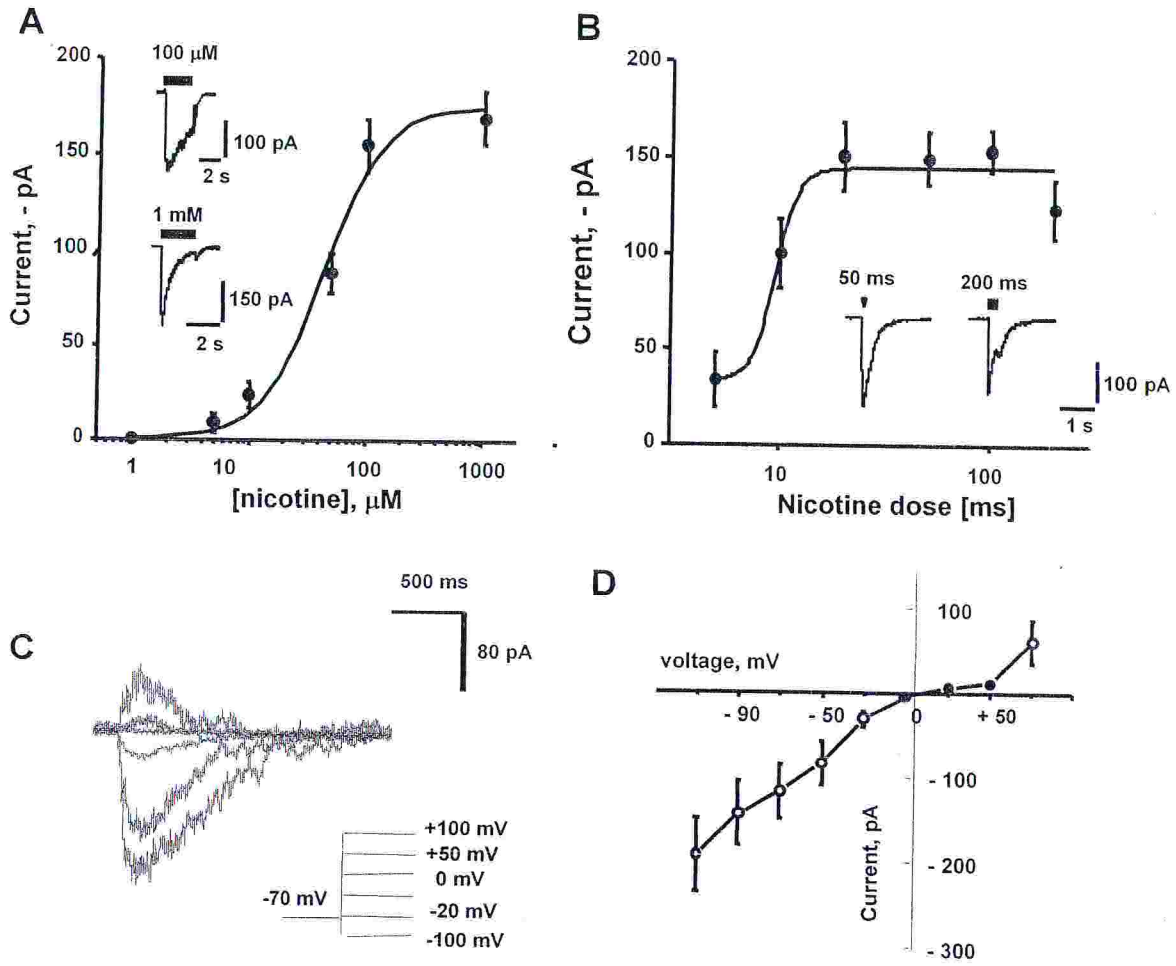

Fig. 14. Properties of native nAChRs expressed on SH-SY5Y cells. (A) Concentration-response curve for nicotine on control cells (n=8 cells). Insets show typical currents evoked by 100 µM or 1 mM concentrations applied by superfusion. Note rebound current at the end of 1 mM nicotine application. (B) 1 mM nicotine puffer pulse-response curve (n=6 cells). Insets demonstrate averaged currents induced by 50 ms or 200 ms pulses with rebound current after the 200 ms application. (C) Examples of currents elicited by 50 ms nicotine pulse at different membrane potentials as indicated in the scheme below with voltage steps from –70 mV holding potential. (D) Current-voltage (I-V) relation for nicotine pulse (50 ms) responses recorded from control cells (n=7).

large concentrations of agonist (Drapeau & Legendre, 2001; Maconochie & Knight, 1992; Uteshev *et al.*, 2002).

Because the rapid current decay was presumably due to receptor desensitization, we performed additional control experiments to find out if nicotine, more focally applied to the recorded cell via a puffer pipette, yielded a different response profile. Varying the puffer pulse duration allowed construction of pulse-response curves (Di Angelantonio & Nistri 2001) as shown in Fig. 14 B.

Fifty ms application of nicotine produced a maximal response of -159 ± 15 pA (n=78), which was then used as a standard nicotine application for most experiments. The maximal current monoexponentially decayed to baseline (time constant, τ, = 150 ± 13 ms; n=19), thus providing an index of the onset of receptor desensitization (Khiroug *et al.*, 1997). Longer nicotine application produced rapidly fading currents followed by a rebound current (see inset to Fig.14 B for the 200 ms pulse of nicotine) in accordance with the data obtained with 1 mM application of nicotine via rapid superfusion (Fig 14 A, inset). The reversal potential for nicotine evoked currents was 6.8 ± 2.0 mV (see Fig. 14 C, D), consistent with the standard notion that receptor activation mediated an increase in non-specific cation conductance.

2. Comparing the acute and chronic effects of the nicotinic antagonist CC4 on nicotine-evoked currents

The next series of experiments were performed to investigate the effects of the cytosine (Cyt) derivative CC4 on nAChRs. Molecular biology experiments have been done by Cecilia Gotti's group in Milan (CNR, Institute of Neuroscience, Cellular and Molecular Pharmacology (see Appendix 1)), whereas our goal was to characterize the principal pharmacological properties of CC4 on nicotine-induced currents in control SH-SY5Y cells and to investigate the effect of chronic CC4 treatment on the nAChR function.

In brief, treatment for 48 hours 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 binding receptors. Preliminary experiments showed that CC1, CC2, CC3, CC6 or CC7 evoked a dose-dependent up-regulation 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 up-regulation, cytisine or CC4 induced the highest level of up-regulation (3.5-4 fold at 1000 μM).

Since the strongest overall up-regulation was obtained with CC4, we decided to investigate the effect of this compound in detail.

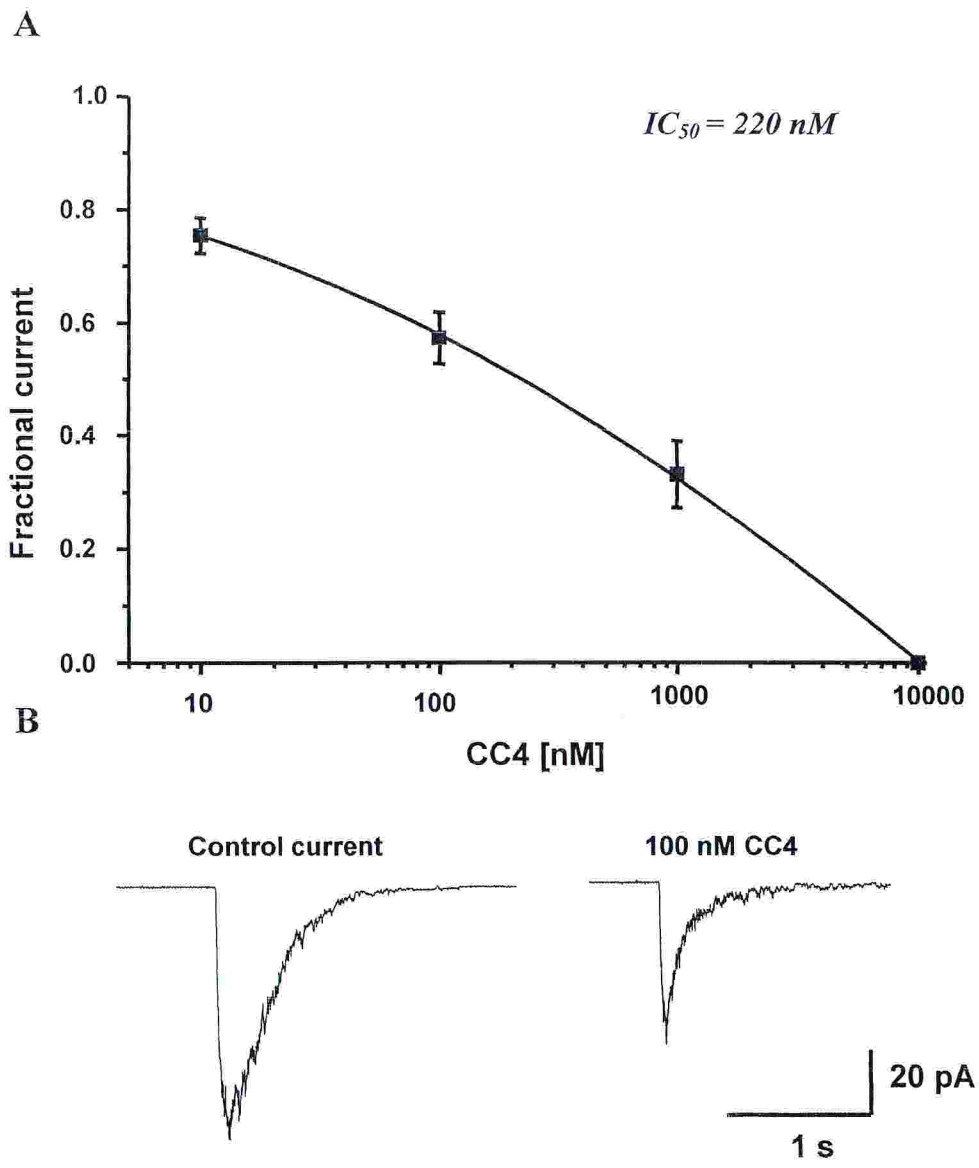

Fig. 15. (A) Fractional reduction in nicotine current amplitude against different logarithmic concentration (from 10 nM to 10 μM) of CC4. Data are from 13 cells. (B) Example of the reduction in current amplitude in the presence of 100 nM CC4. Nicotine was applied by puffer pulses (50 ms).

2.1 Pharmacological characterization of nicotine-induced currents in the presence of acutely applied CC4

First, we characterized the principal pharmacological properties of CC4 on nicotine-induced currents of control SH-SY5Y cells. Fifty millisecond pressure application of nicotine (1 mM) induced an inward current whose peak amplitude was reduced by 35 % after 15 s pre-applied CC4 (100 nM; via the fast superfusion system; Fig. 15 B). This agonist application was selected to produce a maximal response amplitude (see also Fig. 16 A) with a brief pulse in order to minimize nAChR desensitization (Di Angelantonio *et al.*, 2003). 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 could quantify the reduction in the nicotine-evoked currents.

Figure 15 A 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 16 A 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-application), 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). Longer pulse duration of nicotine application could not restore the full response amplitude in the presence of CC4 (Fig. 16 B). 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 non-equilibrium conditions. Overall, these data indicate that CC4 was an effective antagonist of SH-SY5Y nAChRs.

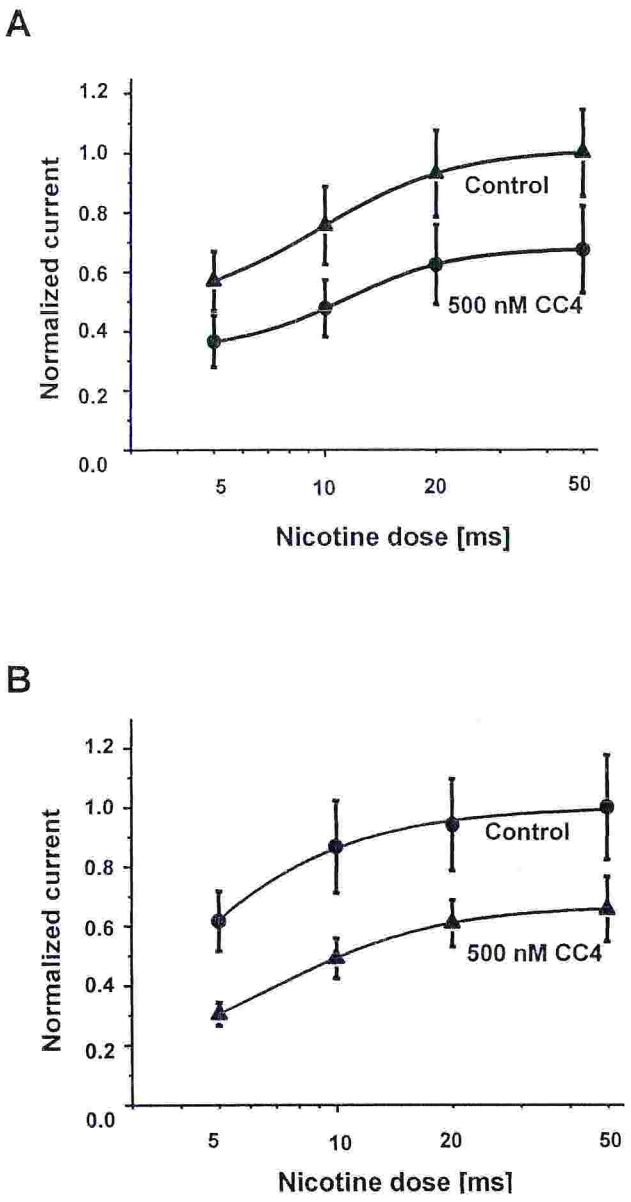

Fig. 16. Plots of nicotine current amplitude vs. nicotine puffer ejection time in control condition and in the presence of 500 nM CC4 (15 s preapplication). A: control cells (n=9); B: cells previously treated for 48 h with 1 mM CC4 (n=9).

2.2 Chronic CC4 treatment changes nicotine-evoked currents

To find out if the up-regulated receptors present on the cell membrane following chronic exposure to CC4 were functional, we applied 1 mM CC4 for 48 hours,

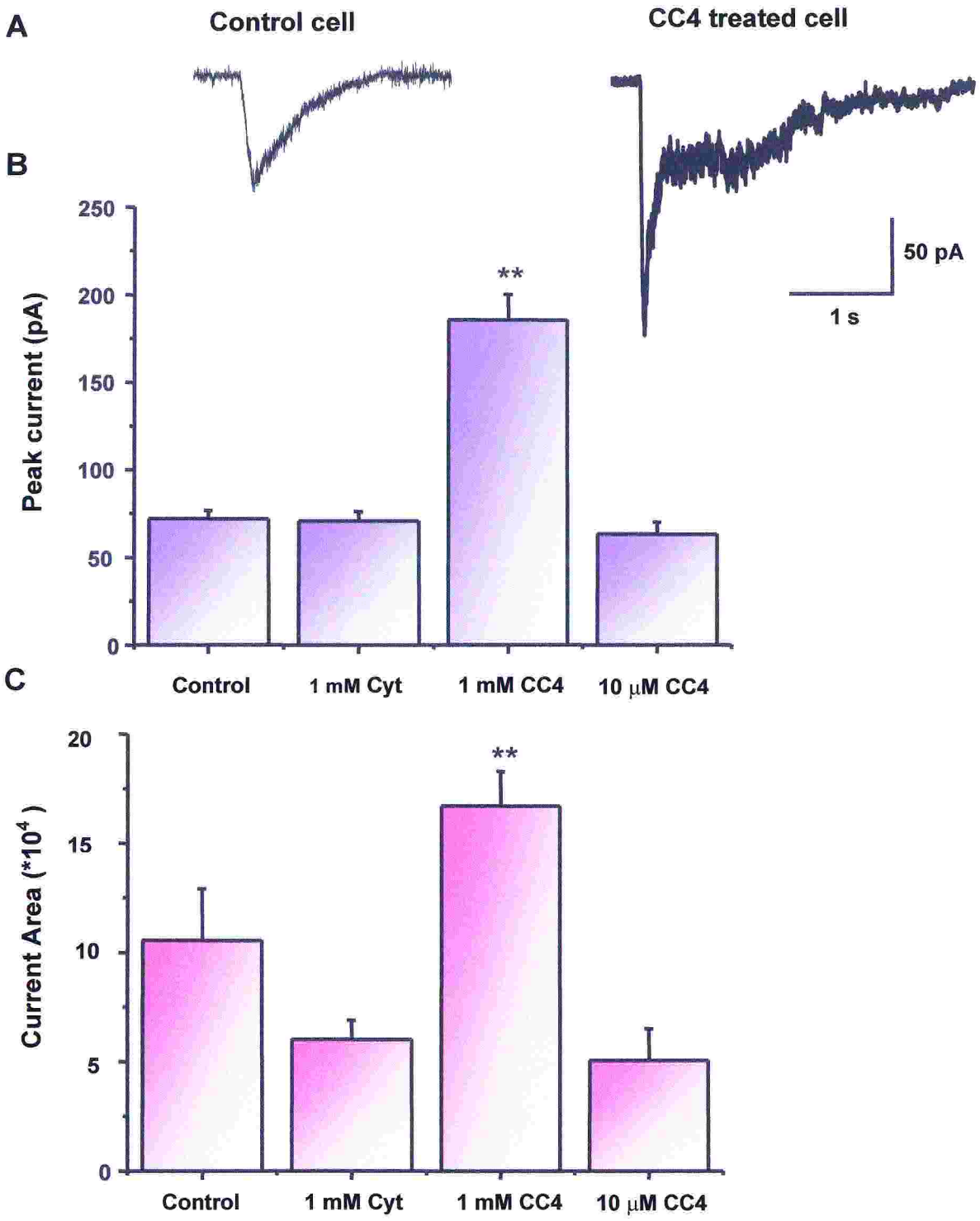

Fig. 17. Effects of the chronic application of CC4 on nicotine-evoked currents. (A) Representative nicotine-evoked current (1 mM, 50 ms) in a control cell and a cell treated with 1 mM CC4. (B) Mean peak amplitude of nicotine-evoked currents in control cells (n=68) and cells treated for 48 h with 1 mM Cyt (n=52), 1 mM CC4 (n=71) or 10 μM CC4 (n=62). Treatment with 1 mM CC4 significantly increased the mean current amplitude ($P<0.05$). (C) Mean area of nicotine-evoked currents in control cells and cells chronically treated with 1mM Cyt, 1 mM CC4 or 10 μM CC4. Treatment with 1mM CC4 significantly increased the mean current area ($P<0.05$).

currents in control cells and cells chronically treated with 1mM Cyt, 1 mM CC4 or 10 μM CC4. Treatment with 1mM CC4 significantly increased the mean current area ($P<0.05$).

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 Fig. 17 B 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 (Fig. 17 C). After normalizing 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 CC4 (n= 62), a concentration, however, sufficient to completely block nicotine-evoked current in acute conditions. We hypothesized that, during a long incubation time, CC4 might have been degradated/inactivated.

Fig. 18 shows that the inhibitory effect of CC4 on nicotine evoked currents was decreased after 48 h incubating this antagonist at 37°C in culture medium: while 1 μM CC4 normally inhibited 67 ± 5.8 % of nicotine evoked current, it had less inhibitory effect (50 ± 2.5 %) after incubation. Ten μM CC4 totally inhibited nicotine evoked currents in normal conditions, but, after incubation of the CC4 solution, a residual current of about 32 ± 1.5 % remained in the presence of 10 μM CC4.

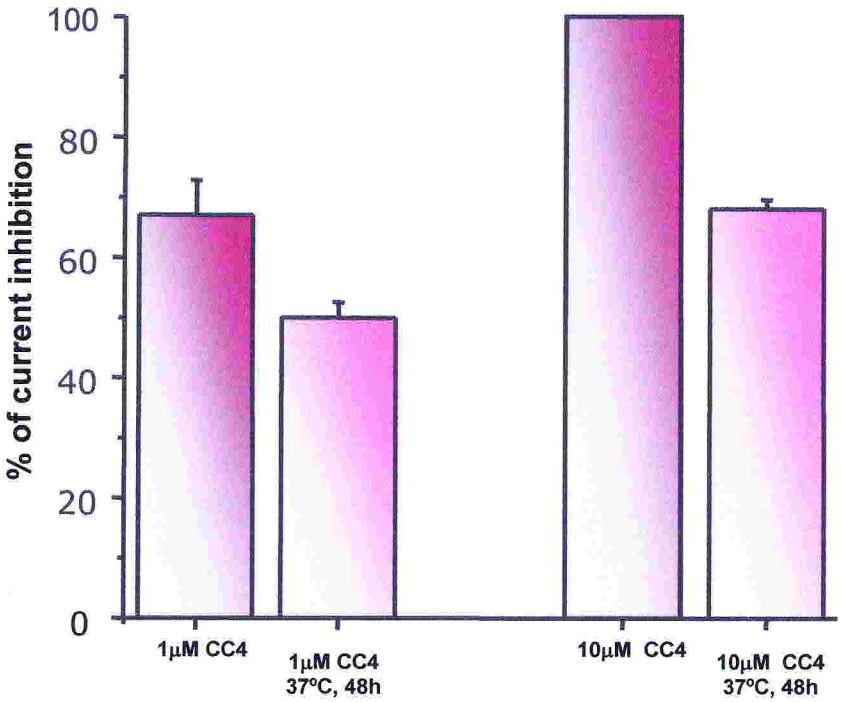

Fig. 18. The graph shows loss of CC4 inhibitory activity on nicotine-evoked currents, when a solution of CC4 was incubated at 37°C for 48 h. Before incubation, 10 μM CC4 completely blocks the nicotine current (100% inhibition), whereas after incubation its ability to block the nicotine current decreases (68%). Data relative to CC4 incubated at 37°C are from 16 cells.

We also studied whether chronic treatment with CC4 (1 mM) altered the ability of acutely applied CC4 to antagonize nAChRs. As shown in Fig. 16 B, 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. Fig. 16 A).

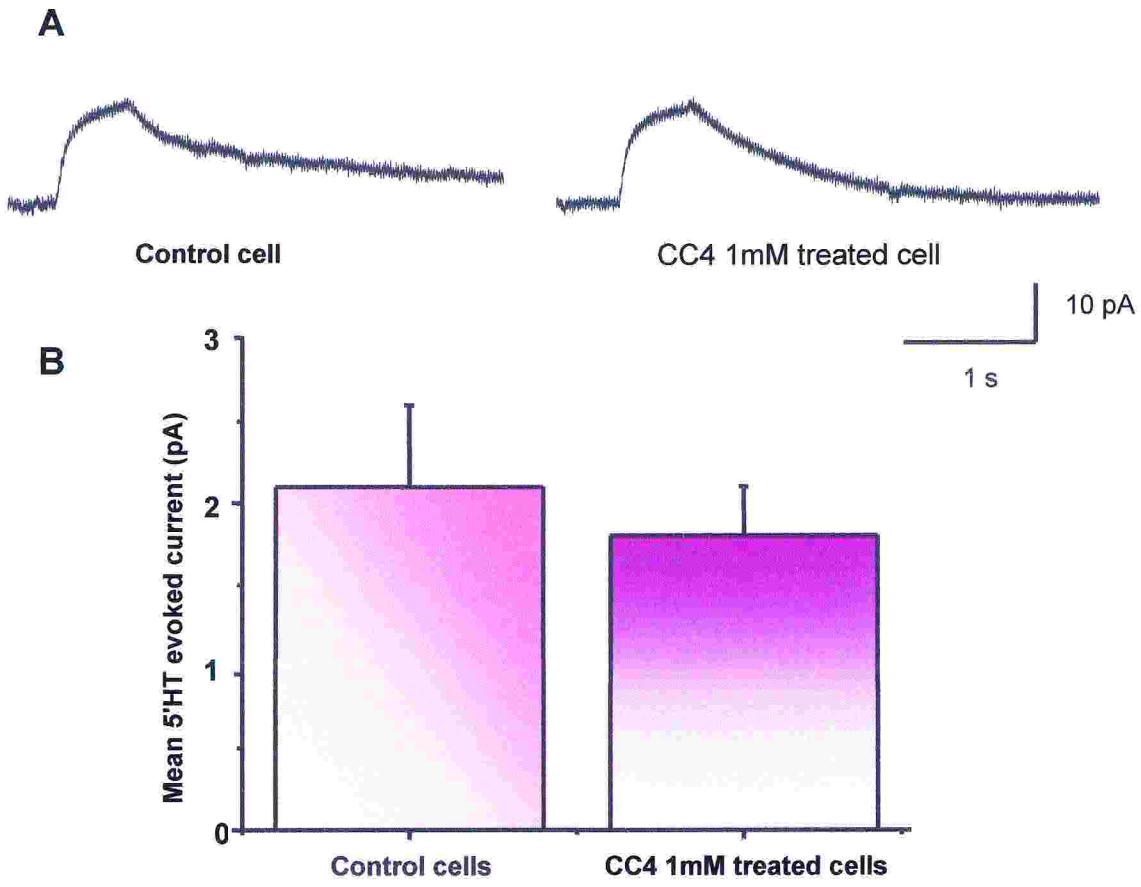

Fig. 19. (A) Example of 5-HT evoked current in a control cell and in a CC4 treated cell. (B) Summary of the effect of CC4 treatment on 5-HT evoked current, in control (n=16) and CC4 treated (n=17) cells (no significant difference).

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 for 500 ms) 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 (Fig. 19 A). The histograms in Fig. 19 B summarize the results obtained from 17 cells to show that chronic CC4 application did not bring about a broad enhancement in membrane receptor function.

2.3 Nicotinic AChR 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 Fig. 17 A) 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 up-regulated α7-containing receptors often responsible for the fast activating and inactivating component of the nicotine-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 Fig. 20 A 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 ± 1 %; n=7) than in control (88 ± 3 %; n=6).

An example of the blocking effect of MLA on nicotine-evoked currents in CC4-treated cells is shown in Fig. 20 B, 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 (see Appendix 1) and suggests a stronger contribution by functional α7 receptors in cells chronically treated with 1 mM CC4.

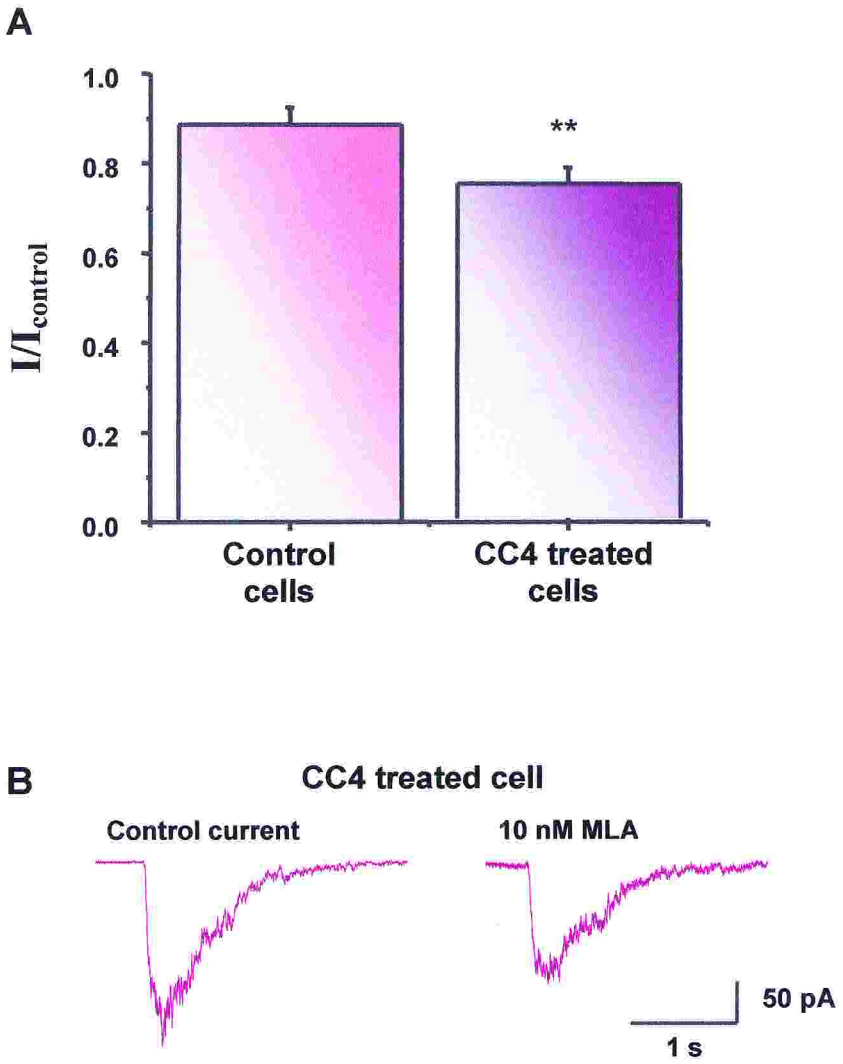

Fig. 20. (A) Fractional reduction in nicotine evoked current in the presence of 10 nM MLA, in control cells (n=6) and in CC4 treated cells (n=7). (B) Example of nicotine evoked current in a CC4 treated cell, in control condition and in the presence of 50 nM MLA.

Because 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 by Gotti *et al.*, 2005) although the latter subunit is not expressed in SH-SY5Y cells (see immunoprecipitation results) (Peng *et al.*, 1997; Ridley *et al.*, 2001; Wang *et al.*, 1998).

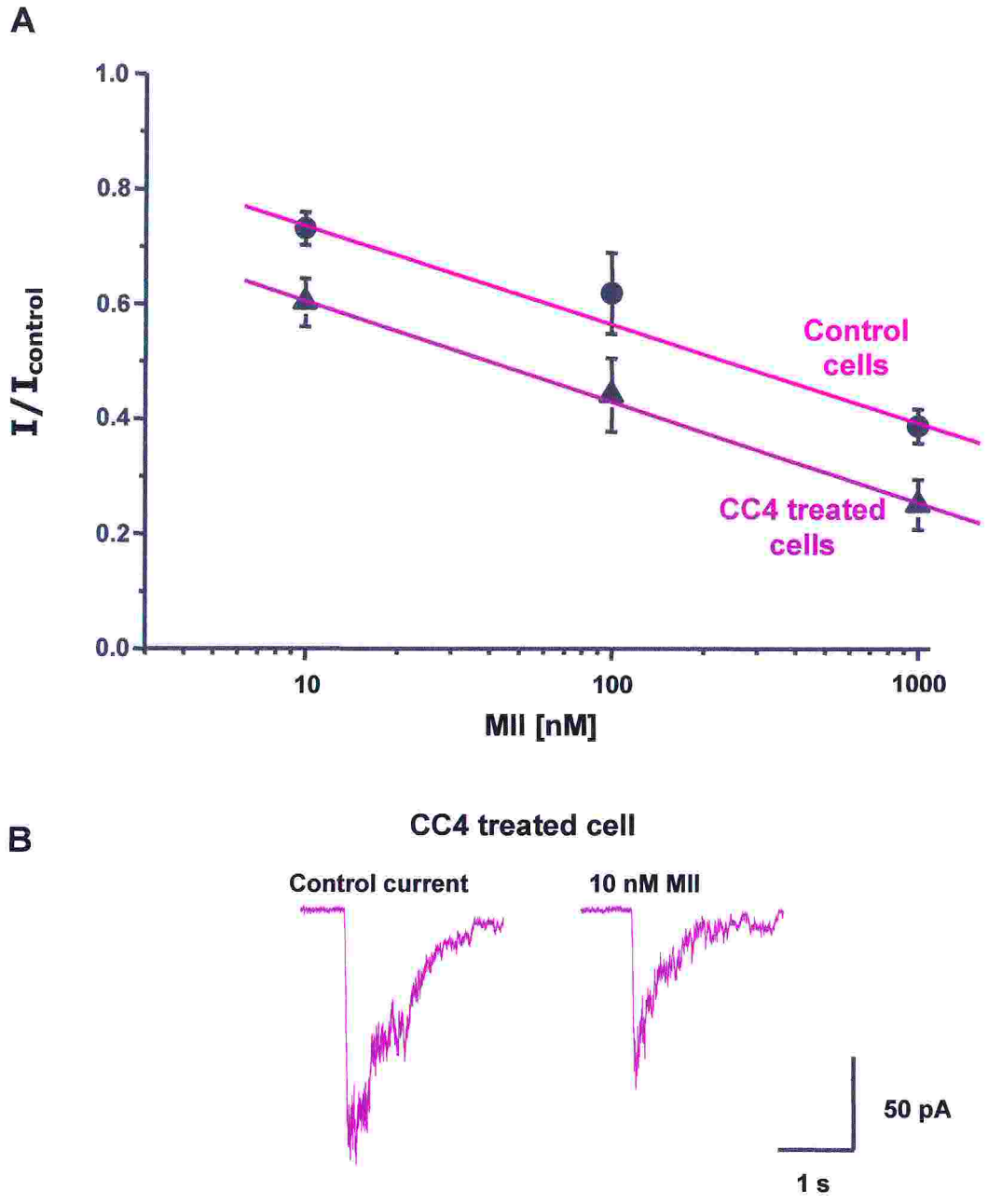

Fig 21. Fractional reduction in current amplitude vs. MII concentration, in control cells (n=6) and in CC4 treated cells (n=6). (B) Example of nicotine evoked current in a CC4 treated cell, in control conditions and in the presence of 10 nM MII.

Figure 21 A 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 21 B 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.

3. Desensitization of nAChRs during short or long exposure to nicotine

3.1 Pharmacological properties of desensitization of nAChRs on SH-5YSY cells

Figure 22 shows the basic characteristics of rapid desensitization following pulse application of nicotine. By varying the time between pulses, it was possible to measure recovery from desensitization. As the shortest interval which could be used for pulse delivery without overlapping current responses was 1 s, at this time point the second current had already recovered to 61 ± 7 % of the first one (n=7), indicating prompt reattainment of nAChR-mediated responses despite their virtually complete current decay (Figure 22).

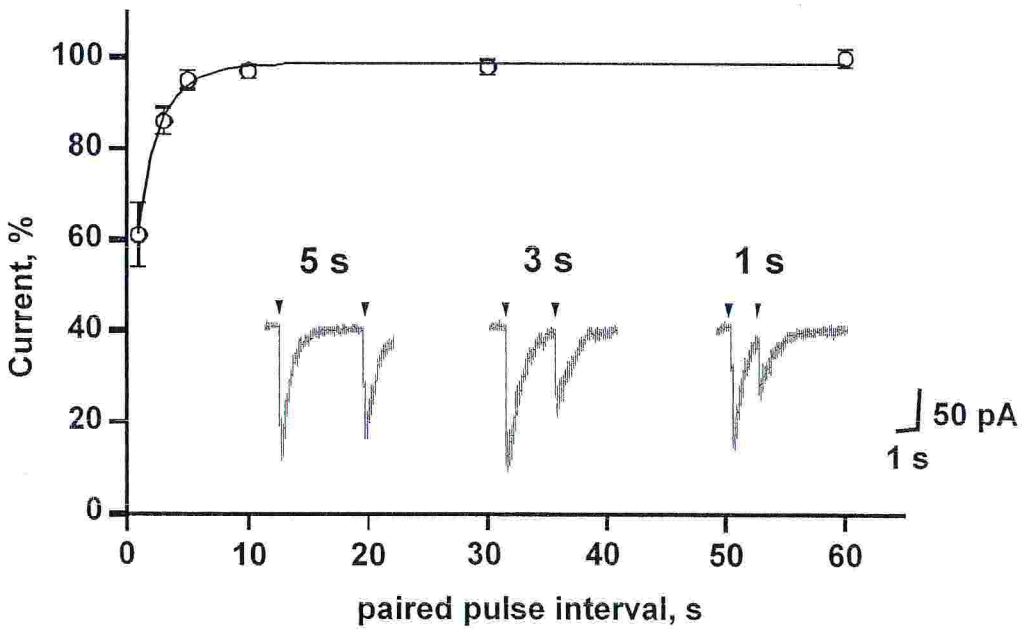

Fig. 22. Quantification of recovery from desensitization investigated with paired-pulse protocol on untreated cells. Ordinate provides amplitude of second response in each pair as per cent of the first one (n=8 cells). Insets show typical pairs of currents induced by 50 ms pulses of nicotine (arrowheads) applied at different time intervals.

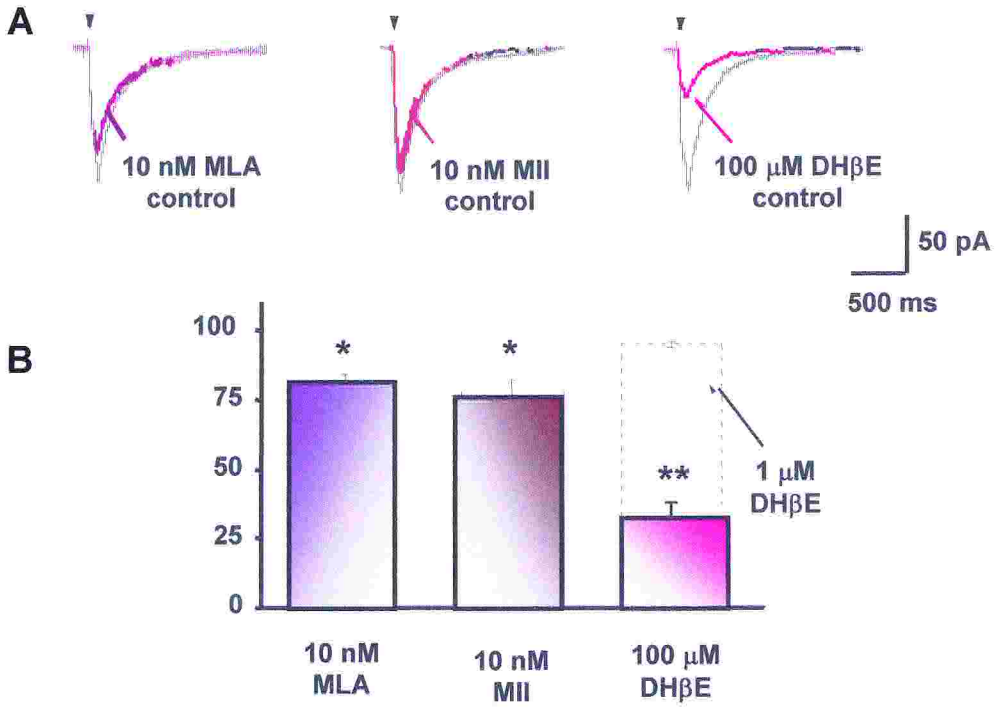

Fig. 23. (A) Examples of averaged currents evoked by 50 ms pulses of nicotine before or during antagonist application. To aid comparison records are superimposed. (B) Histograms with average data on antagonist sensitivity indicating that nAChRs of control cells have limited sensitivity to MLA (selective α7 antagonist; n=11), MII (selective α3/α6 antagonist; n=8) and DHβE (that at 1 μM concentration is preferentially selective for β2-containing receptors; n=5). A large concentration (100 mM) of DHβE, which exerts broad spectrum nAChR antagonism, strongly decreases nicotine responses (n=5). **P<0.01; *P<0.05.

Antagonist sensitivity of nAChRs was tested with methyllycaconitine (MLA) or α-conotoxin MII (selective antagonists of α7- or α3-containing subunits, respectively; Dwoskin & Crooks, 2001; Nicke *et al.*, 2004), which are expressed by SH-SY5Y cells (Peng *et al.*, 1997; Wang *et al.*, 1998; Warpman *et al.*, 1998). Either drug applied at 10 nM concentration for 5-10 min was moderately effective in blocking nicotine induced currents (Fig. 23 A, see also Fig. 20 and 21). As shown in Fig. 23, a high concentration (100 μM) of DHβE (at this concentration lacking subunit-specific effects) largely blocked nicotine induced currents (n=5). At 1 μM concentration DHβE (a specific antagonist of α4β2 and α3β2 receptors; Dwoskin

and Crooks, 2001) did not produce any significant change in the current amplitude (95 ± 1%, n=5, Fig. 23 B, dashed line column).

Overall, these data suggests, under our experimental conditions, potent expression of nAChRs which underwent rapid, strong desensitization with quick recovery. nAChRs were probably heterogeneous in view of the limited block of subtype-selective antagonists.

3.2 Desensitization produced by continuous application of nicotine

In these experiments 10 μM nicotine was continuously superfused (usually for 10 min) to produce desensitization of nAChRs, while responsiveness of these receptors was assessed with 50 ms puffer applications of 1 mM nicotine (see Figure 24 A, left).

As shown in the example in Figure 24 A (right), nicotine (10 μM; filled bar) produced first a peak current (on average -163 pA), which then decayed with a noisy baseline (presumably representing multiple channel openings; Machonochie & Knight, 1992). In 7 out of 10 cells the current evoked by bath-applied nicotine decayed completely, while in the remaining three cells a very small residual current could be recorded up to the end of the 10 min. application (-4.0±0.6 pA).

Figure 24 B presents averaged data on the development of desensitization to puffer pulses of nicotine in the continuous presence of bath-applied nicotine.

The depression of nicotine-mediated responses followed a biexponential time course (first-time constant τ_1=6 ± 2 s; second-time constant τ_2=96 ± 30 s; n=10), which left a puffer pulse current amounting to 6 ± 2 % of control at 490 s from the start of bath application of nicotine. The biphasic nature of desensitization accords with previous reports of fast and slow nAChR desensitization (Khiroug et al., 1997; Maconochie & Knight, 1992). Recovery of puffer currents from nicotine (10 μM)-induced desensitization had gradual onset (see sample trace in Figure 25 A) and biphasic time course (Figure 25 B; τ_1=14 ± 6 s and τ_2=277 ± 120 s; n=7–10 cells) to attain a mean amplitude value of -170 ± 18 pA.

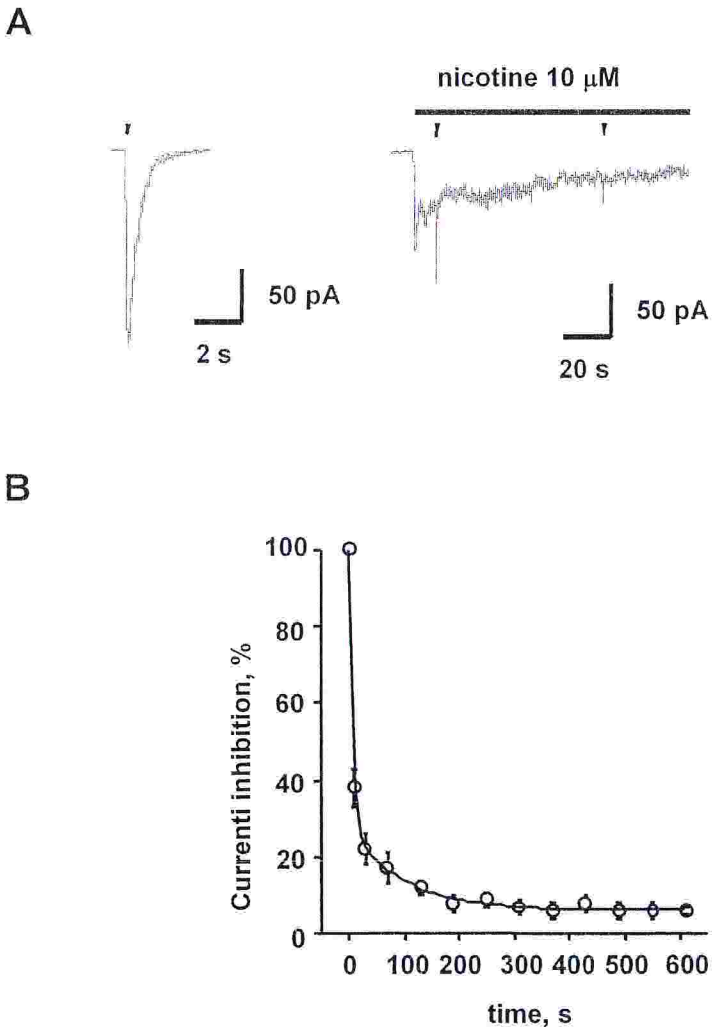

Fig. 24. (A) Left, current recorded in response to nicotine pulse (50 ms; arrowhead) in control conditions; right, sustained current evoked by superfusion (filled horizontal bar) of 10 μM nicotine during which test pulses (arrowheads) of 1 mM nicotine (50 ms) are applied. The first test pulse is applied after 10 s from the start of 10 μM nicotine and then repeated at 1min interval. The current evoked by continuous application of nicotine gradually fades back to baseline (not shown). (B) Plot of inhibition of responses to test pulses of nicotine applied every 60 s starting 10 s from the beginning of sustained 10 μM nicotine application (n=12).

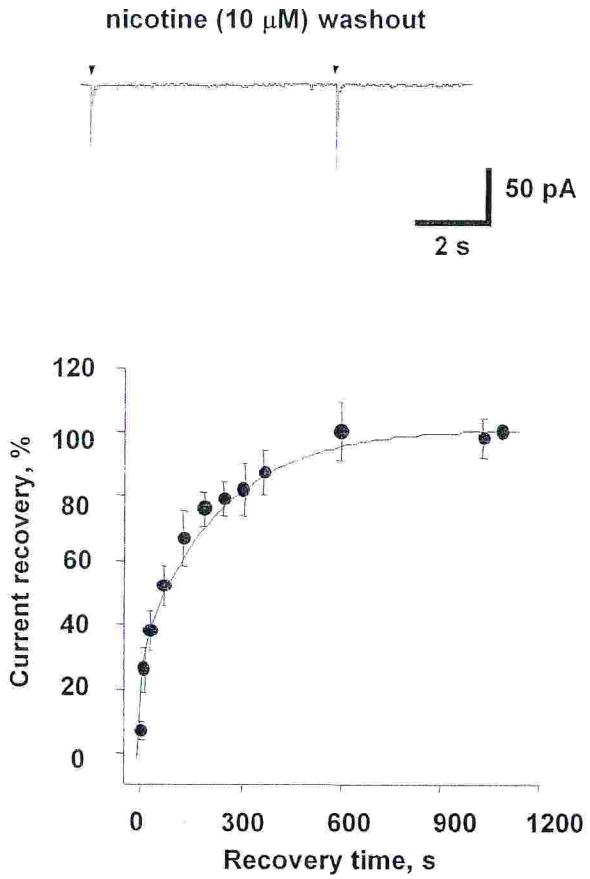

Fig. 25. (A) Rapid return of responses to pulses of nicotine at the end of 10 mM nicotine bath application. The first pulse of nicotine is applied 10 s after washout of bath-applied nicotine. (Different cell from Fig. 24 A). (B) Time course of return of nicotine pulse (50 ms)-induced currents during washout after 10 min nicotine application (10 μM); n=7–10.

3.3 Desensitization produced by chronic application of nicotine

The next series of experiments was designed for two purposes. First, to find out if, during rather prolonged exposure to nicotine, nAChRs preserved a degree of response. This issue was explored by measuring if there was any significant fast inward current elicited by pulses (50 ms) of nicotine maximally effective in control solution. Second, it seemed interesting to study whether, after prolonged nicotine-dependent desensitization, nAChRs could recover their full sensitivity.

SH-SY5Y cells were chronically treated with nicotine (10 μM) for either 8 or 48 h, then patch-clamped and tested without washing out the nicotine-containing culture medium. The majority of patched cells (90 %) surprisingly responded to nicotine pulses with inward currents (see one example in Fig. 26 A top) of -25 ± 3 pA amplitude (n=26) for 8 h nicotine pretreatment, or -22 ± 5 pA amplitude (n=24) for 48 h nicotine pretreatment. Thus, responses to test pulses of nicotine had the same amplitude regardless of the length of sustained exposure to nicotine (10 min, 8 h, or 48 h; see solid columns in Fig. 26 B). When cells were exposed for 48 h to 1 mM nicotine (i.e. protocol used by Ke et al., 1998; Peng et al., 1997; Wang et al., 1998), and then washed for 30 min with standard solution, the amplitude of their responses to nicotine pulses was -201 ± 20 pA (n=32), a value similar to the peak amplitude (-225 ± 20 pA, n=20) recorded with a similar protocol except the use of 10 μM nicotine exposure.

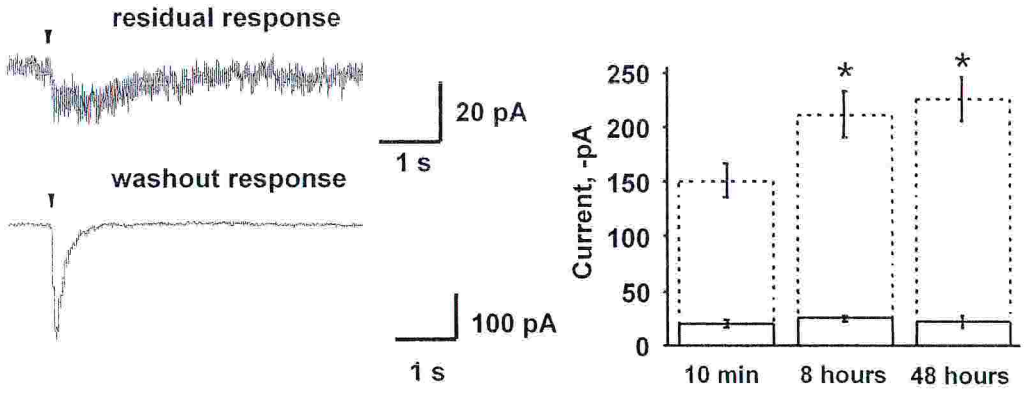

Fig. 26. (A) Examples of currents recorded in response to test pulse (50 ms) of 1 mM nicotine in culture solution containing 10 μM nicotine for 48 h (top) or after 30 min washout (bottom; same cell). (B) Solid lines in the bar chart show average amplitude of currents recorded in the continuous presence of nicotine (10 μM) applied for 10 min, 8 or 48 h in response to test pulse (50 ms) of nicotine (1 mM). Data are from 15 to 64 cells. Dashed columns represent average data of current amplitude after washout of nicotine applied for times indicated below (data are from 26 to 78 cells). Asterisks indicate statistically significant increase in the amplitude of responses from pretreated cells ($P<0.05$).

Standard solution was then used to wash out the nicotine-containing culture medium for at least 30 min, and the amplitude of the responses to repeated test puffer pulses of nicotine (50 ms, 1 mM) was measured. Cells regained quickly their ability to generate robust inward currents as exemplified in Fig. 26 A (bottom). On average, after washing for 30 min nicotine applied for 8 or 48 h, the amplitude of responses to nicotine pulses was significantly larger ($P<0.05$) than the one of the nicotine currents of untreated cells (Fig. 26 B, dashed columns). Continuous presence of nicotine, therefore, produced up-regulation of nicotinic receptors of SH-SY5Y cells in accordance with previous biochemical studies of the same cells (Peng et al., 1997; Ridley et al., 2001).

Note, however, that the τ value of current decay during nicotine pulses was 156 ± 16 ms (n=12), that is virtually the same as in untreated controls.

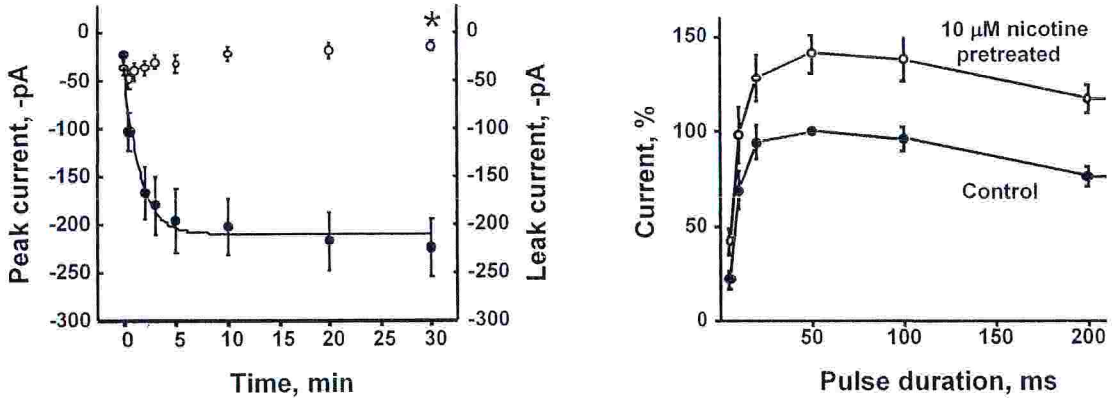

Fig. 27. (A) Time course of simultaneously recorded recovery in amplitude of test nicotine currents (50 ms; filled circles) and baseline leak current (open circles) during 30 min of washout after 48 h nicotine (10 μM) pretreatment; n=6. (B) Plots of current amplitude (as per cent of response to 50 ms of time-matched untreated cells) evoked by pulses of nicotine of varying duration. Open circles refer to cells treated for 8 h with 10 μM nicotine (n=6), while filled circles refer to time-matched untreated controls (n=8).

Fig. 27 A shows that the return of strong nAChR sensitivity following chronic desensitization was relatively fast, so that 90 s after the start of nicotine washout from the culture, the current amplitude (filled symbols) was already 50% of the value reached at steady state (30 min washout period). The increased amplitude of

nicotine currents was not associated with a change in the leak current (open symbols in Fig. 27 B).

Figure 27 B plots the nicotine current amplitude as a function of increasing pulse duration after 8 h treatment with 10 μM nicotine or time-matched controls. It is clear that the two plots differed in the maximum amplitude, indicating functional up-regulation of nAChRs after chronic application of nicotine.

We then tested if nAChRs up-regulated by chronic nicotine treatment changed their functional properties. The reversal potential (6.7 ± 1.8 mV; n=5) of 8 h treated cells was not significantly different from that of control cells, suggesting that the larger responses were not due to increased driving force for the ionic currents.

Once the maximal enhancement of current amplitude had reached steady-state level, the τ value for current decay with 2 s pulses of nicotine was 139 ± 12 ms (n=9), a result similar to the one of control-untreated cells. Recovery from desensitization induced by paired pulses of test nicotine applications was also very similar to the recovery of untreated cells (63 ± 7% at 1 s interval; n=6; $P<0.05$ versus control). When we tested the sensitivity of up-regulated receptors to antagonists, the blocking action by MLA, MII or DHβE (Figure 28 A and B) was not significantly different from the one observed on control cells (see Figure 23). Thus, long-term pretreatment with 10 mM nicotine did not apparently lead to preferential up-regulation of subunits sensitive to MLA, MII or DHβE.

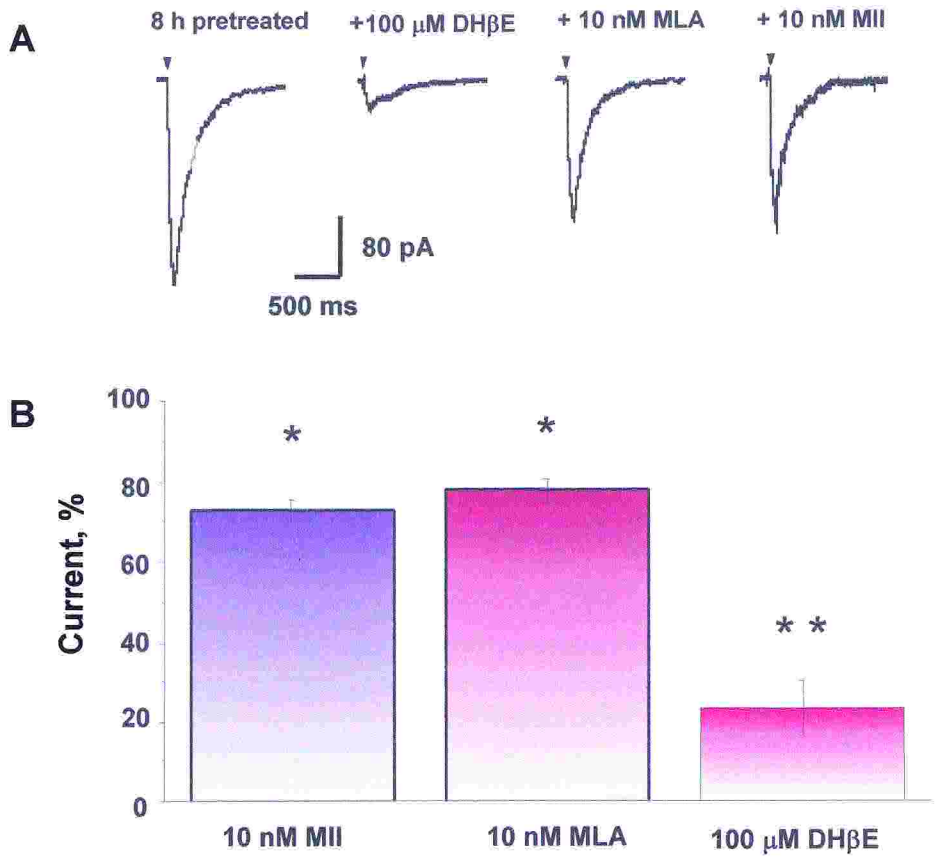

Fig. 28. Antagonist sensitivity of up-regulated nAChRs. (A) After washout of 8 h nicotine (10 μM) application, typical currents are recorded in response to test pulses of nicotine (50 ms) in control solution or in the presence of 10 nM MLA, 10 nM MII, or 100 μM DHβE. (B) Histograms to quantify the extent of antagonist block of nicotine test pulses after 8 h nicotine (10 μM) treatment; **P<0.01; *P<0.05; n=7–20.

DISCUSSION

General relevance of nAChR studies

Neuronal nicotinic acetylcholine receptors (nAChRs) exist in a variety of subtypes depending on their different subunit composition (Dani and Bertrand, 2007; Gotti et al., 2006). Different subtypes have distinct anatomical distribution in the central and peripheral nervous systems (Gotti and Clementi, 2004). By responding to the endogenous neurotransmitter acetylcholine, nAChRs contribute to a wide range of brain activities and influence a number of physiological functions including cognition, learning and memory, arousal, cerebral blood flow and metabolism and a growing list of pathological conditions. Perturbation of cholinergic nicotinic neurotransmission can lead to various diseases involving nAChR dysfunction during development, adulthood and ageing. Some of pathologies resulting from dysfunction of neuronal nicotinic receptors are, for example, Alzheimer's disease, Parkinson's disease, epilepsy, and other mental diseases such as schizophrenia, Tourrette's syndrome, anxiety and depression (Gotti et al., 2006). Xiu et al. (2005) showed that nanomolar concentration of the beta-amyloid peptide (1-42) increase levels of α7, α4 and β2 mRNAs and proteins; it has been hypothesized that this effect might be related to ongoing defensive or compensative mechanisms.

Peripheral nAChRs are involved in the regulation of blood pressure and hypertension (Boccafusco, 1996), in megacystis-microcolon-intestinal hypoperistalsis syndrome (MMIHS) (Wang et al. 2002) and in a syndrome consisting of idiopathic or pareneoplastic autonomic neuropathy, postural tachycardia, as well as in idiopathic gastrointestinal dysmobility (Balestra et al., 2000; Vernino et al., 1998, 2000).

In recent years, it has been discovered that nAChRs are present in a number of non-neuronal cells (Gotti and Clementi, 2004): in muscle, lymphoid tissue, macrophages, their function is still unknown; in human epidermal keratinocytes, nAChRs mediate several skin function, particularly keratinocyte proliferation, apoptosis, differentiation, adhesion and motility; in lung cells, they influence cell proliferation and play a role in stimulating the growth of small-cell lung carcinoma. nAChRs are also expressed in the vascular system, in endothelial cells and vascular smooth muscle: the function of

arterial nAChRs is not yet known, but it has been postulated that they play a role in controlling angiogenesis and smooth cell proliferation, suggesting a therapeutic application in artherosclerosis, tumor growth, and revascularization after ischemic insults. nAChRs are expressed in brain endothelial cells and control the permeability of the blood-brain barrier.

nAChRs are also the target of natural ligands and toxins including nicotine, the most widespread drug of abuse, and are involved in the tolerance/dependence to nicotine in chronic tobacco users (for reviews see Gotti et al., 2006; Gotti and Clementi, 2004; Paterson and Nordberg, 2000).

Therefore, it is important to know the structure, function and pharmacology of these receptors in order to identify and provide chemical substance for dissection of different subtypes and for treatment of those diseases resulting from nAChR dysfunction.

Chromaffin cells, that are neuro-endocrine elements secreting catecholamines, are very useful models to understand the relative contribution of various nAChR subunits to cell function. Our study of rat chromaffin cells was, therefore, addressed to understand what subunits are normally expressed and how they generate functional native receptors in these cells. We subsequently examined the issue of nAChR regulation after chronic exposure to agonist/antagonists.

Characterization of nAChRs of the rat chromaffin cells

The principal finding of the experiments carried out on rat chromaffin cells is the identification of potential subunits which in various combinations can make up native nAChRs and the demonstration of their relative contribution to the genesis of membrane currents induced by nicotine. To date, our understanding of this issue was incomplete despite the fact that chromaffin cells, physiologically excited by acetylcholine released by splanchnic nerve endings, have been widely used as models for pharmacological and physiological studies aiming at characterizing nAChRs (Gandia et al., 1997; Giniatullin et al., 1999; Herrero et al., 2002; Khiroug et al., 1997, 1998; Sala et al., 2002).

Rat chromaffin cells possess the potential to express a large number of nAChR subunits

RT-PCR analysis indicated the putative presence of several nAChR subunits (α2, α3, α4, α5, α7, β2, β4). Transcripts for α6 and β3 subunits were never detected. Single cell RT-PCR experiments confirmed expression of the same range of nAChR subunits and also suggested significant co-expression of α3 with α5 and β4, plus co-expression of α5 with β4. Lack of significant co-expression for other subunit combinations should be viewed with caution, owing to the relatively small sample of cells examined. Of course, the mere presence of mRNA signals indicated ability to synthesize certain proteins rather than their actual expression by cells. For this purpose, immunofluorescence staining and western blot of nAChR subunits were performed. Western blots demonstrated the presence of α3, α4, α5, β2 and β4 subunits, whose molecular mass was in accordance with previously reported values (Arroyo-Jimenez et al., 1999; Balestra et al., 2000; Guan et al., 2000; Jones et al., 2001; Martin-Ruiz et al., 1999; Zhou et al., 2001). However, the β2 antibody occasionally gave a higher molecular weight band of uncertain origin, which might represent a protein complex (like, for example, a β2 hetero- or homo-dimer) resistant to the standard denaturing conditions used for electrophoresis.

Immunofluorescence staining indicated that the mRNAs detected with RT-PCR were translated into cellular proteins, except the α7 subunit. Few cells expressed α2 or α4 containing receptors, while none expressed β3 subunits. Lack of availability of an anti-α6 antibody prevented us from testing this subunit expression. As there was no mRNA signal for this subunit, the chances of its presence must, however, remain very small. While virtually all chromaffin cells expressed α3 subunits, most of them expressed α5 in accordance with the view that this subunit confers special functional properties to autonomic neuron nAChRs (Ramirez-Latorre et al 1996). While β3 subunits could not be detected, most cells expressed β2 and β4 subunits. Double immunofluorescence suggested co-existence of α3α5 and, in a few cases, α4β2 subunits. No cellular segregation of subunit expression was found. Note, however, that the present immunofluorescence tests on permeabilized cells could not indicate whether nAChR

subunits were membrane bound or intracellularly located. Nevertheless, all cells were found immunopositive for at least one α and one β subunits, indicating that all cells express nAChRs, a result that is in line with the electrophysiological data.

A report by Mousavi et al. (2001) suggests that the major population of nAChRs on young rat chromaffin cells comprises α3 and α7 subunits. While our data concur for α3 expression, they differ in terms of α7 detection. It is well established that, although bovine chromaffin cells express α7 homomeric receptors (Garcia-Guzman et al., 1995), on rat chromaffin cells α7 receptors are not demonstrable with receptor binding (Di Angelantonio et al., 2000), and that their selective blocker α-bungarotoxin fails to inhibit responses mediated by nAChRs (Nooney and Feltz, 1995). Furthermore, the present study could not demonstrate staining with the α7 antibody, even if the same antibody can readily stain cells in rat hippocampal slices (Wevers et al., 2000; Zarei et al., 1999). Lack of functional α7 receptors was also reinforced by the present observation that low concentrations of the selective α7 blocker MLA (Séguéla et al., 1993) did not inhibit nicotine mediated currents. Nevertheless, as rat chromaffin cells have the potential to synthesize α7 receptors (see RT-PCR data), it is possible that under certain conditions (perhaps persistent exposure to certain cholinergic agents and/or modulation by local factors), α7 receptors become functionally expressed within the context of adaptive neuronal plasticity.

What native nAChR subunits are assembled to give functional receptors?

Currents induced by nicotine in rat chromaffin cells are potently and reversibly inhibited by mecamilamyne, a selective, broad spectrum nicotinic antagonist (Giniatullin et al., 2000), indicating that these current are entirely due to the activation of nAChRs.

Because the vast majority of cells were immuno-positive for α3 subunits, it seems likely that this subunit was the commonest determinant for nAChR structure. Functional heteromeric receptors also require β subunits since agonist binding sites are thought to be at the interface between α and β subunits (Corringer et al., 1995; Itier and Bertrand, 2001; Lena and Changeux, 1999). Hence, on rat chromaffin cells, it

seems likely that β2 and β4 subunits (readily detected by immunofluorescence in the present study) were co-expressed with α3 subunits (yielding α3β2 and α3β4 assemblies). This possibility could not be tested with double immunostaining in view of the nonselective binding of primary and secondary antibodies from the same animal species. An alternative approach to test this hypothesis was to use subtype-selective blockers like the α-conotoxins MII or AuIB (specific against α3β2 or α3β4, respectively; Cartier et al., 1996; Harvey et al., 1997; Luo et al., 1998). The AuIB toxin is comparatively less potent on α3β4 receptors than the MII toxin on α3β2 receptors (corresponding IC_{50} values are 750 and 8 nM; Cartier et al., 1996; Harvey et al., 1997; Luo et al., 1998). Although the very limited supply of AuIB prevented us from performing extensive tests, the IC_{50} value of AuIB antagonism of toxin-sensitive nicotine current was relatively close to the one reported for recombinant α3β4 receptors (Luo et al 1998), indicating a probable contribution of this subtype to nicotinic current. Low concentrations of the potent blocker MII also antagonized a fraction of the nicotine response, indicating functional α3β2 receptors. The calculated IC_{50} value of MII was 35 nM, which is comparatively similar to the one reported for α3β2 receptors (Cartier et al 1996).

In our experiments, antagonism summation was found when each conotoxin was applied at 10 nM concentration, suggesting distinct contributions by α3β4 and α3β2 receptors. Nevertheless, co-application of AuIB and MII (both at 100 nM concentrations) did not enhance the block obtained with MII alone. A similar occlusion of the two toxin effects has been reported by Fu et al. (1999) and Bibevski et al. (2000) who explained lack of additivity with the presence of heterogeneous receptors comprising co-assembled α3β2β4 subunits targeted by either toxin. Antagonism occlusion might have been also due to non-selective block by high concentrations of MII towards other receptor types (e.g. α4β2, α2β2 as well as the AuIB-sensitive α3β4; see Cartier et al., 1996). It is clear that the MII toxin is not an absolutely specific antagonist of α3β2 receptors because it can also inhibit α6 containing receptors (Champtiaux et al., 2002; Zoli et al., 2002), although their contribution to the present responses can be excluded because no α6 mRNA transcript was detected.

Other subunits that might contribute to nAChRs in rat chromaffin cells are α5, α2 and α4 subunits, whose mRNA and protein signals were detected in the present study. The α5 subunit, which cannot form functional receptors if it is not coexpressed together with another α subunit as well as β subunits (Groot-Kormelink et al., 2001; Ramirez-Latorre et al., 1996), confers certain functional properties. For example, the presence of α5 in α3β4 or α3β2 receptors expressed in *Xenopus* oocytes largely enhances receptor desensitization (Gerzanich et al., 1998; Groot-Kormelink et al., 2001) and increases Ca^{2+} permeability (Gerzanich et al., 1998). Because chromaffin cell nAChRs mediate large rises in Ca^{2+} influx and strong receptor desensitization (Khiroug et al., 1998), it seems likely that such receptors could contain α5 subunits, even though selective pharmacological tools to demonstrate their functional presence are not yet available.

Despite the fact that α4β2 receptors are the most abundant subtype in the mammalian brain (Le Novère et al., 1996) and have been also found in rat adrenal tissue (Mousavi et al., 2001), their contribution to nicotine induced currents on rat chromaffin cells was small as demonstrated by the limited block of nicotine current by DHβE at concentrations selective for α4β2 receptors (Chavez-Noriega et al., 1997). This finding is in agreement with the demonstrably low expression of α4 subunits by rat chromaffin cells. A very large dose (200 μM) of DHβE completely suppresses nicotine responses (Giniatullin et al., 2000), confirming its action as broad spectrum nicotinic antagonist reported nearly 50 years ago for mammalian central neurons (Eccles et al., 1956). It is, therefore, not surprising that DHβE further depressed the residual nicotine current when AuIB and MII were simultaneously applied. The observed cumulative antagonism was less than the expected one based on the sum of the effects of the toxins plus DHβE, suggesting partial occlusion by the toxins of receptor subclasses sensitive to DHβE.

Data interpretation is also complicated by the fact that different nAChRs may have differential affinity for nicotine (Rush et al., 2002). Hence, blocking certain receptor populations could make others more accessible to nicotine and generate non-linearity between antagonist concentration and response amplitude.

In summary, the present data suggest, as a minimum, the following potential combination of receptor subunits: α3β4, α3β2, α3α5β4, and much less commonly α4β2.

Acute and chronic effects of a novel nicotinic drug, 1,2-bisN-cytisinylethane (CC4)

Long-term exposure of nAChRs to nicotine and other nicotinic agonists leads to an increase in receptor number (reviewed by Hogg et al., 2003), whereas chronic exposure to nicotinic antagonists leads to contradictory results (see Table 3). This issue is of potential importance, for example, in understanding the long-term 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 to the effects of chronic nicotinic treatment on α3-containing receptors that are natively expressed in nervous tissue (Gotti & Clementi, 2004).

Because of the importance to find out subunit-selective compounds acting on different nAChRs subtypes, we investigated this aspect using a series of new nicotinic drugs that have recently been synthesized (Carbonnelle et al., 2003) starting from the parent compound cytisine, which has been reported to have partial agonist activity on β2-containing receptors (Chavez-Noriega et al., 1997; Covernton et al., 1994; Houlihan et al., 2001; Papke & Heinemann, 1994; Slater et al., 2003), and both partial (Chavez-Noriega et al., 1997; Houlihan et al., 2001; Slater et al., 2003; Wenger et al., 1997) and full agonist activity on β4-containing receptors (Covernton et al., 1994; Fischer et al., 2005; Wong et al., 1995). 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-bisN-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 (Boido et al., 2003; Canu Boido & Sparatore, 1999) such as hexamethonium, decamethonium, d-tubocurarine,

alkane- and azaalkane-diguanidium, 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). CC4, in fact, showed a rapid, strong antagonist effect on nAChRs.

We used SH-SY5Y neuroblastoma cells as a model because they natively express typical peripheral nAChR subtypes, resemble human foetal sympathetic neurons and may thus have conserved certain endogenous regulatory mechanisms controlling receptor expression perhaps lacking in transfected heterologous cells. Thereafter, we investigated the changes in nicotinic currents after chronic treatment with CC4 showing nicotinic current up-regulation and a differential contribution of different subtypes before and after chronic treatment.

Identity and pharmacological profile of nAChRs on SH-SY5Y cells

Using subunit-specific antibodies, extensively tested in transfected human cell lines for their immunoprecipitation specificity and efficiency, Gotti's group could identify various subtypes of nAChRs. In agreement with previous data (Balestra et al., 2000; Peng et al., 1997; Wang et al., 1998), they 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) α3β4 (46%), α3β2β4 (30%) and α3β2 (24%). Hence, because of their subunit heterogeneity, SH-SY5Y cells offer the advantage of expressing a broad range of native nAChRs constitutively found in the peripheral nervous system of mammals (Peng *et al.*, 1997), although it is uncertain if all of these subunit assemblies are functional.

Nicotine has its strongest potency on α4β2, followed by α4β4, α3β2, α2β4 and α3β4 nAChRs expressed in HEK293 cells (Stauderman *et al.*, 1998). When human nAChRs are expressed in oocytes, nicotine remains most potent on α4β2 and α4β4 receptors, followed by α2β4, α3β4, α3β2 and α7 receptors (Chavez-Noriega *et al.*, 2000). In the present study, the nicotine EC_{50} value was 43 μM, in keeping with the contribution by a heterogeneous receptor population to the observed current, and near the EC_{50} values for α3 or α7 containing receptors in such cells (Peng *et al.*, 1997). Nicotinic currents

reversed near 0 mV, thus confirming that their open channels had the permeability expected for nAChRs (Jones *et al.*, 1999).

Low concentrations of the selective α7 blocker MLA (Gotti et al., 2005) partially blocked nicotinic currents, indicating that a fraction of nAChRs probably included α7 receptors, while a small fraction comprised α3-contaning receptors sensitive to the α-conotoxin MII (the toxin-sensitive α6 subunit is not expressed by SH-SY5Y cells; Ke *et al.*, 1998; Peng *et al.*, 1997; Warpman *et al.*, 1998). Because a low concentration of DHβE could not significantly affect nicotine induced currents, it seems likely that α3β2 (or α4β2) receptors highly sensitive to DHβE (Dwoskin & Crooks, 2001) were, at most, just a very small minority of the global, functional nAChR population.

Plastic changes in receptor activity due to chronic application of nicotinic agents

In analogy with previous studies that had to rely on high concentrations of nicotinic agents largely in excess of the receptor saturating doses (Molinari et al.,1998; Peng et al., 1997; Wang et al., 1998), the CC4 concentration necessary to up-regulate receptors was approximately 1000 times higher than the one necessary to block nicotine-evoked currents. Perhaps up-regulation implied drug binding to yet unidentified accessory receptor sites with low affinity. However, we showed that CC4 in the medium culture, after 48 hours, partially lost its inhibitory effect: we have hypothesized that, during protracted incubation, CC4 was partially inactivated/degraded and this loss of active concentration could be one reason for the need to apply a very high dose in order to observe receptor up-regulation.

Although the precise cause of the discrepancy between blocking and up-regulating concentrations remains unclear, the action of CC4 was not an indiscriminate up-regulation of membrane receptors, because CC4 differentially enhanced native ^{3}H-Epi and ^{125}I-aBgtx sensitive receptors, without affecting the expression of muscarinic receptors (see Appendix) 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 nAChRs, 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 concluded that increased surface nAChRs were functional at single cell level.

Heteromeric nAChR subtypes were up-regulated by chronic application of CC4

Gotti's group showed that chronic CC4 treatment preferentially increased the number of receptors containing the β2 subunits, especially the α3β2 subtype that has a higher affinity for epibatidine. Increased expression of subtypes containing the β2 subunit was confirmed by our 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 (Ki) for transfected human α3β2 and α3β4 receptors of 32 and 1500 nM, respectively (Gotti et al., unpublished results) and its IC_{50} on the oocyte-expressed rat α3β2 subtype is 0.5–2.2 nM, whereas it is 200 times less potent on the α3β4 subtype (Cartier et al., 1996). Although αCntxMII has high affinity and selectivity towards α6β2-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 α3β2 subunits contributing to the overall nicotine evoked currents.

Effects of chronic CC4 treatment on nicotine evoked current

The present report indicates that, unlike standard nAChR blockers, high concentrations of the novel nicotinic antagonist CC4 could differentially up-regulate functional nAChRs: chronic treatment with 1 mM CC4 causes up-regulation of nAChR in SH-SY5Y cells. In particular, nicotine-evoked currents increased by about 150% together with a larger contribution of the α3β2 subtype with respect to control cells. The observation that other antagonists did not change at all the expression level of the nAChRs (Tab. 3) imply that nAChR up-regulation was not an unselective homeostatic process triggered by sustained receptor block with any nicotinic inhibitor.

Desensitization of neuronal nicotinic receptors of human neuroblastoma SH-SY5Y cells during short or long exposure to nicotine

In order to study the role of nAChRs and their contribution to the complexity of the effects of nicotine and nicotinic drugs, it is first important to understand the subunit combinations, distributions and the diversity of physiological and pharmacological properties. Nevertheless, as receptors undergo plastic changes in response to environmental challenges, it is also useful to study how much nAChR function can persist during sustained agonist exposure. As indicated below, a plethora of studies on this subject has not provided a convincing answer. Using a variety of models including expression systems for recombinant nAChRs, most molecular biology and biochemical investigations have shown that up-regulation of neuronal nAChRs occurs after hours or days of exposure to nicotine (Buisson & Bertrand, 2001; Fenster *et al.*, 1999; Kawai & Berg, 2001; Ke *et al.*, 1998; Peng *et al.*, 1997; Ridley *et al.*, 2001; Wang *et al.*, 1998; Warpman *et al.*, 1998). To reach such a conclusion protocols normally involved long incubation in nicotine containing solution and subsequent washout with control buffer. Curiously though, very few studies have directly addressed the question of what happens to nAChRs in the continuous chronic presence of nicotine, whether they can somehow preserve a certain degree of responsiveness, and how rapidly receptors can regain their full sensitivity after agonist washout. We addressed this issue investigating nAChRs in SH-SY5Y cells.

Fast desensitization properties of nAChRs on SH-SY5Y cells

Desensitization of nicotine-evoked currents developed rapidly with a single time constant, indicating that, despite subunit heterogeneity, receptors underwent desensitization in a concerted fashion with full current dissipation within a few hundred ms. Recovery from desensitization was also rapid with full return of cholinergic responsiveness in about 10 s. The relatively fast onset of desensitization coupled to rapid recovery on washout might have outlined a preferential role of α3 subunit containing receptors in determining this property (Quick & Lester 2002), although the results with α-conotoxin MII did not support this notion. Thus, the sensitivity to agonists and antagonists plus the desensitization properties suggest that

the action of nicotine on SH-SY5Y cells involved activation of a heterogeneous class of nAChRs.

A complementary protocol to study desensitization (Katz & Thesleff, 1957) relied on continuously-applied low (i.e. several times < the EC_{50} value) concentration (10 μM) of nicotine that generated an initial current with subsequent full decay to baseline. However, testing receptor sensitivity with a large dose of nicotine demonstrated that most cells exposed to nicotine for 10 min retained a small response and completely recovered after washout.

nAChR sensitivity during sustained exposure to nicotine

Because some theories state that strong desensitization is a necessary requirement for agonist-mediated up-regulation (see Paterson & Nordberg 2000; Buisson & Bertrand 2002), we were interested to find out if nAChRs were fully desensitized during continuous agonist exposure. Functional data concerning how nAChRs of SH-SY5Y cells act during continuous agonist exposure are, however, rare. Ke *et al.* (1998) report that, on such cells, one hour incubation with 10 μM nicotine halves nicotinic responses measured as radioactive Rb^+ fluxes, even though single cell data with higher temporal resolution are lacking. As far as other nAChRs are concerned, there are limited electrophysiological data for α3β2 and α4β2 receptors expressed in oocytes (Hsu *et al.*, 1996), and α4β2 receptors expressed in HEK293 cells (Buisson & Bertrand 2001), all showing small residual responses.

One interesting observation of the present study was that a small, yet clearly measurable response to nicotine remained in SH-SY5Y cells treated for 8 or 48 h with nicotine. To detect this effect, we performed recording from cells in culture medium and we did find small currents (with long decay probably because cells were not be continuously superfused). Although recording such small currents was technically difficult and unsuitable to fully characterize their pharmacology, their amplitude was consistently similar to the one of residual currents to nicotine pulses after 10 min nicotine application under continuous superfusion. This result suggests that either a small, yet finite number of receptors could be activated because less prone to desensitization (Olale *et al.*, 1997; Patterson & Norberg, 2000), or a significant

fraction of desensitized receptors was dwelling in an active state as predicted by a cyclic model of receptor operation (Katz & Thesleff 1957; Paradiso & Steinbach, 2003; Giniatullin et al., 2005).

Washing out the chronically-applied nicotine solution was not followed by an outward shift in leak current. This observation is not incompatible with the possibility that some nAChRs can be persistently activated by ambient ACh (Lester, 2004): if some nAChRs were tonically active, their number was just too small to contribute to the cell conductance at rest.

Up-regulation of nAChRs following chronic exposure to nicotine

While a number of studies show up-regulation on nAChRs due to chronic nicotine application, the identity and the extent of the subunits involved in this phenomenon are controversial.

Radioactive ligand binding studies performed on a number of native or recombinant nAChR subtypes show very large up-regulation of many AChR subtypes including $\alpha4\beta2$ and $\alpha3\beta4$ (Fenster *et al.,* 1999; Gentry *et al.*, 2003), $\alpha3\beta2$ (Wang *et al.*, 1998), and $\alpha7$ (Molinari *et al.* 1998; Kawai & Berg, 2001) receptors in various cells, comprising SH-SY5Y ones (Warpman *et al.*, 1998; Ridley *et al.*, 2001). There are considerable discrepancies concerning the subtype of nAChR mostly up-regulated, because up-regulation largely depends on the expression system (Paterson & Nordberg 2000) and the amount of expressed receptors before applying nicotine (Nahsmi *et al.*, 2003). Furthermore, parallel studies show that nAChRs in expression systems are more strongly up-regulated than the same native receptors of SH-SY5Y cells (Wang *et al.*, 1998). Further complexity is added by the fact that, at least on SH-SY5Y cells, most of the up-regulated $\alpha3$ subunits are intracellular rather than membrane bound (Peng *et al.,* 1997), and that this condition might apply to other subunits and other cells, too. Since there is no change in mRNA of SH-SY5Y cells for any receptor subunit after chronic treatment with nicotine (Peng *et al.*, 1997; Ke *et al.*, 1998), it is likely that post-transcriptional mechanisms govern up-regulation of nAChRs.

Functional studies give results not readily reconciled with binding data. In fact, population studies of intracellular Ca^{2+} levels in SH-SY5Y cells showed that chronic

nicotine treatment actually decreases responses to nicotine itself (Ridley *et al.*, 2002). Likewise, radioactive Rb^+ fluxes from analogous cell pools are largely and persistently depressed (Ke *et al.*, 1998). In either case measured responses are, however, a function of changes in membrane potential. Electrophysiological investigations with recombinant nAChRs expressed by single cells at constant membrane potential do show receptor up-regulation after chronic nicotine administration (Molinari *et al.*, 1998; Buisson & Bertrand 2001), though the extent of enhancement is clearly less than the one found with binding experiments.

The present study is, therefore, the first electrophysiological report that native nAChRs of single SH-SY5Y cells were equi-effectively up-regulated by 10 μM or 1 mM nicotine application. Because these concentrations of nicotine evoked very different activation of nAChRs, yet similar degree of desensitization, it seems likely that desensitization was an important contributor to receptor up-regulation. Up-regulation did not change the receptor sensitivity to antagonists or the current shape or its reversal potential, suggesting that a larger number of receptors with properties analogous to control ones was involved.

Starting from a small residual sensitivity of nAChRs in chronically-applied nicotine solution, washout with standard solution quickly restored normal receptor responsiveness in about 90 s, and later (tens of min) led to the appearance of larger responses. This observation indicates that receptor up-regulation had a genuine delay following the start of washout, because receptor recovery from desensitization had a much faster time course.

It is generally accepted that the phenomenon of up-regulation occurs as a consequence of the deficit in cholinergic function caused by desensitization (and consequent inactivation) of nAChRs following chronic agonist exposure (Paterson & Nordberg 2000). Alkondon and Albuquerque (2005) have recently claimed that a differential sensitivity to the desensitizing action of nicotine corresponds to a differential up-regulation of nAChR subtypes. There is, in fact, a direct relationship between propensity to desensitize and percent up-regulation of the three nAChR subtypes expressed in rat hippocampal slices: α3β4-like receptors appear to be the first to be readily desensitized by smoker's levels of nicotine and are significantly up-regulated *in vivo* by nicotine in less than one day.

In our case, we showed that, on SH-SY5Y cells, desensitization in the continuous presence of nicotine was not complete, a phenomenon explaining why receptor up-regulation in functional terms was significant, but not very large. While the identity of the molecular mechanism(s) responsible for enhanced responses to nicotine remains uncertain, some theories have been proposed to account for this phenomenon.

As far as α4β2-subtype nAChRs are concerned, two schemes are advanced to explain their functional up-regulation (Buisson & Bertrand, 2002). In the first case, receptors might exist in two inter-convertible states (one with high affinity for nicotine, and the other with low affinity for nicotine). Chronic exposure to nicotine would stabilize a large fraction of receptors in the high-affinity state. In the second scheme, chronic exposure to nicotine would slow down receptor endocytosis from the membrane and increase their membrane density by inserting additional, pre-synthesized receptors from a cytoplasmic pool. More recently, Sallette *et al.* (2004) have proposed that chronically-applied nicotine would facilitate maturation processes toward high affinity receptors especially those containing β2 subunits. Darsow et al. (2005), using α4β2 receptors expressed in HEK cells, demonstrated that chronic nicotine treatment induces up-regulation of nAChRs not via modulation of endocytic trafficking or degradation rate, but via a biosynthetic step prior to insertion of α4β2 receptors into the plasma membrane (probably at level of the endoplasmic reticulum which is proposed to store an internal pool of nAChRs relevant to up-regulation). It is currently unknown whether these theories might also be applicable to the up-regulation of native receptors.

Although nicotine is membrane-permeant and can bind to intracellular sites, ligand interaction with surface receptors alone is sufficient to induce up-regulation, since exposure to membrane-impermeant ligands also induce up-regulation (Darsow et al., 2005; Whiteaker et al., 1998). Therefore, it is necessary to assume that a signal initiated by the surface receptor in response to nicotine may activate a second messenger acting intracellularly to drive up-regulation. This pathway is unlikely to be dependent on channel activity, since chronic treatment with competitive antagonists or even the combination of nicotine with channel blockers is sufficient to produce receptor up-regulation (Darsow et al., 2005; Gopalakrishnan et al., 1997; Peng et al.,

1997). Cho et al. (2006) have proposed that regulated exocytosis is a plausible mechanism for the rapid delivery of additional α7 nAChRs to the plasma membrane of both *Xenopus* oocytes and rat hippocampal interneurons when α7 receptors are up-regulated through tyrosine kinases and phosphatases. These authors have observed that a brief exposure to the broad spectrum protein tyrosine kinase inhibitor, genestein, specifically and reversibly potentiated α7 nAChR-mediated responses, whereas the protein tyrosine phosphatase inhibitor, pervanadate, caused their depression: these data implies that the function of a7 nAChRs is augmented under conditions favoring protein tyrosine dephosphoryation. The increase in α7 function is accompanied by an increase in the number of α7 subunits/αBgtx binding sites on the plasma membrane and a concomitant decrease in the intracellular receptor pool, consistent with the notion that tyrosine dephosphorylation can regulate the receptor surface distribution at steady-state. Mutation of α7 tyrosine residues does not prevent up-regulation, indicating that it does not depend on direct phosphorylation/dephosphorylation of the α7 subunit, and that another protein(s) is the target of this process.

Although nAChR up-regulation may be a model to understand some of the effects due to chronic administration of nicotine, it is probable that the complex processes of nicotine tolerance and withdrawal additionally involve alterations in gene expression (Dunckley & Lukas, 2003; Dunckley & Lukas, 2006) and regulatory protein kinases (Dajas-Bailador *et al.*, 2002).

Chronic agonist and antagonist treatments

The present study has shown that both nicotine and CC4, when chronically applied, induced up-regulation of nAChRs in SH-SY5Y cells; as summarized in Table 3, not all nicotinic antagonists could elicit induce up-regulation of nAChRs, whereas nicotinic agonists always did so.

In addition, CC4 has shown preferential up-regulation of the α3β2 subtype, whereas after chronic nicotine treatment the relative contribution of different subtypes to the total current seems identical as in control cells. It is difficult to explain this difference in terms of different agonist/antagonist effects, because a similar differential up-

regulation has been observed, in other cellular systems, after chronic treatment with nicotine (Ridley et al., 2001; Wang et al., 1998).

Future perspectives

Our analysis of the nAChR subtypes expressed in rat chromaffin cells needs further work with a wider range of subunit selective agents. In addition, more extensive single cell PCR data would be useful to validate subunit coexpression by rat chromaffin cells and to firmly identify the nAChR subtypes within the cell population. In general, knowing the subunit composition of nicotinic acetylcholine receptors in a particular neuronal pathway is important for understanding the roles of the native receptors and the rational design of new drugs.

nAChRs are involved in a variety of CNS diseases including drug addiction, neuroendocrine, neuropsychiatric, and neurological diseases, memory and learning disabilities, eating disorders, and the control of pain, as well as cardiovascular and gastrointestinal disorders. For example, schizophrenia and drug abuse have both been associated with hyperactivity of CNS dopaminergic systems, and inhibition of nAChRs may be advantageous in reducing such a hyperactivity. Furthermore, the availability of subtype-selective nAChR antagonists should be valuable for both basic and clinical research, with regard to both treatment and diagnosis of disease. Finally, subtype-selective antagonists will define the role of specific nAChR subtypes in both physiological function and disease states.

Recent studies of how nicotine (or nicotinic drugs) determines the expression of specific subtypes raise the possibility of developing novel strategies aimed at modifying the nAChR subtypes in different brain and cell domains.

Since certain nicotinic antagonists are proposed to help for the treatment of nicotine addiction and require long-term use, 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.

The identification of novel differential enhancers of the nAChR expression might also help in understanding the molecular mechanisms by which they interact with receptors determining up-regulation of certain specific subtypes.

A significant advance in understanding the role of specific subunits has come from the use of "knockout mice", that provide an irreversible loss of function of a specific subunit (Wang et al., 2002). Another potentially interesting method to study subunit function, once they have been identified, is RNA interference (RNAi; Gonzalez-Alegre, 2007), that might permit to switch off a gene without physically cancel it and that might determine a loss of function potentially reversible.

CONCLUSIONS

The main conclusions of the present study are as follows:

1. α3β4 is the principal nicotinic receptor subtype on the rat chromaffin cells; α3β2 receptors are also expressed in these cells and, less commonly, the α4β2 subtype. α5 subunits can be associated with α3β4 and α3β2 receptors. α7-containing receptors are not functionally expressed in rat chromaffin cells;

2. The new pharmacological compound, CC4 is a nicotinic antagonist with a potent, reversible blocking effect on nicotinic currents. Chronic treatment with CC4 induced a significant up-regulation of nicotinic current in SH-SY5Y cells with relative increase in α3β2 and α7 receptor subtypes;

3. The nAChRs of SH-SY5Y cells preserved a degree of responsiveness during chronic application of nicotine, and rapidly recovered on washout to generate larger responses without changes in kinetics or pharmacology.

ACKNOWLEDGEMENTS

First of all, I would like to express my most sincere gratitude to my supervisor, Prof. Andrea Nistri, for his patience, guidance and encouragement at all stages of my work. I wish to thank Dr. Cecilia Gotti for her stimulating collaboration and for supplying me with some of the materials. I would also like to thank Prof. Jerry Yakel for his kind hospitality and Sterling and Leo for the collaboration and help they offered during my stay at the NIEHS.

Many thanks to Massimo Righi and Micaela Grandolfo for their help in cell preparation.

I also wish to thank Drs. Elena Sokolova, Silvia Di Angelantonio and Elsa Fabbretti, with whom I conducted joint experiments, and Alessandra Fabbro for sharing with me the set up and for her friendship. I would also like to thank all my friends, and in particular Dr. Beatrice Pastore, for making this a more pleasant experience.

REFERENCE LIST

Alkondon M., Albuquerque E.X. (1993) Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. J Pharmacol Exp Ther. 265: 1455-1473.

Alkondon M., Albuquerque E.X. (2005) Nicotinic receptor subtypes in rat hippocampal slices are differentially sensitive to desensitization and early in vivo functional up-regulation by nicotine and to block by bupropion. J Pharmacol Exp Ther. 313: 740-750.

Alkondon M., Pereira E.F.R, Almeida L.E.F., Randall W.R., Albuquerque E.X. (2000) Nicotine at concentration found in cigarette smokers activates and desensitizes nicotinic acetylcholine receptors in CA1 interneurons of rat hippocampus. Neuropharmacology 39: 2726-2739.

Amador M., Dani J.A. (1995) Mechanism for modulation of nicotinic acetylcholine receptors that can influence synaptic transmission. J. Neurosci. 15: 4525-4532

Anderson D.J. (1993) Cell fate determination in the peripheral nervous system: the sympathoadrenal progenitor. J Neurobiol. 24: 185-198. Review.

Arroyo-Jimenez M.M., Bourgeois J.P, Marrubio L.M., Le Sourd A.M., Ottersen O.P., Rinvik E., Fairen A., Changeux J.P., (1999) Ultrastructural localization of the α4 subunit of the neuronal acetylcholine nicotinic receptors in the rat substantia nigra. J. Neurosci. 19: 6475-6487.

Balestra B., Moretti M., Longhi R., Mantegazza R., Clementi F., Gotti C. (2000) Antibodies against neuronal nicotinic receptor subtypes in neurological disorders. J Neuroimmunol. 102: 89-97.

Barrantes G.E., Rogers A.T., Lindstrom J., Wonnacott S. (1995) alpha-Bungarotoxin binding sites in rat hippocampal and cortical cultures: initial characterisation, colocalisation with alpha 7 subunits and up-regulation by chronic nicotine treatment. Brain Res. 672: 228-236.

Bate L., Gardiner M. (1999) Molecular genetics of human epilepsies. Exp. Rev. Mol. Med. Dec 10: 1-22.

Bertrand D., Valera S., Bertrand S., Ballivet M., Rungger D. (1991) Steroids inhibit nicotinic acetylcholine receptors. Neuroreport. 2: 277-280.

Bencherif M., Fowler K., Lukas R.J., Lippiello P.M. (1995) Mechanisms of up-regulation of neuronal nicotinic acetylcholine receptors in clonal cell lines and primary cultures of fetal rat brain. J Pharmacol Exp Ther. 275: 987-994.

Benwell M.E., Balfour D.J., Anderson J.M. (1988) Evidence that tobacco smoking increases the density of (-)-[^{3}H]nicotine binding sites in human brain. J Neurochem. 50: 1243-1247.

Bibevski S., Zhou Y., McIntosh J.M., Zigmond R.E. & Dunlap M.E. (2000) Functional nicotinic acetylcholine receptors that mediate ganglionic transmission in cardiac parasympathetic neurons. J. Neurosci. 20: 5076–5082.

Boccafusco J.J. (1996) The role of central cholinergic neurons in the regulation of blood pressure and in experimental hypertension. Pharmacol Rev. 48: 179-211. Review.

Boido, C.C., Tasso, B., Boido, V., Sparatore, F. (2003). Cytisine derivatives as ligands for neuronal nicotine receptors and with various pharmacological activities. Farmaco 58: 265–277.

Brandt B.L., Hagiwara S., Kidokoro Y., Miyazaki S. (1976) Action potentials in the rat chromaffin cell and effects of acetylcholine. J Physiol. 263: 417-439.

Buisson B., Bertrand D. (2001) Chronic exposure to nicotine upregulates the human (alpha)4(beta)2 nicotinic acetylcholine receptor function. J Neurosci. 21: 1819-1829.

Campos-Caro A., Smillie F.I., Dominguez del Toro E., Rovira J.C., Vicente-Agullo F., Chapuli J., Juiz J.M., Sala S., Sala F., Ballesta J.J., Criado M. (1997) Neuronal nicotinic acetylcholine receptors on bovine chromaffin cells: cloning, expression, and genomic organization of receptor subunits. J Neurochem. 68: 488-497.

Canu Boido, C., Sparatore, F. (1999). Synthesis and preliminary pharmacological evaluation of some cytisine derivatives. Farmaco 54: 438–451.

Carbonnelle E., Sparatore F., Canu-Boido C., Salvagno C., Baldani-Guerra B., Terstappen G., Zwart R., Vijverberg H., Clementi F., Gotti C. (2003) Nitrogen substitution modifies the activity of cytisine on neuronal nicotinic receptor subtypes. Eur J Pharmacol. 471: 85-96.

Cartier G.E., Yoshikami D., Gray W.R., Luo S., Olivera B.M., McIntosh J.M. (1996) A new alpha-conotoxin which targets alpha3beta2 nicotinic acetylcholine receptors. J Biol Chem. 271: 7522-7528.

Champtiaux, N., Han, Z.Y., Bessis, A., Rossi, F.M., Zoli, M., Marubio, L., McIntosh, J.M. & Changeux, J.P. (2002) Distribution and pharmacology of α6-containing nicotinic acetylcholine receptors analyzed with mutant mice. J. Neurosci. 22: 1208–1217.

Changeux J.P. (1990) The TiPS lecture. The nicotinic acetylcholine receptor: an allosteric protein prototype of ligand-gated ion channels. Trends Pharmacol Sci. 11: 485-492. Review.

Chavez-Noriega L.E., Crona J.H., Washburn M.S., Urrutia A., Elliott K.J., Johnson E.C. (1997) Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h alpha 2 beta 2, h alpha 2 beta 4, h alpha 3 beta 2, h alpha 3 beta 4, h alpha 4 beta 2, h alpha 4 beta 4 and h alpha 7 expressed in Xenopus oocytes. J Pharmacol Exp Ther. 280: 346-356.

Cho C.H., Song W., Leitzell K., Teo E., Meleth A.D., Quick M.W., Lester R.A. (2005) Rapid upregulation of alpha7 nicotinic acetylcholine receptors by tyrosine dephosphorylation. J Neurosci. 25: 3712-3723.

Cimino M., Marini P., Fornasari D., Cattabeni F., Clementi F. (1992) Distribution of nicotinic receptors in cynomolgus monkey brain and ganglia: localization of alpha 3 subunit mRNA, alpha-bungarotoxin and nicotine binding sites. Neuroscience. 51: 77-86.

Conroy W.G, Berg D.K. (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. J Biol Chem. Mar 270: 4424-4431.

Corringer, P.J., Galzi, J.L., Eisele, J.L., Bertrand, S., Changeux, J.P. & Bertrand, D. (1995) Identification of a new component of the agonist binding site of the nicotinic α7 homooligomeric receptor. J. Biol. Chem. 270: 11749-11752.

Corringer P.J., Sallette J., Changeux J.P. (2006) Nicotine enhances intracellular nicotinic receptor maturation: a novel mechanism of neural plasticity? J Physiol Paris. 99:162-171. Review.

Court D.K., Clementi F. (1995) Distribution of nicotinic subtypes in human brain. Alzheimer Dis Assoc Disord. 9 Suppl 2: 6-14.

Covernton, P.J., Kojima, H., Sivilotti, L.G., Gibb, A.J., Colquhoun D. (1994). Comparison of neuronal nicotinic receptors in rat sympathetic neurones with subunit pairs expressed in *Xenopus oocytes*. J. Physiol., 481: 27–34.

Criado M., Alamo L., Navarro A. (1992) Primary structure of an agonist binding subunit of the nicotinic acetylcholine receptor from bovine adrenal chromaffin cells. Neurochem Res. 17 :281-287.

Cuevas J., Berg D.K. (1998) Mammalian nicotinic receptors with α7 subunits that slowly desensitize and rapidly recover from α-bungarotoxin blockade. J. Neurosci. 18: 10335-10344.

Dajas-Bailador F.A., Soliakov L., Wonnacott S. (2002) Nicotine activates the extracellular signal-regulated kinase 1/2 via the alpha7 nicotinic acetylcholine receptor and protein kinase A, in SH-SY5Y cells and hippocampal neurones. J Neurochem. 80: 520-530.

Dani J.A. (2001) Synaptic plasticity and nicotine addiction. Neuron. 31: 349-52. Review.

Dani J.A., Heinemann S. (1996) Molecular and cellular aspects of nicotine abuse. Neuron. 16: 905-908.

Dani J.A., Bertrand D. (2007) Nicotinic Acetylcholine Receptors and Nicotinic Cholinergic Mechanisms of the Central Nervous System. Annu. Rev. Pharmacol. Toxicol. 47: 699-729.

Darsow T., Booker T.K., Pina-Crespo J.C., Heinemann S.F. (2005) Exocytic trafficking is required for nicotine-induced up-regulation of alpha 4 beta 2 nicotinic acetylcholine receptors. J Biol Chem. 280: 18311-18320.

De Koninck P., Cooper E. (1995) Differential regulation of neuronal nicotinic ACh receptor subunit genes in cultured neonatal rat sympathetic neurons: specific induction of alpha 7 by membrane depolarization through a Ca^{2+}/calmodulin-dependent kinase pathway. J Neurosci. 15: 7966-7978.

Di Angelantonio S., Nistri A., Moretti M., Clementi F., Gotti C. (2000)Antagonism of nicotinic receptors of rat chromaffin cells by N,N, N-trimethyl-1-(4-trans-stilbenoxy)-2-propylammonium iodide: a patch clamp and ligand binding study. Br J Pharmacol. 129: 1771-1779.

Di Angelantonio S., Nistri A. (2001) Calibration of agonist concentrations applied by pressure pulses or via rapid solution exchanger. J Neurosci Methods. 110:155-161.

Di Angelantonio S., Giniatullin R., Costa V., Sokolova E., Nistri A. (2003) Modulation of neuronal nicotinic receptor function by the neuropeptide CGRP and substance P on autonomic nerve cells. Br. J. Pharmacol. 134: 789-796.

Dougherty J.J., Wu J., Nichols R.A. (2003) Beta-amyloid regulation of presynaptic nicotinic receptors in rat hippocampus and neocortex. J Neurosci. 23:6740-6747.

Drapeau P., Legendre P. (2001) Neuromuscular transmission on the rebound. Receptors Channels. 7: 491-496. Review.

Dunckley T, Lukas R.J. (2003) Nicotine modulates the expression of a diverse set of genes in the neuronal SH-SY5Y cell line. J Biol Chem. 278: 15633-15640.

Dunckley T., Lukas R.J. (2006) Nicotinic modulation of gene expression in SH-SY5Y neuroblastoma cells. Brain Res. 1116: 39-49.

Dwoskin L.P., Crooks P.A. (2001) Competitive neuronal nicotinic receptor antagonists: a new direction for drug discovery. J Pharmacol Exp Ther. 298: 395-402. Review.

Eccles, J.C., Eccles, R.M., Fatt, P. (1956) Pharmacological investigations on a central synapse operated by acetylcholine. J. Physiol. (Lond.) 131: 154–169.

El-Bizri H., Clarke P.B. (1994) Blockade of nicotinic receptor-mediated release of dopamine from striatal synaptosomes by chlorisondamine administered in vivo. Br J Pharmacol. 111: 414-418.

Elgoyhen A.B., Johnson D.S., Boulter J., Vetter DE., Heinemann S. (1994) Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell. 79: 705-715.

Fenster C.P., Whitworth T.L., Sheffield E.B., Quick M.W., Lester R.A. (1999) Upregulation of surface alpha4beta2 nicotinic receptors is initiated by receptor desensitization after chronic exposure to nicotine. J Neurosci. 19: 4804-4814.

Fisher, H., Orr-Urtreger, A., Role, L.W., Huck, S. (2005). Selective deletion of the alpha5 subunit differentially affects somatic-dendritic versus axonally targeted nicotinic ACh receptors in mouse. J. Physiol. 563: 119–137.

.

Flores C.M., Rogers S.W., Pabreza L.A., Wolfe B.B., Kellar K.J. (1992) A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. Mol Pharmacol. 41: 31-37.

Flynn D.D., Mash D.C. (1986) Characterization of L-[^{3}H]nicotine binding in human cerebral cortex: comparison between Alzheimer's disease and the normal. J Neurochem. 47: 1948-1954

Frazier C.J., Buhler A.V., Weiner J.L., Dunwiddie T.V. (1998) Synaptic potentials medicated via α-Bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal interneurons. J. Neurosci. 18: 8228-8235.

Fu, Y., Matta, S.G., McIntosh, J.M., Sharp, B.M. (1999) Inhibition of nicotineinduced ippocampal norepinephrine release in rats by α-conotoxins MII and AuIB microinjected into the locus coeruleus. Neurosci. Lett. 266: 113–116.

Galzi J.L., Changeux J.P. (1995) Neuronal nicotinic receptors: molecular organization and regulations. Neuropharmacology. 34: 563-582.

Gandia L., Villarroya M., Sala F., Reig J.A., Viniegra S., Quintanar J.L., Garcia A.G., Gutierrez L.M. (1996) Inhibition of nicotinic receptor-mediated responses in bovine chromaffin cells by diltiazem. Br J Pharmacol. 118: 1301-1307.

Gandia L., Vitale M.L., Villaroya M., Ramirez-Lavergne C., Garcia A.G., Trifaro J.M. (1997) Differential effects of forskolin and 1,9-dideoxi-forskolin on nicotinic receptor- and K^+- induced responses in chromaffin cells. Eur. J. Pharmacol. 329: 189-199.

Gentry CL, Lukas RJ. Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. (2002) Curr Drug Targets CNS Neurol Disord. 1: 359-385. Review.

Gerzanich V, Wang F, Kuryatov A, Lindstrom J. (1998) Alpha 5 Subunit alters desensitization, pharmacology, Ca^{2+} permeability and Ca^{2+} modulation of human neuronal alpha 3 nicotinic receptors. J Pharmacol Exp Ther. 286: 311-320.

Gonzalez-Alegre P. (2007) Therapeutic RNA interference for neurodegenerative diseases: From promise to progress. Pharmacol Ther. 2007 Jan 25

Gahring L.C., Rogers S.W. (2006) Neuronal acetylcholine receptor expression and function on nonneuronal cells. APPS J. 7: 885-894.

Giniatullin R., Di Angelantonio S., Marchetti C., Sokolova E., Khiroug L., Nistri A. (1999) Calcitonin-gene related peptide rapidly downregulates nicotinic receptor function and slowly raises intracellular Ca^{2+} in rat chromaffin cells in vitro. J. Neurosci. 19: 1945-2953.

Giniatullin R.,, Nistri A., Yakel J.L. (2005) Desensitization of nicotinic ACh receptors: shaping cholinergic signalling. Trends Neurosci. 28: 371-378.

Gonzalez-Alegre P. (2007) Therapeutic RNA interference for neurodegenerative diseases: From promise to progress. Pharmacol Ther. 114: 34-55.

Gopalakrishnan M., Molinari E.J., Sullivan J.P. (1997) Regulation of human alpha4beta2 neuronal nicotinic acetylcholine receptors by cholinergic channel ligands and second messenger pathways. Mol Pharmacol. 52: 524-534.

Gotti C., Fornasari D., Clementi F. (1997) Human neuronal nicotinic receptors. Prog Neurobiol. 53: 199-237. Review

Gotti C., Clementi F. (2004) Neuronal nicotinic receptors: from structure to pathology. Prog Neurobiol. 74: 363-396. Review.

Gotti C., Riganti L., Vailati S. (2005) Brain neuronal nicotinic receptors as new targets for drug discovery. Current Pharmaceutical Design.

Gotti C., Zoli M., Clementi F. (2006) Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol Sci. 27: 482-491. Review.

Groot-Kormelink P.J., Boorman J.P., Sivilotti L.G. (2001) Formation of functional alpha3beta4alpha5 human neuronal nicotinic receptors in Xenopus oocytes: a reporter mutation approach. Br J Pharmacol. 134: 789-796.

Guan Z.Z., Zhang X., Ravid R, Nordberg A. (2000) Decreased protein levels of nicotinic receptor subunits in the hippocampus and temporal cortex of patients with Alzheimer's disease. J. Neurochem, 74, 237-243.

Harvey S.C., McIntosh J.M., Cartier G.E., Maddox F.N., Luetje C.W. (1997) Determinants of specificity for α-conotoxin MII on α3β2 neronal nicotinic receptors. Mol. Pharmacol. 51: 336-342.

Herrero C.J., Ales E., Pintado A.., Lopez M.G., Garcia-Palomero E., Mahata S.K., O'Connor D.T., Garcia A.G., Montiel C. (2002) Modulatory mechanism of the endogenous peptide catestatin on neuronal nicotinic acetylcholine receptors and exocytosis. J. Neurosci. 22: 377-388.

Hogg R.C., Raggenbass M., Bertrand D. (2003) Nicotinic acetylcholine receptors: from structure to brain function. Rev Physiol Biochem Pharmacol. 147: 1-46.. Review.

Houlihan, L.M., Slater, Y., Guerra, D.L., Peng, J.H., Kuo,Y.P., Lukas, R.J., Cassels, B.K., Bermudez, I. (2001). Activity of cytisine and its brominated isosteres on recombinant human alpha7, alpha4beta2 and alpha4beta4 nicotinic acetylcholine receptors. J. Neurochem. 78: 1029–1043.

Hsu Y.N., Amin J., Weiss D.S., Wecker L. (1996) Sustained nicotine exposure differentially affects alpha 3 beta 2 and alpha 4 beta 2 neuronal nicotinic receptors expressed in Xenopus oocytes. J Neurochem. 66: 667-675.

Itier, V., Bertrand, D. (2001) Neuronal nicotinic receptors: from protein structure to function. *FEBS Lett.*, 504: 118–125.

Jones G.M., Sahakian B.J., Levy R., Warburton D.M., Gray J.A. (1992) Effects of acute subcutaneous nicotine on attention, information processing and short-term memory in Alzheimer's disease. Psychopharmacology (Berl). 108: 485-494.

Jones I.W., Bolam J.P, Wonnacott S. (2001) Presynaptic localization of the nicotinic acetylcholine receptor β2 subunit immunoreactivity in rat nigrostriatal dopaminergic neurons. J. Comp. Neurol. 439: 235-247.

Kaiser S.A., Soliakov L., Harvey S.C., Luetje C.W., Wonnacott S. (1998) Differential inhibition by alpha-conotoxin-MII of the nicotinic stimulation of [^{3}H]dopamine release from rat striatal synaptosomes and slices. J Neurochem. 70: 1069-1076.

Kao P.N., Karlin A. (1986) Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. J Biol Chem. 261: 8085-8088.

Kasa, P. (1986) The cholinergic systems in brain and spinal cord. Prog Neurobiol. 26: 211-272. Review

Katz B., Thesleff S. (1957) A study of the desensitization produced by acetylcholine at the motor end-plate. J Physiol. 138: 63-80.

Kawai H., Berg D.K. (2001) Nicotinic acetylcholine receptors containing alpha 7 subunits on rat cortical neurons do not undergo long-lasting inactivation even when up-regulated by chronic nicotine exposure.J Neurochem. 76: 1367-1378.

Ke L., Eisenhour C.M., Bencherif M., Lukas R.J. (1998) Effects of chronic nicotine treatment on expression of diverse nicotinic acetylcholine receptor subtypes. I. Dose- and time-dependent effects of nicotine treatment. J Pharmacol Exp Ther. 286: 825-840.

Khiroug L., Giniatullin R., Sokolova E., Talantova M., Nistri A. (1997) Imaging of intracellular calcium during desensitization of nicotinic acetylcholine receptors of rat chromaffin cells. Br J Pharmacol. 122: 1323-1332.

Khiroug L., Sokolova E., Giniatullin R., Afzalov R., Nistri A. (1998) Recovery from desensitization of neuronal nicotinic acetylcholine receptors of rat chromaffin cells is modulated by intracellular calcium through distinct second messengers. J Neurosci. 18: 2458-2466.

Klink R., de Kerchove D.A., Zoli M., Changeux J.P. (2001) Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. J. Neurosci. 21: 1452-1463.

Kuryatov A., Luo J., Cooper J., Lindstrom J. (2005) Nicotine acts as a pharmacological chaperone to up-regulate human alpha4beta2 acetylcholine receptors. Mol Pharmacol. 68: 1839-1851.

Lena C., Changeux J.P. (1993) Allosteric modulations of the nicotinic acetylcholine receptor. Trends Neurosci. 16: 181-186. Review.

Le Novère, N., Zoli, M. & Changeux, J.P. (1996) Neuronal nicotinic receptor α6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. Eur. J. Neurosci. 8: 2428–2439.

Lindstrom J. (1997) Nicotinic acetylcholine receptors in health and disease. Mol Neurobiol 15: 193-222.

Lipton S.A., Kater S.B. (1998) Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. Trends Neurosci. 12: 265-270.

Lopez-Hernandez G.Y., Sanchez-Padilla J., Ortiz-Acevedo A., Lizardi-Ortiz J., Salas-Vincenty J., Rojas L.V., Lasalde-Dominicci J.A. (2004) Nicotine-induced up-regulation and desensitization of alpha4beta2 neuronal nicotinic receptors depend on subunit ratio. J Biol Chem. 279: 38007-38015.

Luetje C.W., Wada K., Rogers S., Abramson S.N., Tsuji K., Heinemann S., Patrick J. (1990) Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations. J Neurochem. 55: 632-640.

Luetje C.W., Patrick J. (1991) Both alpha- and beta-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. J Neurosci. 11: 837-845.

Lukas R.J. (1993) Expression of ganglia-type nicotinic acetylcholine receptors and nicotinic ligand binding sites by cells of the IMR-32 human neuroblastoma clonal line. J Pharmacol Exp Ther. 265: 294-302.

Luo S., Kulak J.M., Cartier G.E., Jacobsen R.B., Yoshikami D., Olivera B.M., McIntosh J.M. (1998) Alpha-conotoxin AuIB selectively blocks alpha3 beta4 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. J Neurosci. 18: 8571-8579.

Maconochie D.J., Knight D.E. (1992) A study of the bovine adrenal chromaffin nicotinic receptor using patch clamp and concentration-jump techniques. J Physiol. Aug;454:129-53.

Marks M.J., Pauly J.R., Gross S.D., Deneris E.S., Hermans-Borgmeyer I., Heinemann S.F., Collins A.C. (1992) Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. J Neurosci. 12: 2765-2784.

Marks M.J., Farnham D.A., Grady S.R., Collins A.C. (1993) Nicotinic receptor function determined by stimulation of rubidium efflux from mouse brain synaptosomes. J Pharmacol Exp Ther. 264: 542-552.

Martin-Ruiz C.M., Court J.A., Molnar E., Lee M., Gotti C., Mamalaki A., Tsouloufis T., Tzartos S, Ballard C., Perry R.H, Perry E.K. (1999) α4 but not α3 and α7 nicotinic acetylcholine recepror subunits are lost from the temporal cortex in Alzheimer's disease. J. Neurochem. 73: 1635-1640.

Miles K., Greengard P., Huganir R.L. (1989) Calcitonin gene-related peptide regulates phosphorylation of the nicotinic acetylcholine receptor in rat myotubes. Neuron. 2: 1517-1524.

Molinari E.J., Delbono O., Messi M.L., Renganathan M., Arneric S.P., Sullivan J.P., Gopalakrishnan M. (1998) Up-regulation of human alpha7 nicotinic receptors by chronic treatment with activator and antagonist ligands. Eur J Pharmacol. 347: 131-139.

Mousavi M., Hellstrom-Lindahl E., Guan Z.Z., Bednar I., Nordberg A. (2001) Expression of nicotinic acetylcholine receptors in human and rat adrenal medulla. Life Sci. 70: 577-590.

Mugnaini M., Garzotti M., Sartori I., Pilla M., Repeto P., Heidbreder C.A., Tessari M. (2006) Selective down-regulation of [(125)I]Y0-alpha-conotoxin MII binding in rat mesostriatal dopamine pathway following continuous infusion of nicotine. Neuroscience. 137: 565-572.

Mulle C., Lena C., Changeux J.P. (1992) Potentiation of nicotinic receptor response by external calcium in rat central neurons. Neuron. 8: 937-945.

Nashmi, R., Dickinson, M.E., McKinney, S., Jareb, M., Labarca, C., Fraser, S.E., Lester, H.A. (2003). Assembly of α4β2 nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons. J. Neurosci. 23: 11554 –11567.

Nicke, A., Wonnacott, S., Lewis, R.J. (2004). α-conotoxins as tools for the elucidation of structure and function of neuronal nicotinic acetylcholine receptor subtypes. Eur. J. Biochem. 271: 2305-2319.

Nooney J.M., Feltz A. (1995) Inhibition by cyclothiazide of neuronal nicotinic responses in bovine chromaffin cells. Br J Pharmacol. 114: 648-655.

Olale F., Gerzanich V., Kuryatov A., Wang F., Lindstrom J. (1997) Chronic nicotine exposure differentially affects the function of human alpha3, alpha4, and alpha7 neuronal nicotinic receptor subtypes. J Pharmacol Exp Ther. 283: 675-683.

Orr-Urtreger A., Goldner F.M., Saeki M., Lorenzo I., Goldberg L., De Biasi M., Dani J.A., Patrick J.W., Beaudet A.L. (1997) Mice deficient in the alpha7 neuronal nicotinic acetylcholine receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents. J Neurosci. 17: 9165-9171.

Pake, R.L., Heinemann, S.F. (1994). Partial agonist properties of cytisine on neuronal nicotinic receptors containing the beta 2 subunit. Mol. Pharmacol. 45: 142–149.

Papke R.L. (1993) The kinetic properties of neuronal nicotinic receptors: genetic basis of functional diversity. Prog Neurobiol. 41: 509-531. Review.

Paradiso K., Zhang J., Steinbach J.H. (2001) The C terminus of the human nicotinic alpha4beta2 receptor forms a binding site required for potentiation by an estrogenic steroid. J. Neurosci. 21: 6561–6568

Paradiso K.J., SteinbachJ.H. (2003) Nicotine is highly effective at producing desensitization of rat alpha4beta2 neuronal nicotinic receptors. Nicotine is highly effective at producing desensitization of rat alpha4beta2 neuronal nicotinic receptors. J Physiol. 553: 857-871.

Paterson D., Nordberg A. (2000) Neuronal nicotinic receptors in the human brain. Prog Neurobiol. 61: 75-111.

Paton, W.D., Zaimis, E.J. (1949). The pharmacological actions of polymethylene bistrimethyl-ammonium salts. Br. J. Pharmacol. 4: 381–400.

Pauly J.R., Marks M.J., Gross S.D., Collins A.C. (1991) An autoradiographic analysis of cholinergic receptors in mouse brain after chronic nicotine treatment. J Pharmacol Exp Ther. 258: 1127-1136.

Peng X., Katz M., Gerzanich V., Anand R., Lindstrom J. (1994) Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in Xenopus oocytes. Mol Pharmacol. 45: 546-554.

Peng X., Gerzanich V., Anand R., Wang F., Lindstrom J. (1997) Chronic nicotine treatment up-regulates alpha3 and alpha7 acetylcholine receptor subtypes expressed by the human neuroblastoma cell line SH-SY5Y. Mol Pharmacol. 51: 776-784.

Pereira E.F., Reinhardt-Maelicke S., Schrattenholz A., Maelicke A., Albuquerque E.X. (1993) Identification and functional characterization of a new agonist site on nicotinic acetylcholine receptors of cultured hippocampal neurons. J Pharmacol Exp Ther. 265: 1474-1491.

Pugh P.C., Berg D.K. (1994) Neuronal acetylcholine receptors that bind alpha-bungarotoxin mediate neurite retraction in a calcium-dependent manner. J Neurosci. 14: 889-896.

Quick M.W., Ceballos R.M., Kasten M., McIntosh J.M., Lester R.A. (1999) Alpha3beta4 subunit-containing nicotinic receptors dominate function in rat medial habenula neurons. Neuropharmacology. 38: 769-783.

Quick M.W., Lester R.A. (2002) Desensitization of neuronal nicotinic receptors. J Neurobiol. 53: 457-478. Review.

Quik M., Geertsen S., Trifaro J.M. (1987) Marked up-regulation of the beta-bungarotoxin site in adrenal chromaffin cells by specific nicotinic antagonists. Mol Pharmacol. 31: 385-391.

Quik M., (1995) Growth related role for nicotinic α-Bungarotoxin receptors. In: Clarke P.B., Quik M., Adlkofer F., Thurau K. (Eds.), Advences in Pharmacological Sciences, Effects of Nicotine on Biological Systems II. Birkhauser Verlag, Basel. Pp 145-150.

Quik M., Bordia T., Forno L., McIntosh J.M. (2004) Loss of alpha-conotoxin MII- and A85380-sensitive nicotinic receptors in Parkinson's disease striatum. J. Neurochem. 8: 668-679.

Quik M., Choremis J., Komourian J., Lukas R.J., Puchacz E. (1996) Similarity between rat brain nicotinic alpha-bungarotoxin receptors and stably expressed alpha-bungarotoxin binding sites. J Neurochem. 67: 145-154.

Ramirez-Latorre J., Yu C.R., Qu X., Perin F., Karlin A., Role L. (1996) Functional contributions of alpha5 subunit to neuronal acetylcholine receptor channels. Nature. 380: 347-351.

Ridley D.L., Rogers A., Wonnacott S. (2001) Differential effects of chronic drug treatment on alpha3* and alpha7 nicotinic receptor binding sites, in hippocampal neurones and SH-SY5Y cells. Br J Pharmacol. 133: 1286-1295.

Role L.W., Berg D.K. (1996) Nicotinic receptors in the development and modulation of CNS synapses. Neuron 16: 1077-1085.

Rush, R., Kuryatov, A., Nelson, M.E., Lindstrom, J. (2002) First and second transmembrane segments of α3, α4, β2, and β4 nicotinic acetylcholine receptor subunits influence the efficacy and potency of nicotine. Mol. Pharmacol. 61: 1416–1422.

Sala F., Mulet J., Choi S., Jung S.Y., Rhim H., Valor L.M., Criado M., Sala S. (2002) Effects of ginsenoside Rg2 on neuronal nicotinic acetylcholine receptors. J. Pharmacol. Exp. Ther. 301: 1052-1059.

Sallette J., Bohler S., Benoit P., Soudant M., Pons S., Le Novère N., Changeux J.P. and Corringer PJ (2004) An extracellular protein microdomain controls up-regulation of neuronal nicotinic acetylcholine receptors by nicotine. J Biol Chem 279: 18767-18775.

Seguela P., Wadiche J., Dineley-Miller K., Dani J.A., Patrick J.W. (1993) Molecular cloning, functional properties, and distribution of rat brain α7: a nicotinic cation channel highly permeable to calcium. J. Neurosci. 13: 596-604.

Severance E.G., Zhang H, Cruz Y, Pakhlevaniants S, Hadley SH, Amin J, Wecker L, Reed C, Cuevas J. (2004) The alpha7 nicotinic acetylcholine receptor subunit exists in two isoforms that contribute to functional ligand-gated ion channels. Mol Pharmacol. 66: 420-429.

Schmuck, K., Ullmer, C., Engels, P. & Lubbert, H. (1994). Cloning and functional characterization of the human 5-HT2B serotonin receptor. FEBS Lett., 342: 85–90.

Schwartz R.D., McGee R. Jr, Kellar K.J. (1982) Nicotinic cholinergic receptors labeled by [^{3}H]acetylcholine in rat brain. Mol Pharmacol. 22: 56-62.

Sheffield E.B., Quick M.W., Lester R.A. (2000) Nicotinic acetylcholine receptor subunit mRNA expression and channel function in medial habenula neurons. Neuropharmacology. 39: 2591-2603.

Simmons L.K., Schuetze S.M., Role L.W. (1990) Substance P modulates single-channel properties of neuronal nicotinic acetylcholine receptors. Neuron. 4: 393-403.

Slater, Y.E., Houlihan, L.M., Maskell, P.D., Exley, R., Bermudez, I., Lukas, R.J., Valdavia, A.C., Cassels, B.K. (2003). Halogenated cytisine derivatives as agonists at human neuronal nicotinic acetylcholine receptor subtypes. Neuropharmacology 44: 503–515.

Sudweeks S.N., Yakel J.L. (2000) Functional and molecular characterization of neuronal nicotinic ACh receptors in rat CA1 hippocampal neurons. J Physiol. 527: 515-28.

Svensson A.L. (2000) Tacrine interacts with different sites on nicotinic receptor subtypes in SH-SY5Y neuroblastoma and M10 cells. Behav Brain Res. 113: 193-197.

Tachikawa E., Mizuma K., Kudo K., Kashimoto T., Yamato S., Ohta S. (2001) Characterization of the functional subunit combination of nicotinic acetylcholine receptors in bovine adrenal chromaffin cells. Neurosci Lett. 312: 161-164.

Uteshev V.V., Meyer E.M., Papke R.L. (2002) Activation and inhibition of native neuronal alpha-bungarotoxin-sensitive nicotinic ACh receptors. Brain Res. 948: 33-46.

Valenzuela C.F., Dowding A.J., Arias H.R., Johnson D.A. (1994) Antibody-induced conformational changes in the Torpedo nicotinic acetylcholine receptor: a fluorescence study. Biochemistry. 33: 6586-6594.

Vernino S., Amador M., Luetje C.W., Patrick J., Dani J.A. (1992) Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. Neuron. 8: 127-134.

Vernino S., Rogers M., Radcliffe K.A., Dani J.A.. (1994) Quantitative measurement of calcium flux through muscle and neuronal nicotinic acetylcholine receptors. J Neurosci. 14: 5514-5524.

Vernino S., Adamski J., Kryzer T.J., Fealey R.D., Lennon V.A. (1998) Neuronal nicotinic ACh receptor antibody in subacute autonomic neuropathy and cancer-related syndromes. Neurology. 50: 1806-1813.

Villaroya, M., Gandia, L., Lopez, M.G., Garcia, A.G., Cueto, S., Garcia-Navio, J.L., Alvarez-Builla, J. (1996). Synthesis and pharmacology of alkanediguanidinium compounds that block the neuronal nicotinic acetylcholine receptor. Bioorg Med. Chem. 4: 1177–1183.

Visanji N.P., Mitchell S.N., O'Neill M.J., Duty S. (2006) Chronic pre-treatment with nicotine enhances nicotine-evoked striatal dopamine release and alpha6 and beta3 nicotinic acetylcholine receptor subunit mRNA in the substantia nigra pars compacta of the rat. Neuropharmacology. 50: 36-46.

Wang F., Nelson M.E., Kuryatov A., Olale F., Cooper J., Keyser K., Lindstrom J. (1998) Chronic nicotine treatment up-regulates human alpha3 beta2 but not alpha3 beta4 acetylcholine receptors stably transfected in human embryonic kidney cells. J Biol Chem. 273: 28721-28732.

Wang N., Orr-Urtreger A., Korczyn A.D. (2002) The role of neuronal nicotinic acetylcholine receptor subunits in autonomic ganglia: lessons from knockout mice. Prog Neurobiol. 68: 341-360. Review.

Warpman U., Friberg L., Gillespie A., Hellstrom-Lindahl E., Zhang X., Nordberg A. (1998) Regulation of nicotinic receptor subtypes following chronic nicotinic agonist exposure in M10 and SH-SY5Y neuroblastoma cells. J Neurochem. 70: 2028-2037.

Wenger, B.W., Bryant, D.L., Boyd, R.T., McKay, D.B. (1997). Evidence for spare nicotinic acetylcholine receptors and a beta 4 subunit in bovine adrenal chromaffin cells: studies using bromoacetylcholine, epibatidine, cytisine and mAb35. J. Pharmacol. Exp. Ther. 281: 905–913.

Wevers, A., Burghaus, L., Moser, N., Witter, B., Steinlein, O.K., Schutz, U., Achnitz, B., Krempel, U., Nowacki, S., Pilz, K., Stoodt, J., Lindstrom, J., De Vos, R.A., Jansen Steur, E.N. & Schroder, H. (2000) Expression of nicotinic acetylcholine receptors in Alzheimer's disease: postmortem investigations and experimental approaches. Behav. Brain Res. 113: 207–215.

Whiteaker P., Sharples C.G., Wonnacott S. (1998) Agonist-induced up-regulation of alpha4beta2 nicotinic acetylcholine receptors in M10 cells: pharmacological and spatial definition. Mol Pharmacol. 53: 950-962.

Wong, E.T., Holstad, S.G., Mennerick, S.J., Hong, S.E., Zorumski, C.F., Isenberg, K.E. (1995). Pharmacological and physiological properties of a putative ganglionic nicotinic receptor, alpha 3 beta 4, expressed in transfected eucaryotic cells. Brain Res. Mol. Brain Res. 28: 101–109.

Wonnacott S, Thorne B. (1990) Separation of pre- and post-synaptic receptors on Percoll gradients. Biochem Soc Trans. 18: 885-886.

Wonnacott S. (1990) The paradox of nicotinic acetylcholine receptor upregulation by nicotine. Trends Pharmacol Sci. 11: 216-219. Review.

Wonnacott S., Wilkie G.I., Soliakov L., Whiteaker P. (1995) Presynaptic nicotinic autoreceptors and heteroreceptors in the CNS. In: Clarke P.B., Quik M., Adlkofer F., Thurau K. (Eds.), Advances in Pharmacological Sciences. Effects of Nicotine on Biological Systems II. Birkhauser Verlag, Basel, pp. 87-94.

Wonnacott S. (1997) Presynaptic nicotinic ACh receptors. Trends Neurosci. 20: 92-98. Review.

Woolf N.J. (1991) Cholinergic systems in mammalian brain and spinal cord. Prog Neurobiol. 37: 475-524. Review.

Wooltorton J.R., Pidoplichko V.I., Broide R.S., Dani J.A. (2003) Differential desensitization and distribution of acetylcholine receptor subtypes in midbrain dopamine areas. J. Neurobiol. 53: 457-478.

Xiu J., Nordberg A., Zhang J.T., Guan Z.Z. (2005) Expression of nicotinic receptors on primary cultures of rat astrocytes and up-regulation of the alpha7, alpha4 and beta2 subunits in response to nanomolar concentrations of the beta-amyloid peptide(1-42). Neurochem Int. 47: 281-290.

Zarei, M.M., Radcliffe, K.A., Chen, D., Patrick, J.W. & Dani, J.A. (1999) Distributions of nicotinic acetylcholine receptor $\alpha 7$ and $\beta 2$ subunits on cultured hippocampal neurons. Neuroscience 88: 755–764.

Zhang X., Gong Z.H., Hellstrom-Lindahl E., Nordberg A. (1995) Regulation of alpha 4 beta 2 nicotinic acetylcholine receptors in M10 cells following treatment with nicotinic agents. Neuroreport. 6: 313-317.

Zheng J.Q., Felder M., Conor J.A., Poo M.M. (2004) Turning of nerve growth cones induced by beurotransmitters. Nature 368: 140-144.

Zhou Y., Generis E., Zigmond R.E. (2001) Nicotinic acetylcholine receptor subunit proteins α7 and β4 decrease in the superior cervical ganglion after axotomy. J. Neurobiol. 46, 178-192.

Zoli M., Lena C., Picciotto M.R., Changeux J.P. (1998) Identification of four classes of brain nicotinic receptors using beta2 mutant mice. J Neurosci. 18: 4461-4472.

Zoli M., Moretti M., Zanardi A., McIntosh J.M., lementi F., Gotti C. (2002) Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. J. Neurosci. 22: 8785-8789.

APPENDIX 1

Molecular and cellular biology data produced by our collaborators (Dr. C. Gotti et al.) concerning the action of CC4 and reported in our joint publication: Riganti L., Matteoni C., Di Angelantonio S., Nistri A, Gaimarri A., Sparatore F., Canu-Boido C., Clementi F., Gotti C. (2005) "Long-term exposure to the new nicotinic antagonist 1,2-bisN-cytisinylethane upregulates nicotinic receptor subtypes of SH-SY5Y human neuroblastoma cells" British Journal of Pharmacology 146, 1096–1109. Details of the methods are supplied therein.

Up-regulation of ^{3}H-Epibatidine sensitive receptors

Initial experiments explored the CC4 concentration and time dependence for changes in receptor expression of SH-SY5Y cell homogenates. The highest level of up-regulation (5 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 . For this reason further studies relied on 1 mM CC4 that yielded a nearly 4 fold increase in ^{3}H-Epi binding. The up-regulation effect started after 6 hours and was strongly developed at 48 hours, the time point selected for subsequent experiments.

In order to differentiate between increased receptor binding affinity and increased number of binding sites, saturation binding analyses were performed, which showed the maximum level of ^{3}H-Epi sensitive receptors in the CC4-treated cells to be 417.9±80.8 fmol/mg of protein, a value significantly larger ($n=7$, $P<0.005$) that the one (117.6 ± 18.8 fmol/ mg of protein) of control cells. Conversely, the apparent dissociation constant (Kd) 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%).

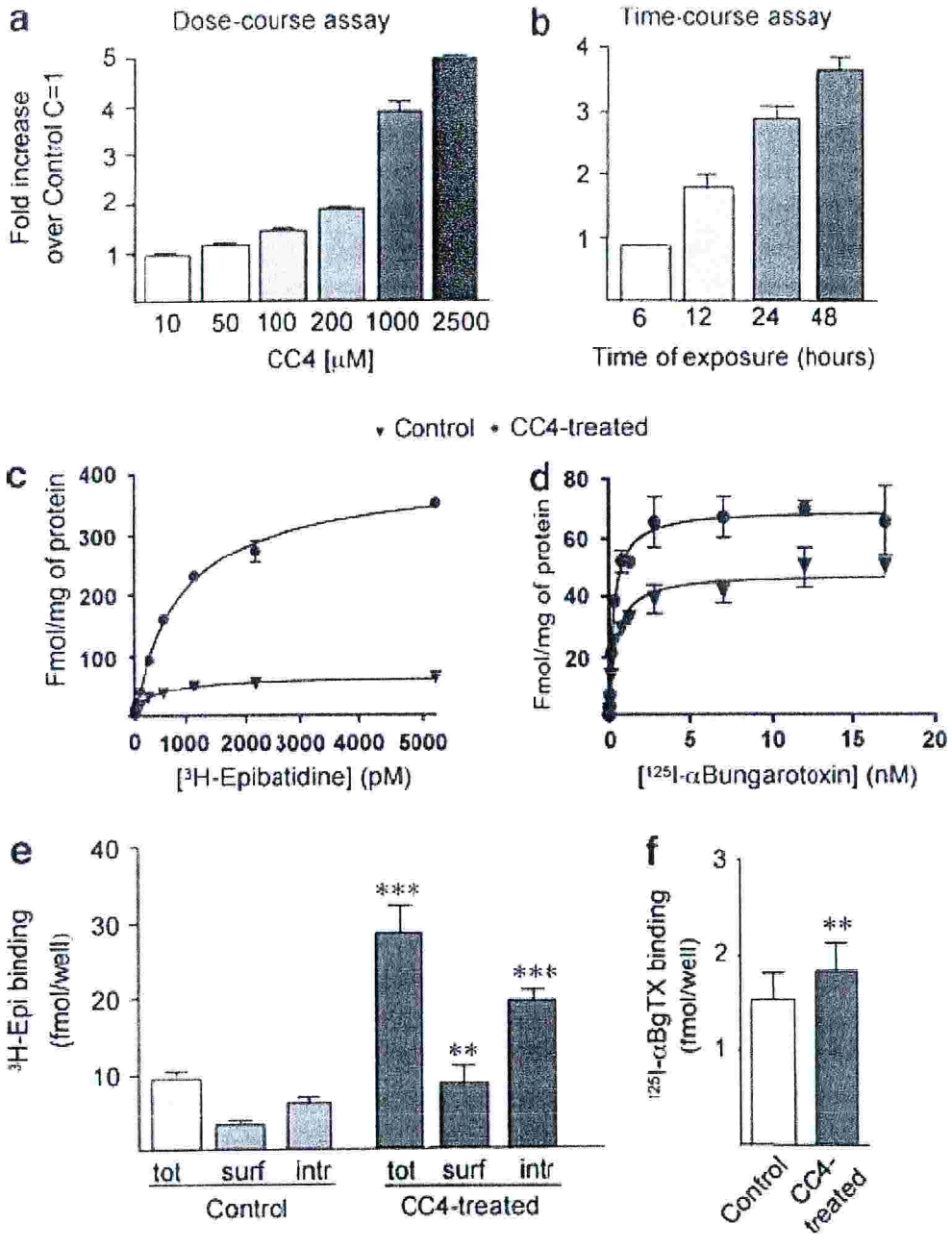

Fig.1. CC4-induced up-regulation of nAChRs in SH-SY5Y cells. Dose (a) and time dependence (b) of CC4-induced up-regulation of ^{3}H-Epi receptors in SH-SY5Y cells. ^{3}H-Epi labelling of nAChRs was measured on membrane homogenates preincubated with 1 mM αBgtx. The data are expressed as fractional increase (mean±s.e.m.) over control (n=3–14 experiments) with the control value being 117.6 ± 18.8 fmol of 3H-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 2nM ^{3}H-Epi (in the presence or absence of 250 nM unlabelled Epi) in the absence or presence of carbamylcholine (5mM 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-aBgtx to surface receptors expressed as fmol/well was measured using 8 nM ^{125}I-αBgTx (in the presence or absence of 1 mM unlabelled aBgTx). Paired t-test **P<0.001.

The saturation binding curves of control and CC4 treated cells were also analysed (Fig. 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, competition binding experiments on intact cells were performed, 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 ± 2.6%; (n=11), whereas at the same concentration it displaced (Ki 1.6 μM) almost the whole fraction of ^{3}H-Epi binding in cell homogenates (90 ± 3%; n=5), suggesting that >50% of nAChRs were intracellular.

Using the carbamylcholine displacement method, 8.7 ± 0.6 fmol (mean±SE, n=10) of ^{3}H-Epi receptors/well were found in control cells (3.1±0.4 fmol on the cell surface and 5.6±0.5 in the intracellular pool; Fig 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.

^{3}H-Epi receptor binding up-regulation induced by other nicotinic drugs at 1 mM concentration was also investigated (see Table 1): nicotine and cytisine strongly up-

regulated ($P<0.0001$) membrane-bound and intracellular receptors, mecamylamine only slightly increased their expression ($P<0.05$), whereas carbamylcholine, d-tubocurarine, dihydrobetaerythroidine, 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 up-regulation 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 versus 1.21 ± 0.19 fold for cells treated with 100 μM CC4 plus 1.5 mM d-tubocurarine (n=5).

Up-regulation of ^{125}I-αBungarotoxin receptors

Like the case of ^{3}H-Epi sensitive receptors, the dose-dependent effect of CC4 on αBgtx receptors in SH-SY5Y homogenates (Bmax=68.4 ± 18 fmol/mg of protein) was first determined; the highest up-regulation was obtained with 1 mM CC4 (Bmax= 82.9 ± 18 fmol/mg 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 Bmax values with no change in Kd values (Fig 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; Fig 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 hours to 1mM 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 non incubated and 48 hour-incubated samples, indicating that the up-regulation 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 Kd value of was 117 pM (CV 17%) with Bmax=493 ± 66 fmol/mg of protein, against an apparent Kd value of 86 pM (29%) and Bmax=426 ± 83.6 fmol/mg of protein for the CC4-treated cells: these differences were not statistically significant.

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

We tested whether the CC4-evoked up-regulation 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 α3 mRNA, 1.1 ± 0.1 for β2 mRNA, and 0.98 ±0.1 for β4 mRNA. These results indicate that CC4 did not up-regulate the steady-state amounts of mRNA for the α3, β2 and β4 subunits, and that up-regulation 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 up-regulation of the α4β2 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 up-regulation 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 hour 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 hours, respectively, n=3).

While the protein synthesis inhibitor puromycin decreases nAChR expression by bovine chromaffin cells (Free *et al.*, 2005), the protein synthesis inhibitor cycloexamide 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 Epibatidine 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 Fig. 2A, nearly all receptors contained the α3 subunit, the large majority contained the β4 subunit, and a smaller fraction contained β2 and/or α5 subunits. There were no receptors containing the α4, α2, or α6 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

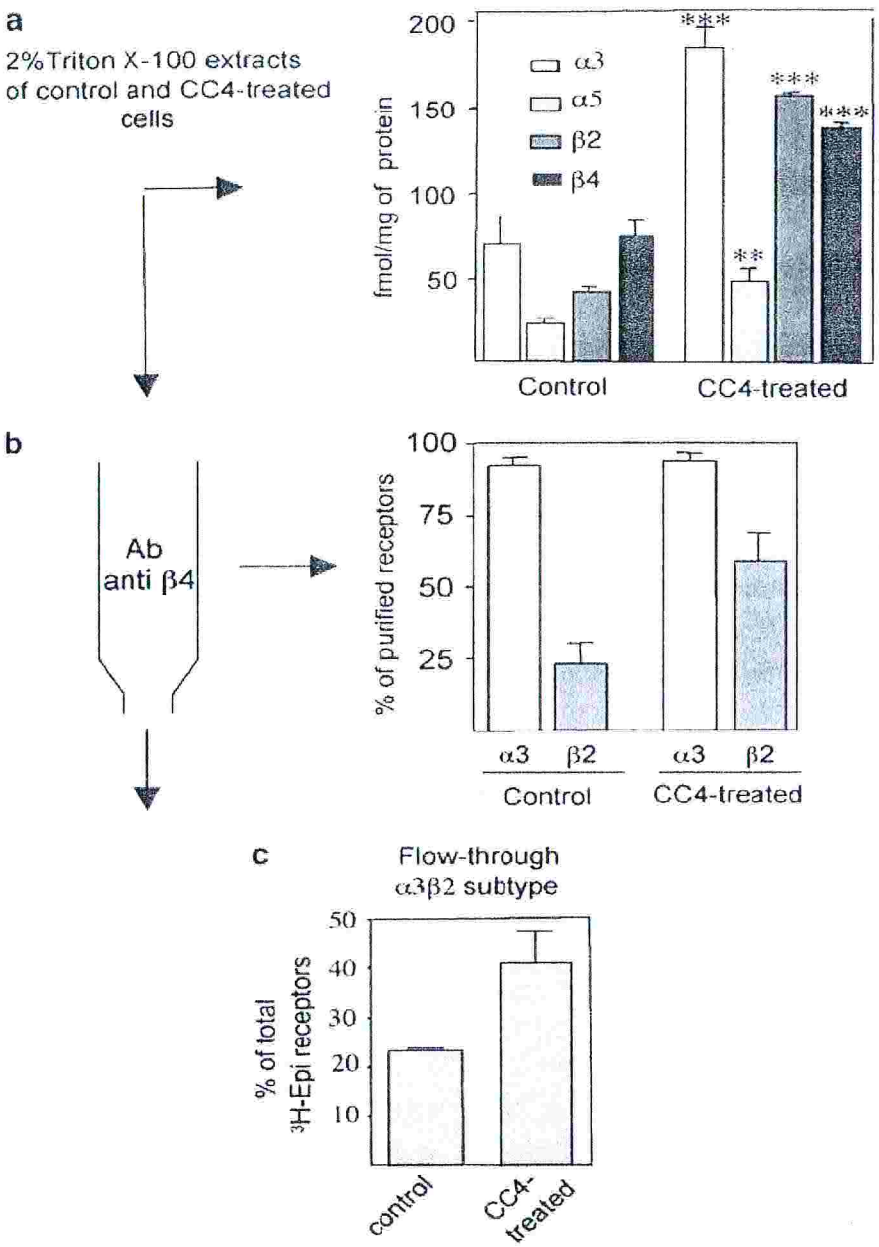

Fig. 2. Immunoprecipitation of ^{3}H-Epi-labelled receptors and receptor subtype immunopurification from control and CC4-treated SH-SY5Y cell extracts. (a) An aliquot of the extracts was labelled with 2 nM ^{3}H-Epi and incubated overnight with a saturating concentration of affinity-purified IgG. The results of immunoprecipitation are expressed as mean±s.e.m. of fmol ^{3}H-Epi labelled immunoprecipitated receptors/mg of proteins (n=4). The increase in immunoprecipitation for the α3, α5, β2 and β4 subunits in CC4-treated cells were statistically significative for all four subunits ($P<0.001$). (b) The remaining extracts were incubated three times with 5ml of Sepharose-4B with bound human anti-β4 Abs in order to immunodeplete the β4 containing-subtypes. The bound receptors were eluted by competition with the corresponding β4 peptide, labelled with 2 nM ^{3}H-Epi, and then immunoprecipitated by the indicated subunit-specific Abs. Immunoprecipitation was carried out using saturating (20–30 mg) 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 β4-containing receptors present in the solution before immunoprecipitation. Each data point is the mean value±s.e.m. of three determinations performed in triplicate. (c) Binding and

immunoprecipitation analyses of the flow through of the anti-b4-affinity column revealed the presence of ^{3}HEpi receptors containing the α3 and β2 subunits. This population, respectively, represented 23.470.3% and 41.573.9% 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 (α3β2) population. The results are the mean value±s.e.m. of three determinations.

the α3 (from 69.9 ± 15.4 to 185.3 ± 11.6, P<0.0001), α5 (from 22.0 ± 3.5 to 47.8 ± 8.6, P<0.0001), β2 (from 41.6 ± 1.1 to 156.7 ± 3.1, P<0.0001) and β4 (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 immunoimmobilized on anti-β2 and anti-β4 Abs bound to microplates. In control cells, the β2-containing receptors had a Kd of 41 pM (CV 27%) and the β4- containing receptors had an affinity of 130 pM (CV 21%; n=3). In CC4 treated cells the β2-containing receptors had a Kd of 16.5 pM (CV 33%) and the β4-containing receptors had an affinity of 45 pM (CV 41%; n=3).

We then used immunopurification studies to identify the subtypes of the receptors up-regulated 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 (Fig. 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 ± 8 % of purified receptors in control cells, and rising to 59 ± 9% in CC4-treated cells (n=3, P <0.05) (Fig. 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 α3β2 subtype (23.4 ± 0.3% of ^{3}H-Epi sensitive receptors in the extracts from control cells growing to 41.5 ± 3.9% in the CC4-treated cells, n=3, P <0.05, Fig. 2C). Saturation binding experiments performed on these flow-through receptors (containing only the α3β2 subtype) immunoimmobilized on anti-β2 Abs gave ^{125}I-Epi Kd 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 (α3β2 and α3β2β4).

Furthermore, binding experiments performed on immunoimmobilized subtypes confirmed that receptor populations with different affinities for ^{125}I-Epi were present in control and CC4 treated cells. In particular, the α3β2 subtype present in the flow-through of the 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 α3β2β4 receptor subtype (immuno-immobilized by anti-β2 Abs) present in a variable ratio with the α3β2 subtype in control and CC4 treated cells.

The presence of α3β2β4 subunits immunoimmobilized 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.

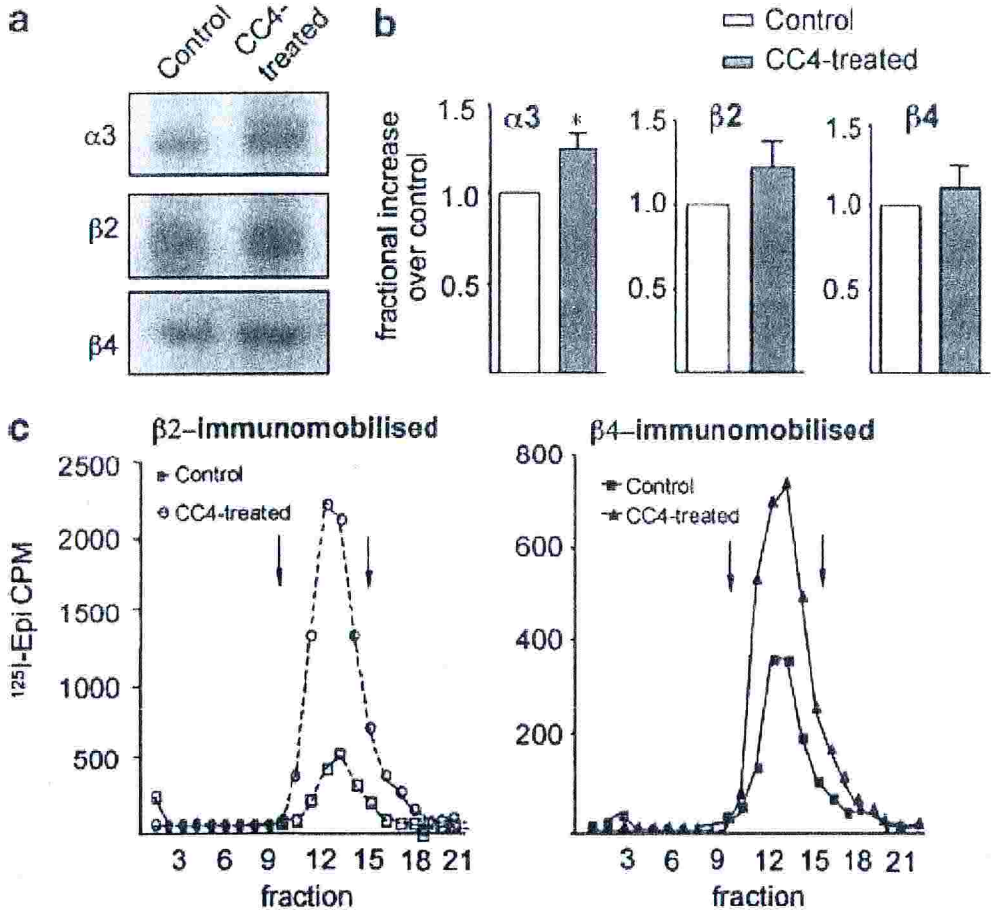

Fig.3. Western blot analysis and quantification of extracts from control and CC4-treated cells (a, b) and sucrose gradient analysis of the β2 * and β4 *-containing ^{3}H-receptors present in the extracts of control and CC4-treated cells (c). (a) Of extracts 20 μg were separated on 9% acrylamide SDS gel, electrotransferred to nitrocellulose, and probed with 10 μg/ml 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±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 1mM PMFS, and centrifuged for 14 h at 40,000 r.p.m. in a Beckman rotor at 41°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 a standard, ^{125}I-aBgtx labelled Torpedo AChRs were subjected to sucrose gradient centrifugation in parallel, the fractions were collected, and the radioactivity determined by g counting. The arrows indicate the position of the Torpedo monomer and dimer in each gradient.

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 Fig. 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 (Fig. 3B).

Sucrose gradient analysis of ^{3}H-Epibatidine 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 Fig. 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 α3β2β4 subtype.