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XXV CYCLE

NICOTINE-INDUCED SUBUNIT STOICHIOMETRY AFFECTS THE STABILITY AND THE INTRACELLULAR TRAFFICKING OF THE α3β4 NICOTINIC RECEPTOR

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ABBREVIATIONS

A- AChBP: Aplysia ACh binding protein

aa: amminoacid

ACh: acetylcholine

AChBP: ACh binding protein

BEC: bronchial epithelial cell

CFTR: cystic fibrosis transmembrane regulator

CNS: central nervous system

COP II: coat protein complex II

ECD: extracellular domain

ELIC: pLGIC *Erwinia chrysanthemi*

ER: endoplasmic reticulum

ERAD: ER-associated proteasome degradation

ERES: ER exit site

GARAP: GABA-$_A$ receptor-associated protein

GLIC: pLGIC *Gloeobacter violaceus*

GlyR: glycine receptor

IND: intracellular domain

L- AChBP: Lymnea stagnalis ACh binding protein

LBD: ligand-binding domain

pLGIC: pentameric ligand gated ion channel

mAChR: muscarinic acethilcholine receptor

MLA: methyllycaconitine

nAChR: nicotinic acethilcholine receptor

NMJ: neuromuscular junction

pLGIC: procariotic ligand gated ion channel

PNS: peripheral nervous system

PRIP: Phospholipase-C-related catalytically inactive proteins

TMD: transmembrane domain

UPS: ubiquitin-proteasome systems

α-Btgx: α-bungarotoxin

α-Cbtx: α-cobratoxin

α-Ctxs: α-conotoxins

ABSTRACT

Nicotine –induced subunit stoichiometry affects the stability and the intracellular trafficking of the α3β4 nicotinic receptor

The α3β4 is a heteromeric nicotinic acetylcholine receptor (nAChR) implicated in the risk of nicotine dependence, smoking, and lung cancer. Little is known about the effect of nicotine on this subtype, so to study it, the α3β4 subunits were expressed in epithelial Hela and neuroblastoma SH-SY5Y cells: in both cells the receptor is very inefficiently exported from the Endoplasmic Reticulum (ER) to plasmamembrane, indicating that a stringent quality control is present at the level of ER to avoid the exit of unfolded/misassembled protein. In this project, It has been demonstrated that nicotine, in a dose dependent manner, is able to up-regulate the newly-synthesized receptor and to increase the percentage of receptor at the plasmamembrane. Furthermore, It has been revealed that an ER export motif (LFM) present in the M3-M4 intracellular loop of the β4 subunit is necessary for ER export of the receptor. The presence of nicotine during receptor synthesis facilitates the assembly of a pentameric receptor with a (α3)2(β4)3 stoichiometry. The effect of nicotine is double:1) on receptor degradation, that is reduced, and 2) on receptor trafficking,that it is increased, through favoring the assembly of (α3)2(β4)3 receptors. The same effect is obtained with other nicotinic agonists and/or antagonists providing that they are cell permeable and, like nicotine, bind the orthosteric binding site. The receptor with this stoichiometry is less prone to proteasome degradation and thanks to the presence of more export signals can more easily exit from the ER. To confirm the importance of the orthosteric binding site for the nicotine upregulation, the amino acid tryptophan, located in the binding site of the α3 subunit sequence, was mutated to alanine. This mutated α3*β4 nAChR is no more up-regulated by nicotine but is efficiently exported to plasmamembrane, suggesting a gain of function effect of the mutation.

1 INTRODUCTION

The cholinergic system is one of the most important and filogenetically oldest nervous pathway. The neurotransmitter Acetylcholine (ACh) is the neurotransmitter that is synthesised, stored and released by cholinergic neurons, and exerts its effects on the central nervous system (CNS) and peripheral nervous system (PNS) through two distinct types of receptor: the muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively). The five mAChR subtypes M1-M5 belong to the superfamily of G-protein-coupled receptors and mediate the slow metabolic responses to ACh via coupling to second messenger cascades, whereas the nAChRs are ligand-gated ion channels that mediate the fast synaptic transmission of the neurotransmitter. The nAChRs are involved in a wide range of physiological and pathophysiological processes: e.g. the post synaptic nAChR present at the neuromuscular junction has a key role in the electrical transmission responsible for the skeletal muscle tone. nAChRs are also widely expressed in the brain, both at presynaptic and postsynaptic densities, where they are involved in a number of processes connected to cognitive functions, learning and memory, arousal, reward, motor control, and analgesia. nAChRs are also the target of nicotine, the most diffuse drug of abuse. The different behavioral effects elicited by nicotine depends on the different neuronal pathway involved that express different subtypes of nicotinic acetylcholine receptors. The nicotine effects in the CNS are not only due to receptor activation but also to the receptor desensitisation, indeed, a fundamental property of nAChRs is their susceptibility to desensitization, inactivation and channel opening. The equilibrium between all these processes was originally described by Changeux and colleagues who proposed an allosteric model of nAChR states, that has been refined for different nAChR subtypes using experimental measurements of the rate constants for the transition between these states. The balance between nicotine-mediated activation and desensitisation of specific subtypes of nAChRs can influence the functional and behavioral responses to nicotine exposure.

1.1 THE CYS-LOOP RECEPTORS SUPERFAMILY: NACHRS

nAChR is a pentameric ligand gated ion channel (pLGIC) that show high permeability to cation. pLGIC, that comprises trimeric cation channels opened by ATP, is a large family of

ionic channels that comprises three families: the Cys-loop receptor family, the GluR gene family (that comprises AMPA, kainite and NMDA receptors) and P2X family.

The best well characterized family is the Cys-loop ion channel receptor family that include neuronal nAChRs, muscle-type nAChRs, $GABA_A$, glycine and serotonin ($5HT_3$) receptors. All of them share a lot of common features in their structure, such as five identical or different subunits, arranged around the central axis that makes the ion channel (organized symmetrically in the case of homo-pentamers or pseudo-symmetrically in the case of hetero-pentameters). The polypeptide chain of each subunit shows a similar organisation: a long N-terminal extracellular fragment that consists of about 200 amino acid residues, four transmembrane fragments (TM1-TM4), long cytoplasmic loop of 100 residues or more between the fragments TM3 and TM4 and a short C-terminal fragment at the extracellular surface. All five subunits together contribute with their N-terminal fragments to make a ligand-binding domain (LBD) with sites for agonists and competitive antagonists. Cys-loop receptors take their name from a 13 amino acid loop within the extracellular domain that is enclosed by a pair of disulfide bonded cysteine (Cys) residues (corresponding to Cys 128 and 142 of the α_1 subunit of the muscle-type nAChR**).** The others important receptor domains are the transmembrane domain (TMD) and the intracellular domain (IND): the TMD is made up by all TM1-TM4 fragments of five subunits, in particular five transmembrane fragments TM2 (one from each subunit) line the channel. The IND is made up by the five long cytoplasmic loops together (Tsetlin V, 2011).

1.1.1 MOLECULAR STRUCTURE OF NACHRS

Our knowledge of the nAChR structure and in more general of the Cys-loop receptors, has been obtained using different approaches in different systems (Albuquerque EX, 2009). In particular, most information have been obtained by studying : A) detailed electron microscopy analyses of the muscle-type nicotinic receptors, which are easily purified in large quantities from the electric organs of Torpedo; B) X-ray crystallography of ACh binding proteins (AChBPs), pentameric soluble proteins that are homologous to the extracellular domain (ECD) of nAChRs identified in various snail species. C) X-ray crystallography of bacterial pentameric procariotic ligand gated ion channels (pLGICs) obtained from the proteobacteria *Erwinia chrysanthemi* (ELIC) and cyanobacteria *Gloeobacter violaceus* (GLIC) (Brejc K, 2001).

Studies of the Torpedo nAChR in its different channel states (closed and open states) give us information about size and shape of nAChRs, the location of the ligand-binding sites, and the organization of the ion channel (Unwin, N,1995).

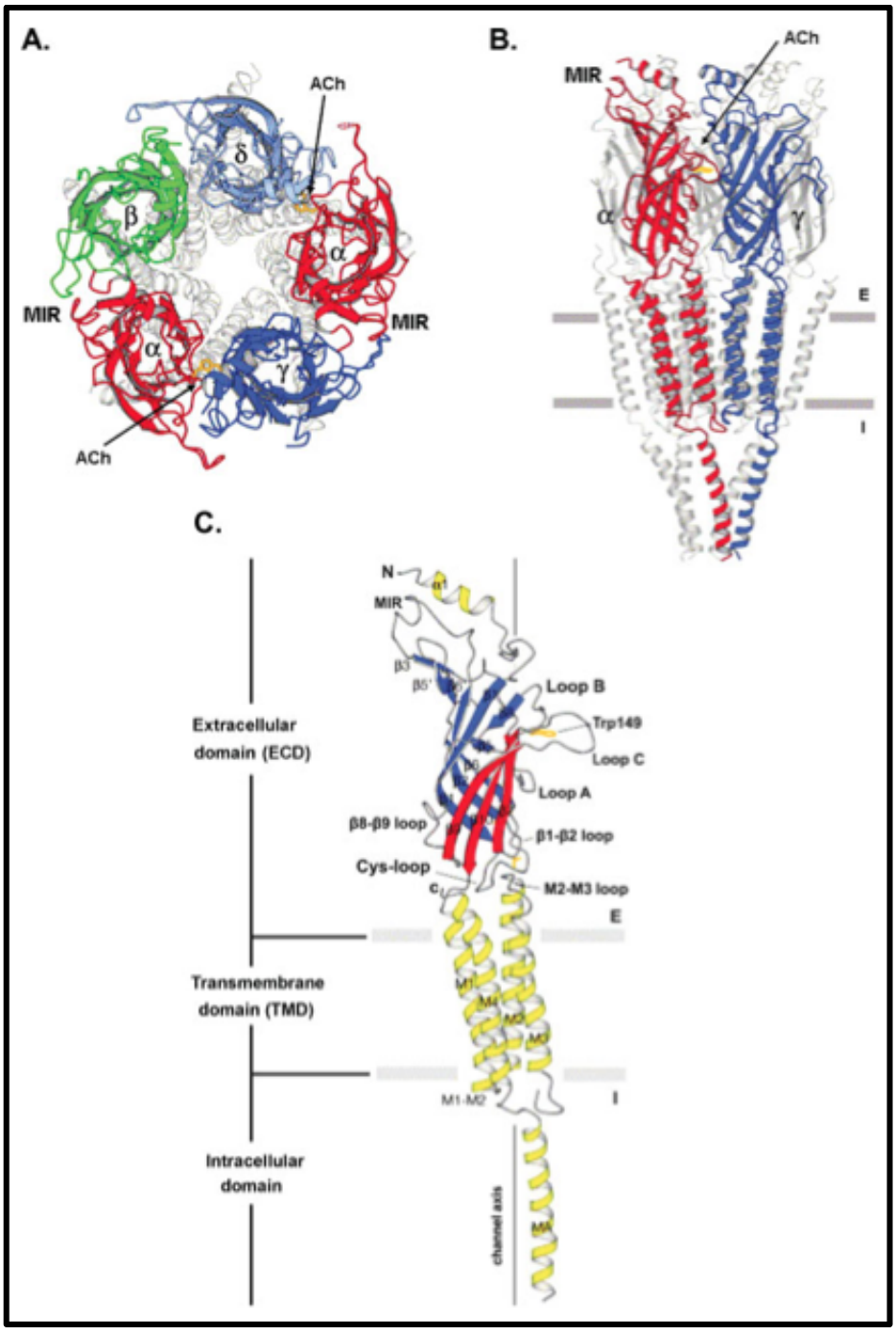

Fig. 1 Electron microscopy structure of the Torpedo nAChR.

Ribbon diagrams of the whole nAChR as viewed (A) from the synaptic cleft and (B) parallel to the membrane plane. The locations of the ACh-binding sites, the side chain of the highly conserved aTrp149 residue (gold) in the principal side of the ligand-binding site, and the MIR epitope of the α subunit are shown. (C) Ribbon diagram of the side-view of the α subunit in an orientation with the central axis of the pentamer at the back. Loops A, B, and C form the principal side of the ACh-binding site, and $\beta1$–$\beta2$ loop and the Cys-loop are in contact with the M2–M3 loop of the TMD, thus coupling the conformational changes occurring in the ECD on binding of ACh or other nAChR

agonists to the TMD. (Cell membrane: between the horizontal bars; E: extracellular; I: intracellular) (from Zouridakis M, 2009).

Furthermore, studies in atomic detail of *Lymnaea stagnalis*, *Bulinus truncates* snails, and the saltwater mollusc *Aplysia californica* provide information about the receptor binding sites: the AChBP is a soluble homo-pentamer made by five subunits, each subunit is 210 amino acid long and aligns well with the ECD of the pentameric ligand gate ion channels (pLGICs). Like the nAChR, AChBP assembles into a homo-pentamer, unlike the nAChR, however, it lacks the domain that forms the transmembrane ion channel, so any comparison made between AChBP and nAChR implicates only the ECD of the nAChR. AChBP differs from the nAChR also for the function, as it plays a modulatory role in molluscan synaptic transmission: in the molluscan synaptic cleft, in response to active neurotransmission, there is an increased concentration of the presynaptically ACh release. ACh binds to nAChRs present on the perisynaptic glial cells and determine the secretion of AChBP in the synaptic cleft where this soluble protein captures ACh molecules, therefore decreasing or terminating synaptic transmission. So far, no orthologs of AChBP have been found in other animal phyla (Sixma, T, 2003).

1.1.1.1 EXTRACELLULAR DOMAIN (ECD)

The ECD contains the ACh-binding site: two separate parts are involved in the formation, one is called “principal component” or plus side, the other is called the “complementary component” or minus side. Every component is made by several loop regions that are critical for receptor function: loops A, B and C of the α subunit (principal component) and loops D, E and F of the adjacent non-α subunit. The residues of neighbouring subunits in the ECD are oppositely charged, in particular the inner wall of the extracellular vestibule is negatively charged. This promotes a cation-stabilising environment and allows ions flow thanks to the electrochemical gradient when the channel is opened (Corringer PJ, 2000).

1.1.1.2 TRANSMEMBRANE DOMAIN (TMD)

The four membrane-spanning α–helical domains (TM1-TM4) of each subunit contribute to the formation of the TMD. The TM2 domains form the central conduction channel with residues charged that create rings with chemically distinct properties: among them, the

extracellular ring is negatively charged and influence ion transport when the channel is open by facilitating the transport of cations at the extracellular entry of the channel. When the channel is closed, the TM2 residues are tilted inward the pore: this creates a tight hydrophobic girdle that represents the gate of the channel and functions as an energetic barrier to ion permeation. The TM1-TM3 and TM4 domains form an outer ring that preserve the inner ring of the TM2 domains from the membrane lipids (Gotti C, 2004).

1.1.1.3 INTRACELLULAR DOMAIN (IND)

The IND is made up by the large cytoplasmic loop between TM3-TM4 and its length differs among the different subunits (from 110 to 270 aa). The aa of all subunits in the IND, aa that are negatively charged, shape the wall of the pore and together with the aa of the extracellular and intracellular vestibules determine the charge and size of the "selectively filter" of the nAChR channel, facilitating the transport of small cations and preventing the flow of anions and large ions. The IND has also other import roles: it affects the assembly of subunits, the electrophysiological properties of nAChRs, the regulation of nAChR trafficking and interactions with the cytoskeleton, which induce nAChR clustering, through phosphorylation in different sites.

1.1.1.4 GLYCINE, GABA-$_A$ AND OTHER MAMMALIAN CYS-LOOP RECEPTORS

AChBP is not only an excellent LBD model for all nAChR subtypes, but may play a similar role of model for other Cys-loop receptors (Grudzinska J. 2005; Harrison NJ, 2006).
Alignment between the amino acid sequences of the binding sites of ACh in AChBPs with those of neurotransmitter binding in Cys-loop receptors showed a 15-20% sequence homology with $GABA_AR$, Gly-R and 5HT-3, and 25% and homology with nAChR (**Fig. 2**). Almost all of the conserved residues in the members of the Cys-loop receptor family are present in AChBPs, including those that are important for ligand binding. This led to the hypothesis, and subsequent demonstration, that ligand binding sites have a common structure. The binding site of the neurotransmitter (ACh, GABA, glycine and serotonin) in Cys-loop receptors lies at the interface between two adjacent subunits, one of which provides the principal component and the other the complementary component, as mentioned before.

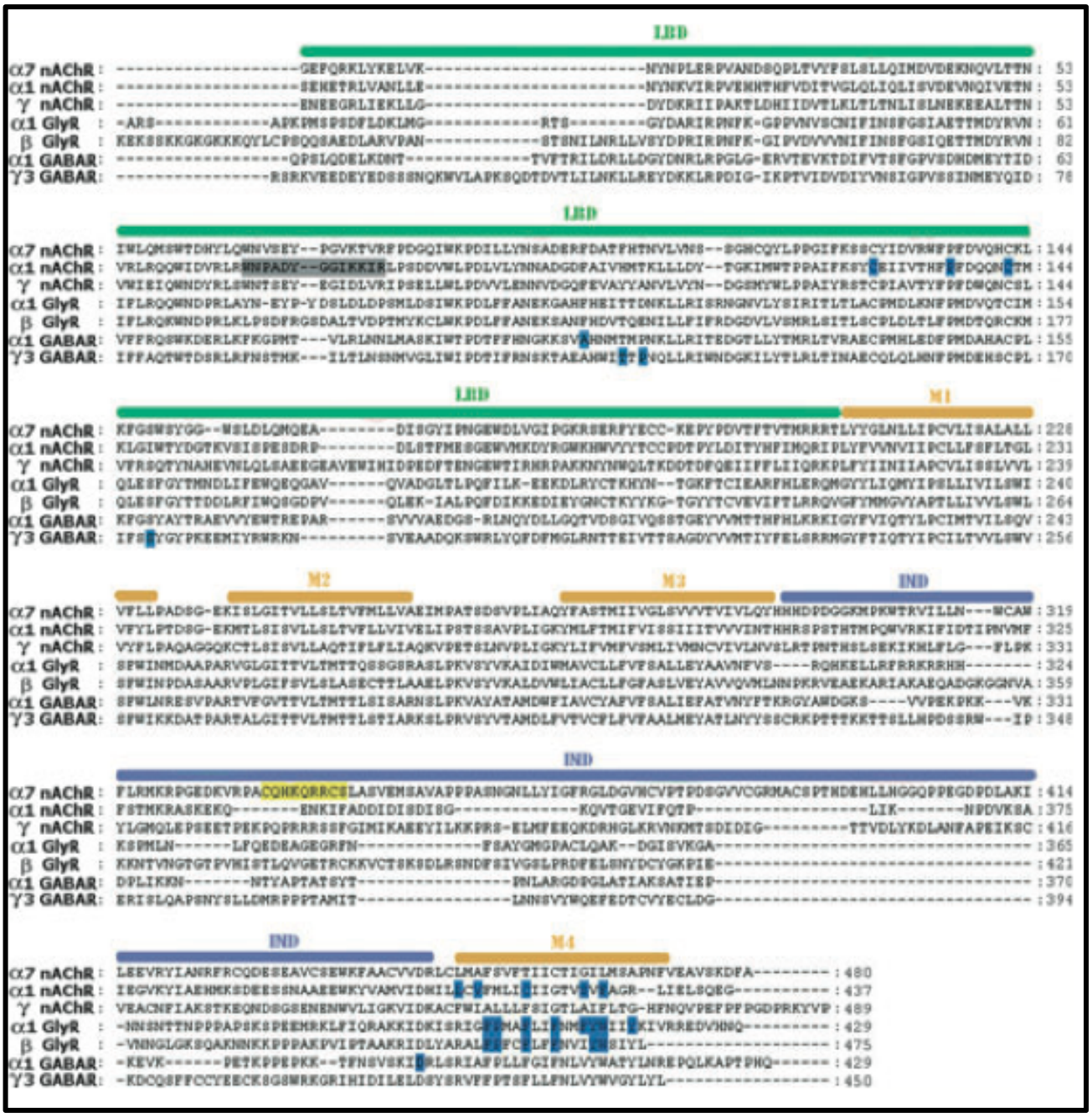

Fig. 2 Alignment of the subunit sequences for several representatives of the Cys-loop receptors.

Some of the residues important for protein folding and clustering are labeled in blue; those important for function in yellow; main immunogenic region (MIR) is labeled in gray (from Tsalin V, 2011)

Furthermore, the rings of leucines in position 251 and valines in position 255 in the TMD2 restrict the channel by forming a hydrophobic girdle that does not allow the flow of hydrated ions. The ring of leucines is conserved in all members of the Cys-loop family, and the replacement of leucine with hydrophilic residues induces in the various receptors to open more easily after neurotransmitter binding, thus indicating that the presence of this

ring stabilizes the closed state and explaining why the principles of assembly and in particular the role of LBD, IND and TMD are in general very similar for the whole family of Cys-loop receptors. Differently, we do not have yet crystals of the extracellular domains of homomeric or heteromeric GlyR due to the protein aggregates formation. However, we know that, similarly to nAChRs, homo-pentameric GlyRs can be formed by α subunits, whereas the β subunits only assemble with α ones. GlyRs, GABA-A or 5HT-3 serotonin receptors share other features with nAChRs, as oligomerization and N-glycosylation, that are important for all these receptors for their transport from the Endoplasmic Reticulum (ER) to the Golgi (Kuhse J, 1993; Thompson AJ, 2006).

1.1.1.5 AGONIST LIGANDS

Information of interactions between agonists and nAChR at the atomic scale is provided by studies of the crystal structures of the Lymnaea stagnalis AChBP (L-AChBP) complexed with nicotine or carbamylcholine and the Aplysia californica AChBP (A-AChBP) complexed with lobeline or epibatidine. Many amino acid residues of loops A, B and C (the principal component) and D, E and F (the complementary component) contribute to the ACh binding site. Inside the compact structure of AChBP, in the middle of the interface of two adjacent subunits in the LBD, loops A, B, D and F provide a hydrophobic pocket in which agonists bind, pocket that is closed by loop C.

So in all agonist-bound AChBP structures, the agonist is fully enveloped by the protein, through chemical bounds among residues like hydrogen bonds, π-cation, dipole-cation, and van der Waals forces, especially in loop C. In particular, the highly conserved Trp143, located in the principal face of the subunit, makes strong aromatic π-cation interactions with all four agonists. (Tsetlin V, 2011).

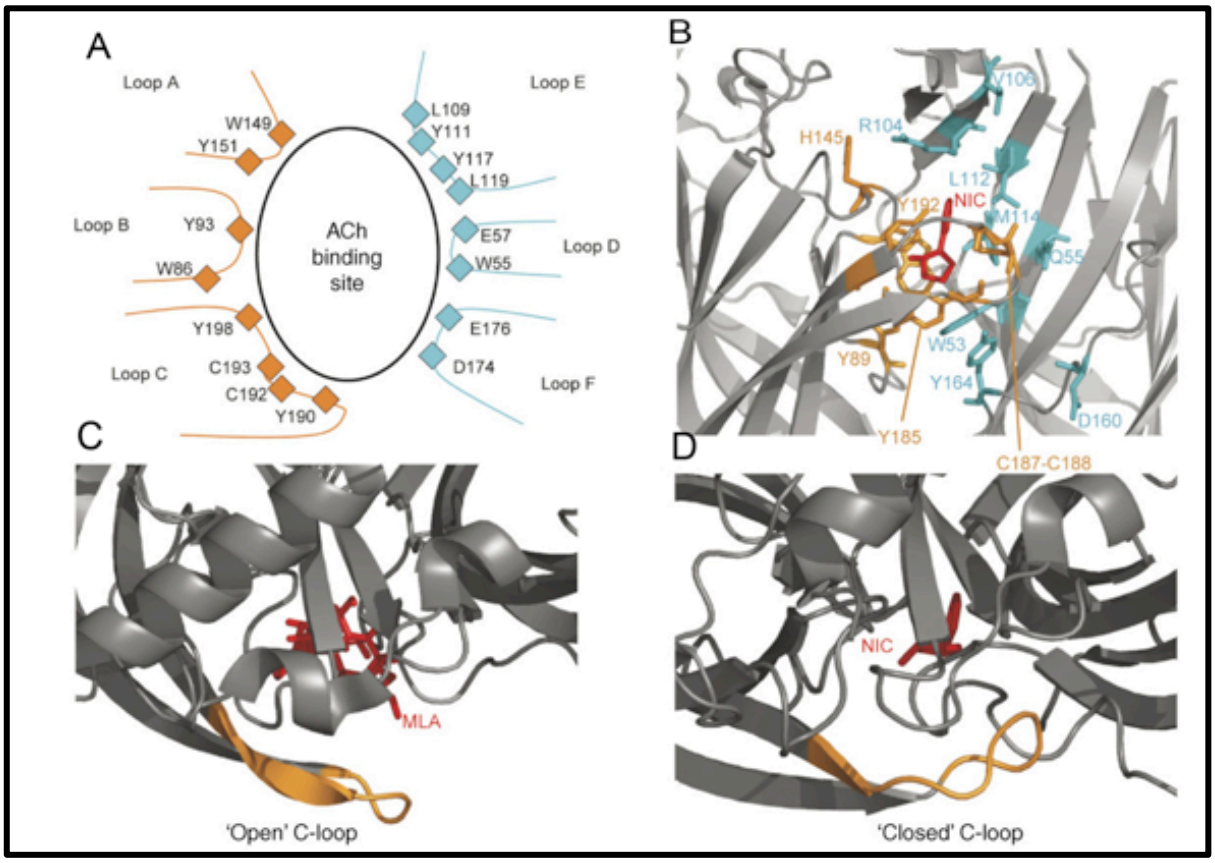

Fig. 3 Structure of the ACh binding site

A) Schematic illustration of the ACh binding site, showing the amino acids in the loops that participate in its formation. Loops A, B, C are provided by the α subunit, and loops D, E, F by the adjacent subunit. B), C) and D) Analyses of the X-ray structure of the binding site of the AChBP co-crystallographed in the presence of nicotine (B and D) and the antagonist *METHYLLYCACONITINE* (MLA) (C). This analysis shows that the agonists bind in a pocket containing the binding site that promotes extensive contact between ligand and protein. In yellow is shown loop C and as in the case of other antagonists, the binding of *METHYLLYCACONITINE* leads to an open conformation of loop C that interferes with receptor activation and channel opening (C).

The aromatic residues and di-sulfide bridge of the ACh binding site are negatively charged: this lead to neutralisation of the positive charge carried by the majority of nicotinic ligands. The binding of ACh therefore gives rise to a non-covalent interaction between the cation and the electron-rich π system of the W residue in B loop. Generally, the tertiary or quaternary ammonium ligands of nicotinic ligands bind in the middle of the pocket created by the hydrophobic aromatic residues of loops A, B, C and D. This interaction is highly conserved in the family of Cys-loop receptors, although the loops involved, differ among receptors: loop B in the case of AChRs, glycine and serotonin receptors; loop A in the case of $GABA_A$ receptors.

Studies of chimera, obtained by the fusion between AChBPs with the transmembrane regions of the serotonin (5-HT3) receptor, provided information about the role of C loop in receptor activation: this chimera functions as an ACh receptor, shows that ACh binding lead to the closure of loop C and the activation of the receptor.

Furthermore, an electrophysiology study demonstrated that between moving loop C and the opening channel there is a feedback communication. In a modified receptor, mutated to opens spontaneously even in the absence of agonist, the formation of covalent bond between opposite cysteines in the loops C and E, leads to a closed C loop. This study also demonstrated that the binding of one molecule of ACh opens the channel for a short time, whereas the binding of two molecules increase the time opening (Miyazawa A, 2003). It seems also that the closed conformation of C loop induces a state of channel pre-activation and increases the duration of channel opening depending on the number of closed conformation loop C in the receptor. Single-channel analyses of ACh and glycine receptors showed that full agonist binding, easily allows the transition from pre-activation to complete activation of the receptor and the consequent closing of loop C. At the single channel level, it was found that full agonist, such as ACh, at saturating concentrations activates the channels to near 100% open probability whereas partial agonist is less efficacious and evokes less total current than the full agonist. Partial agonists are also called “dualist ligands” because as they bind at the same site and compete with full agonists when they are co-incubated with full agonists, they reduce the response induced by full agonists. Furthermore, partial agonists are less effective in inducing the conformational changes that lead to channel opening in comparison to full agonists.

1.1.1.6 ANTAGONIST LIGANDS

Antagonists are nicotinic ligands with very low efficacy (e.g. <5%) in comparison with full agonists. Antagonists that bind at or near the LBD and compete with agonists, are called competitive antagonists whereas antagonists that bind in other domains of the nAChR and block channel function are called non-competitive antagonists.

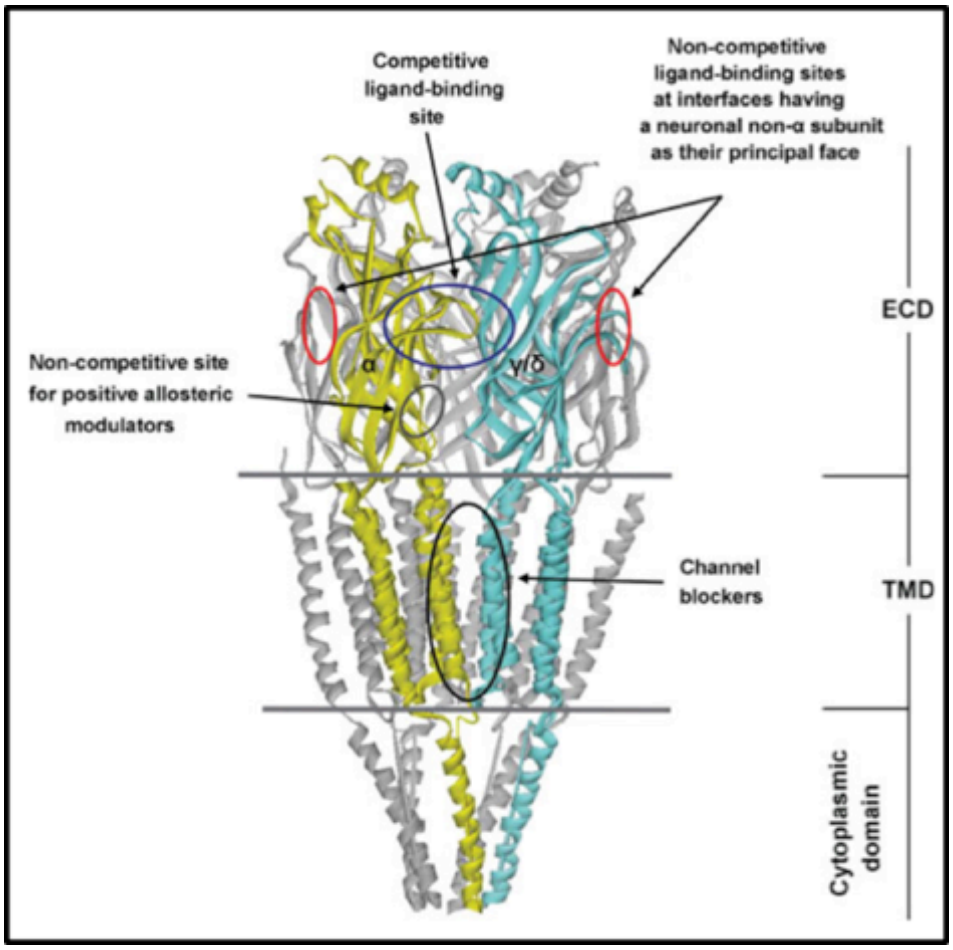

Fig.4 Schematic representation of the location of competitive and noncompetitive ligand-binding sites in nAChRs

The red ovals indicate the positions of noncompetitive (allosteric) ligand-binding sites on heteromeric neuronal nAChRs, in which a non-α neuronal subunit takes the place of the χ or δ subunit. Competitive ligand-binding sites (blue ovals) are also formed at the interfaces between some neuronal α subunits (in homomeric α7, α8, and α9 nAChRs) and between several neuronal α (α2–4 or α6) and β subunits (β2 or β4) in heteromeric neuronal nAChRs (from Tsetlin V, 2011).

Competitive antagonists, like snake neurotoxins and snail conotoxins, have been used for the isolation and biochemical characterization of nAChRs. Also the use of synthetic peptides and studies of the crystal structures of the complexes of L-AChBP, A-AChBP, mouse nAChR α1-ECD and A-AChBP with respectively α-cobratoxin (α-Cbtx), α-conotoxins (α-Ctxs), α-bungarotoxin (α-Btgx), and with the small alkaloid antagonist methyllycaconitine (MLA) identified at the atomic level the residues involved in toxin/antagonist binding and allowed to know the properties and pharmacology of binding sites in both neuronal and muscle receptors. These structures showed that the binding of competitive antagonists with LBD involved several interactions, especially hydrogen bonds and Van der Waals contacts. This binding involves the principal side with highly conserved

amino acids residues in loop C and the complementary side with the less conserved amino acids, especially those present in loop F. X-ray studies of AChBPs showed that, in the absence of agonist or in the presence of the antagonist, loop C does not cover the hydrophobic pocket. In the presence of the agonist, loop C covers the LBD and assume a closed conformation (Tsetlin V, 2011).

1.1.1.7 ALLOSTERIC LIGANDS

The allosteric ligands comprise all the ligands that, differently from ACh or α–Bgtx, do not bind the orthosteric binding site, but another site called allosteric binding site. They could be either positive or negative allosteric modulators, and respectively increasing or decreasing agonist-induced activity. The allosteric ligand-binding sites have several localisations: in the ion pore, where they may act as channel blockers, or in the extracellular, cytoplasmic and transmembrane domains. X-ray studies of resolved crystal structures of the complexes of the A-AChBP with the positive allosteric nAChR ligand galanthamine and negative allosteric ligand cocaine indicate that the binding does not involved the C loop like for the competitive nAChRs ligands. It is still unknown the amino acid residues that contribute to the allosteric site in neuronal nAChRs, several studies suggest that they are likely located at the interface between α and non α subunits (Tsetlin V, 2011).

The physiological significance of the allosteric sites is still not well understood and surely a lot of allosteric binding sites for endogenous substances are still unknown. Recently, a family of proteins, secreted by neuronal cells, that shares significant structural similarity with the α-Bgtx it has been discovered. Among these proteins Lynx inhibits α4β2 nAChR receptor function by inducing a decrease in ACh affinity and by slowing the recovery from desensitisation.

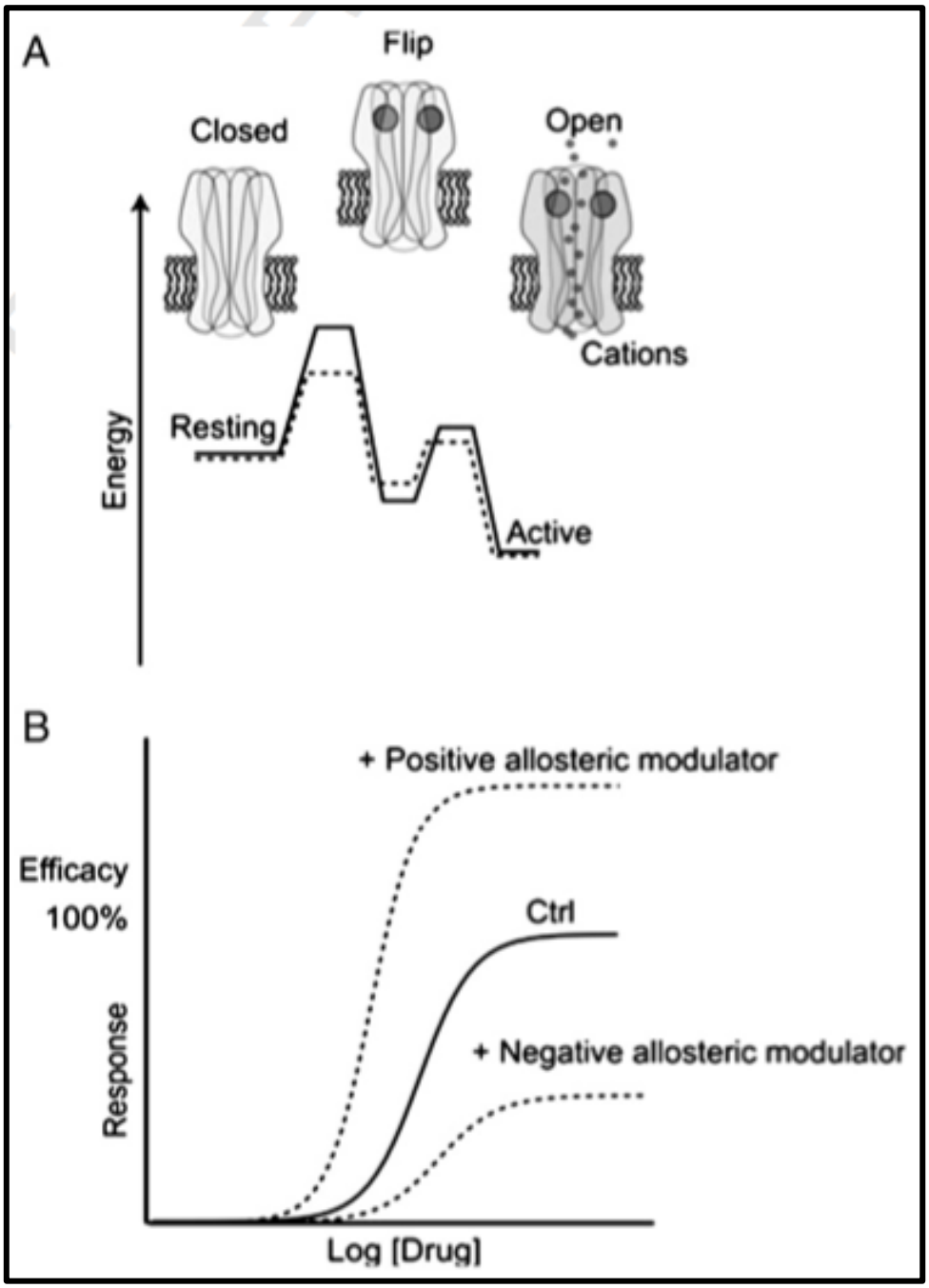

Fig. 5 Allosteric modulators alter the energy associated with transitions between functional states.

Upper panel illustrates the energy barriers and wells associated with conformational change from resting (closed) to open states of the receptor. The dashed line illustrates how a positive allosteric modulator can enhance channel opening by lowering the energy barriers associated with the transition from the resting to the open conformation. The lower panel illustrates the effect of positive or negative allosteric modulator on the concentration response relationship between agonist and channel opening. A positive allosteric modulator reduces the concentration of agonist required to achieve channel opening whereas a negative allosteric modulator will increase the concentration of agonist required for channel activation. In addition, allosteric modulators can increase or decrease the maximum level of channel activity (Hurst R, 2012).

1.1.2 FUNCTIONAL STATES AND BIOPHYSICAL PROPERTIES

Ligand gated ion channel may exist in three different functional states: closed (or resting), open or desensitized. When the receptor is open or desensitized, ligands are bound to the receptor, but in the desensitized state there is no ion flux. When the receptor is closed, no ligands are bound no ion flux is permitted. The equilibrium between the different conformational states of the receptors is influenced by receptor's stoichiometry and by the different ligands (agonist, antagonist, allosteric modulators). nAChRs and other fast ligand-gated ion channels show low affinity for the physiological ligand and after ligand binding, fast transition from closed to the open state. These features are essential for the fast, high frequency signalling of the excitatory synaptic transmission present in autonomic ganglia and at the neuromuscular junction, where agonist must quickly dissociate and interrupt postsynaptic signalling. When receptor is desensitized, it assume a conformation in which agonist is yet bound, its affinity is high (pM - nM) but the ion channel is closed. Desensitization lead to a conformational transition characterized by relatively stable state and influenced by parameters such the type of agonist, the type of α and β subunits present, and the phosphorylation or dephosphorylation state of the receptor. We can distinguish between two types of desensitization: fast (I) or slow (D) desensitisation. Fast desensitisation (I) immediately follows the peak response and has a time-course of milliseconds, slow desensitisation (D) is a progressive reduction of the response, takes place in the time-course of seconds or minutes and is observed in the presence of low agonist concentration.

nAChR channels transit from open to non-conducting state also by the dissociation of the agonist from the receptor, process known as deactivation. The rate of deactivation determines the fraction of channels in the open state: the faster the deactivation rate is, the greater will be the drive from the open state back to the closed resting state. Deactivation is particularly crucial during sustained exposure to low agonist concentrations, when steady-state macroscopic currents must be controlled (Hurst R, 2012)

1.1.3 RECEPTOR STOICHIOMETRY

The nAChRs family consists of receptors that significantly differ for subunit composition, stoichiometry and pharmacological properties. The first classification is made by nicotinic ligands affinity:

1. The αBgtx-insensitive receptors: they bind nicotinic ligands with nM affinity, they could be only heteromeric, made up by a combination of α (α2–α6) and β (β2–β4) subunits.
2. The α-Bgtx-sensitive receptors: they bind nicotinic ligands with μM affinity and α-Bgtx with nM affinity, they could be both homomeric (made up of α7, α8, α9, subunits) and heteromeric (α7–α8, or α9–α10).

The heteromeric receptor has two LBD, at the interface between one α subunit with the primary component and one non α subunit with the complementary component, as mentioned before. The homomeric receptor has five LBD, carried by the five α subunits of the pentameric receptor that contribute with both primary and complementary components.

Heteromeric αBgtx-insensitive receptors could have also subunits that do not directly participate in LBD formation, like the α5 and β3, and for this reason these subunits are called accessory subunits. The presence of an accessory subunit modifies the receptor's pharmacological and biophysical properties, affinity to allosteric modulators and sensitivity to up-regulation. For example the $(\alpha4\beta2)_2\alpha5$ subtype in comparison with the $(\alpha4\beta2)_2\beta2$ subtype, shows higher Ca^{2+} permeability, lower affinity for ACh and nicotine, lower sensitivity to nicotine desensitisation but higher sensitivity to the galantamine allosteric modulation. The effect of α5 subunits is different among different subtypes, for example in α3β2 and α3β4 subtypes, it increase desensitisation, Ca^{2+} permeability and the response to agonist (Brown R, 2007). The β3 subunit assembles preferentially with α6 subunits: in β3 KO mice, the level of α6 is reduced both in the cell bodies and nerve terminals of dopaminergic neurons, demonstrating the dominant negative effect of the β3 subunit and its importance in the nAChR assembly, degradation and trafficking (Gotti C, 2005).

α4β2 subtype

This subtype is the most widespread in the CNS and is composed by the α4 subunit, encoded by gene CHRNA4 on human chromosome 20, and the β2 subunit, encoded by gene CHRNB2 on the human chromosome 1. This receptor could be found in two different stoichiometries that differ not only for the ratio between α/β, but also for pharmacological properties: the $(\alpha4)_2(\beta2)_3$ shows a maximal ACh-evoked current, while $(\alpha4)_2(\beta2)_3$ shows a smaller current but a 65 fold higher affinity for ACh. These results are independent of the expression system, as demonstrated by studies in oocytes injected with α4 and β2 in different ratio (Zwart R,1998) and studies in HEK293 cells transfected with the human α4 and β2 subunits in different ratio (Buisson B, 2001).

α6 complex

This subunit, encoded by the gene CHRNA6 on human chromosome 8 , is abundant in VTA and mesolimbic system. When α6 is assembled with α4 and β2 subunits, this complex shows enhanced ACh sensitivity and lower nicotine-induced desensitization. The importance of α6 subunit is confirmed by studies in KO mice that show alteration in nicotine-induced behaviour (Champtiaux N, 2002) and studies in mice with α6 mutated that display a gain of function feature (Drenan RM, 2008).

α7 subtype

The α7 subunit, encoded by the gene CHRNA7 on human chromosome 15, has a unique gene structural organization with several introns and splicing sites. Its structure displays high similarity with the ancestor genes, preserving specific properties such as high calcium permeability and fast desensitization (Yu CR, 1998). These subunits are known to be able to form a homopentamer, but it is thought to potentially co-assemble with other subunits, such as β2 subunits, even if the exact stoichiometry and the pharmacological properties of this heteromeric receptor remain to be clarified.

α9–10 subtype

The α9 subunit, encoded by the gene CHRNA9 on human chromosome 4, is expressed in the outer hair cells of the inner hear (Elgoyhen AB, 1994) and probably in several areas of CNS where it may play a role in pain modulation (McIntosh JM, 2009). It displays a pharmacological properties somewhere between that of nicotinic and muscarinic receptors

(Elgoyhen AB, 1994), it forms functional hetero-oligomers with the α10 subunits, encoded by the human gene CHRNA10 on chromosome 11.

α3β4 subtype

The α3, β4 with the α5 subunits are encoded by the same gene cluster on chromosome 15 in humans, with the transcription of CHRNB4 and CHRNA3 in the same direction, whereas the transcription of CHRNA5 is in the opposite sense. This cluster is highly conserved throughout evolution, suggesting a close interaction and regulation of these subunits. The receptor stoichiometry of α3β4 and α5 nAChR remains to be elucidated: studies performed using different expression system, such as HEK-293 cells or oocytes transfected with different ratios of α3 and β4 subunits, show that complex stoichiometry of the α3β4 receptors may be present (Krashia P, 2010). It is known from a long time that the α4β2 nAChR may exist in two stoichiometries, which contain either two or three copies of the α4 subunit, that differ in their pharmacological properties: the two-α form is activated at much lower ACh concentrations, has a different sensitivity to agonists and does not display enhancement of submaximal ACh responses in the presence of low Zn^{2+} concentrations. Also for the α3β4 nAChR studies in oocytes has demonstrated that may exist with different stoichiometry: when injections are carried out with equal amounts of α3 and β4 cDNA, the putative two-α form of the pentamer predominates in oocytes, whereas the three-α form is more abundant in HEK cells. The α3β4 nAChR with two α subunits shows both potentiation and inhibition in response to Zn^{2+} whereas only inhibition is observed in the three-α form. Moreover, the two-α form has long-burst and low conductance whereas the three-α form has short-burst and high conductance. So, in conclusion, depending on the α3 to β4 cDNA ratio used, oocytes and HEK293 cells can express receptors with different stoichiometry: two-α form when oocytes are injected with the 1:9 α:β ratio, three-α form when oocytes are injected with the 9:1 α:β ratio (Krashia P, 2010).

1.1.3.1 LOCALISATION OF NATIVE NACHR SUBTYPES

nAChR are widely distributed in the brain and their subunit composition is cell and region specific. The most abundant subtype in the CNS is the α4β2 that account for 90%, whereas the α3β4 one is the major subtype in the PNS. Most of our knowledge about

nAChRs distributions is due to studies in KO and KIn mice with *in situ* hybridisation studies, immuno-precipitation and immuno-purification approaches.

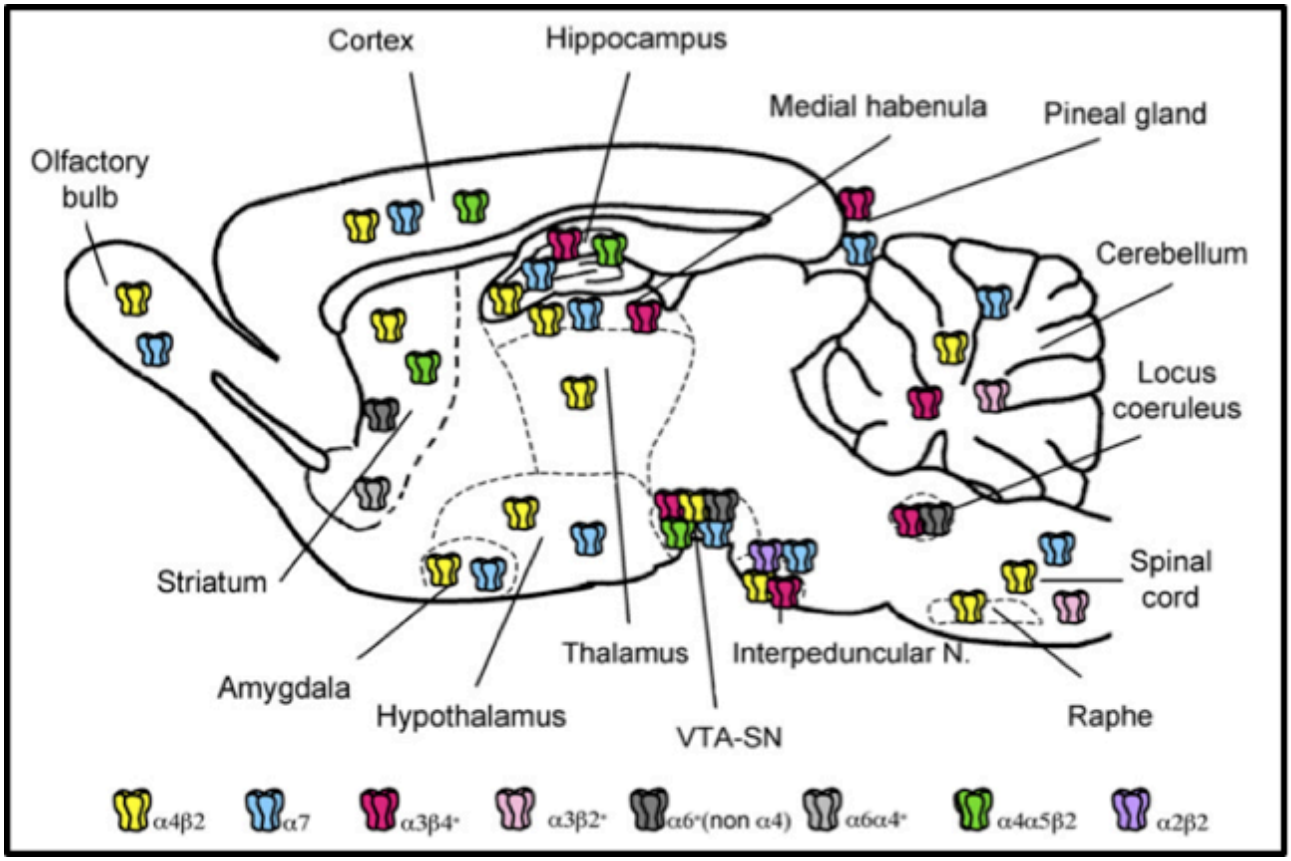

Fig. 6 Summary of the data on nAChR subtype localisation in rodent CNS.

The data shown bring together results of binding, immunoprecipitation and/or immunopurification experiments in the cortex, cerebellum, hippocampus, interpeduncular nucleus, medial habenula and pineal gland, and the results of in situ hybridisation, single-cell PCR or binding studies of the amygdala, hypothalamus, locus coeruleus, olfactory bulb, raphe nuclei, spinal cord, substantia nigra–ventral tegmental area and thalamus obtained from rat and/or WT and/or nAChR subunit Ko mice (from Gaimarri A, 2007).

α4β2 subtype: is the major subtype in the CNS, is localised in the cortex, olfactory bulb, striatum, hippocampus, medial habenula, thalamus, hypothalamus, amygdala, cerebellum, spinal cord, interpeduncular nuclei, and Ventral tegmental Area (VTA)-Substantia Nigra (SN).

α6 complex: when associated with α4 subunits, is found in the mesostriatal and visual pathways when associated with non α4 subunits, is found in striatum, VTA-SN and locus coeruleus. The α6 nAChR are expressed at higher concentration on nerve terminals than in the cell body/dendrite compartment

α2β2 subtype: is localised in the interpeduncular nucleus

α7 subtype: is the second most abundant nAChR in the CNS, it is localised in the olfactory bulb, cortex, hippocampus, hypothalamus, amygdala, VTA-SN, interpeduncular nucleus, spinal cord, pineal gland and cerebellum

α8 subtype: is present only in the avian CNS as homomeric receptor or heteromeric receptor with α7 subtype

α9–10 subtype: is localised in the cochlea, dorsal root ganglia, the pars tuberalis of the pituarity gland, lymphocytes and keratinocytes

α3β4 subtype: is the major subtype in the autonomic ganglia and adrenal medulla as well as in medial habenula, pineal gland and retina. In particular, autoradiography analyses find α3β4 in the pathways that link the habenula to the interpeduncular nucleus that likely is connect with the limbic system (Bianco IH, 2009). The α3 subunits are expressed in all autonomic ganglia with other subunits such as α5, α7, β2 and β4. The importance of α3 subunits in autonomic ganglia is demonstrated by studies in α3 KO mice. These mice die early after birth or within 6-8 weeks. Postganglionic axotomomy of adult rat produces a decrease of more than 80% in ganglion nAChR mRNA, including α3 subunit mRNA and protein and chick treated with α3 antisense oligonucleotides shows a significantly decrease of 80% of the ACh-induced current (Wang N, 1998). Even if other subunits, such as α5, α7 and β4 subunits are normally present in nAChRs of autonomic ganglia, KO mice lacking each of these subunits grow normally and without any significant physical, neurological or autonomic deficit. The expression of α3β4 in oocytes resemble those from native autonomic ganglia in macroscopic pharmacology but they differ significantly at single-channel level: they show much longer burst than native receptors. The α3β4 receptors are found not only in central and peripheral nervous system, but also in lung tissue. Indeed, developing lung expresses multiple nAChR subtypes in airway epithelial cells, airways fibroblasts and pulmonary type II cells that form an intrinsic, non-neuronal cholinergic paracrine signalling system. Monkey bronchial epithelial cells (BECs) synthesize and secrete ACh, choline acetyltransferase, the vescicular ACh transporter and different subunits such as the α3 and β4 nAChR subunits, measured by RT-PCR (Fu XW, 2008).

nAChRs have both a presynaptic and postsynaptic localisation. When they are present on presynaptic membranes, their activation is involved in the modulation of the release of several neurotransmitters such as ACh, noradrenaline, dopamine, glutamate, and GABA; when they are expressed on postsynaptic membranes, their activation triggers intracellular

signalling mechanisms important for cell excitability, gene expression, cell differentiation and survival. Less information is available about the subcellular distribution of nAChRs in the single neuron, surely it is known that the intracellular loop between TMD3-TMD4 is critical for the targeting of proper folded receptor from the ER towards the plasma membrane, as demonstrated for the $\alpha 7$, $\alpha 3$ and $\alpha 4\beta 2$ subtypes.

The nAChRs are expressed also in areas different from SNC, such as skin, leukocytes, kidney….(Hurst R, 2012) where they play an important role and their presence must be considered in therapeutic and side effects associated with compounds that interact with nAChRs.

1.1.4 NACHR SUBTYPE ASSEMBLY

There are a lot of information about the structure, the function and the pharmacological properties of nAChRs, but little is known about the mechanisms that control and guide the assembly, trafficking and degradation of them (Gaimarri A, 2007).

Much of our knowledge is based on the studies performed on muscle-type AChR. The muscle-type receptor subunits are translated by ER membrane-associated ribosomal complexes and co-translationally inserted into the ER membrane via the translocon. The nascent subunit polypeptide subsequently undergoes cleavage of its signal sequence, oxidation of its disulfide bonds, proline isomerisation and N-glycosylation of specific residues. The rapid co-translational events are followed by slower folding reactions where chaperones, such as binding protein BiP and calnexin, promote proper folding and maturation of nAChR subunits. All these processes occur in the ER, which provides “quality control” by identifying and delivering any misassembled or misfolded proteins to the ER-associated proteasomal degradation machinery (ERAD). Only when subunits are folded in a proper pentamer, AChRs are exported in coat protein complex II (COPII) vesicles from the ER to the Golgi and then transported to the plasma membrane (Wanamaker CP, 2003). The assembly of muscle-type nAChR is particularly complex, not only because of its size and its 20 membrane-spanning domains, but also because the finished product must have the correct oligomeric arrangement as well as subunit stoichiometry for proper function. Consequently, nAChR assembly is both slow and inefficient. Muscle-type nAChR subunits assemble into pentamers with a $t_{1/2}$ of more than 90 min and only ~30% of the synthesized α subunits are assembled. Unfortunately, ion

channels do not assemble in a test tube or by use of in vitro translation methods but only within the environment of a cell. Further, some nicotinic receptors are properly assembled only in cells of neuronal origin and appear to require additional factors for properly assembly, like for α7 nAChR (Cooper ST, 1997). Furthermore, different cell lines infected with adenoviruses encoding the α7, α4 and β2 subunits produce the appropriate mRNA, but have very different levels of α7 and α4β2 subtype expression and the ratio between surface and intracellular receptors may be very different for the same subtype (Gotti C, 2009).

1.1.4.1 INTRACELLULAR CHAPERONES AND ADAPTOR PROTEINS

Over the past few years much has been done to characterize the chaperone proteins: this class of proteins are not only responsible for helping nascent polypeptides folding but they are also important in retaining the immature polypeptides within the ER. This retention increases the probability that the immature polypeptide will be correctly folded and that it does not prematurely enter the secretory pathway. At the end, the prolonged retention of the misfolded or incompletely assembled polypeptide leads to its degradation. The first nicotinic receptor interactor identified was the protein rapsyn over 30 years ago. Rapsyn promotes clustering of nAChRs at the postsynapse of the neuromuscular junction, allowing to achieve high density of receptors in that site. Rapsyn does not interact with neuronal α3β2 or α4β2 nAChRs in the CNS.

The protein APC was demonstrated to be able to clusterize and stabilize α3-containing nAChRs and to form a complex with proteins EB1 (microtubule binding protein) and IQGAP1 (cytoskeletal macrophin proteins). The interaction between α3 and APC is not direct but mediated by the adaptor protein 14-3-3, that acts as a chaperone and scaffold protein. The α3 subunit has in its intracellular loop, between TMD3 and TMD4, a consensus binding sequence for the 14-3-3 protein (Jones AK, 2010).

Other identified proteins, such as ubiquilin-1, can influence significantly the assembly and trafficking of nAChRs. Ubiquilin-1 is an ubiquitin like protein that can interact with both the proteasome and ubiquitin ligase and has an important role in regulating nicotine-induced up-regulation. Binding of ubiquilin-1 to the α3 or α4 subunits sequesters them on proteasome, thus decreasing the availability of subunits for the assembly/trafficking of mature receptors (Ficklin MB, 2005).

In two hybrid screening studies the calcium sensor protein VILIP-1 8 (visinin-like protein) (Lin L, 2002) and UBXD4 (Rezvani K, 2010) were found to interact with the intracellular loop between TMD3-4 of $\alpha 3$ and $\alpha 4$ subunits and these interactions significantly increase the $\alpha 4$ subunits steady-state levels. The greater availability of $\alpha 4$ subunit increases the formation of the $\alpha 4\beta 2$ subtype, thus leading to an increase in surface $\alpha 4\beta 2$ receptors. It has been demonstrated that UBXD4 is expressed both in neuronal and non-neuronal cells where it increases the amount of $\alpha 3$-containing receptors at the plasma membrane, possibly by preventing their degradation.

Also the RIC-3 (Resistance to Inhibitors of Cholinesterase) protein interacts with a number of nAChR subtypes and enhances their functional response depending on the subtype or the host system (Millar NS, 2008). It is been demonstrated that RIC-3, first identified in the nematode C. Elegans, increases the expression of certain nAChR subtype such as $\alpha 7$, and in the absence of RIC-3, in certain host cell types, fail to generate functional receptors. RIC-3 probably associates with unassembled receptor subunits in the ER facilitating their receptor folding and assembly.

1.1.4.2 THE TRAFFICKING OF CYS-LOOP RECEPTORS

In the case of protein channels, a proper protein folding and assembly in the ER favour an efficient trafficking of the proteins from ER to their final destination and determines how many of them can reach the plasma membrane. The best known disease characterized by misfolding/ misassembling of ion channels is the cystic fibrosis. Mutation on the cystic fibrosis transmembrane regulator (CFTR) leads to the ER retention of the protein, consequently degradation by proteasome, and lack of delivery of CFTR to the cell surface. Similarly, many efforts have been made to increase the expression of muscle-type nAChR in patients with Myasthenia Gravis, a disease characterized by significant decrease of nAChR at the neuromuscular junction (NMJ): every nAChR controls the flow of billion of ions per second, so this explain why an improper targeting of nAChR could be extremely dangerous for the maintenance of the muscle fibers. Among the nicotinic receptors, the assembly and trafficking of muscle-type nicotinic receptor is the best characterized. Like other nicotinic receptors, muscle-type nAChR is a pentamer with a stoichiometry of $\alpha_2\beta\gamma\delta$ and a molecular weight of 270 KDa. The folding of membrane proteins are more complex than soluble proteins in eukaryotic, indeed the nAChR assembly is both slow and inefficient: only 30% of the synthesized α subunits are assembled. After the subunits

assembly and pentamer folding, processes that required a lot of time, the receptors exit from the ER and rapidly reach the plasma membrane. The exit from the ER is the limiting step within the secretory pathway, pointing to an accumulation of receptors in the ER, as demonstrated for muscle-type AChRs (Merlie JP, 1983) and neuronal $\alpha4\beta2$ AChRs (Sallette J, 2005). nAChRs that pass quality control, are exported in coat protein complex II vesicles from the ER to Golgi, presumably after masking specific ER retention signals (Keller SH, 2001, Wang N, 2002) and recognition of ER export motifs in properly folded and oligomerized nAChR subunits (Srinivasan R, 2011).

The presence of ER retention motif was demonstrated for the α-subunit of muscle-type nAChR: the mutation of Arg^{313}-Lys^{314} sequence in the cytoplasmic loop between TMD3-TMD4 to K314Q, increases the trafficking of the mutated unassembled α-subunit from the ER to Golgi apparatus in transfected HEK-293 cells. This mutated α-subunit interact less efficiently with the component of COP I coats γ–COP, protein implicated in the retrograde transport from Golgi to ER, indicating that usually the Arg^{313}-Lys^{314} sequence is masked when the α subunits are assembled into pentamer, allowing ER to Golgi transport of the α-subunit. The unassembled mutated α-subunit accumulate in the Golgi apparatus, but do not leave it and reach the cell surface, indicating that another quality control mechanism is present at post-Golgi level. Removal of the ER retention sequence is not sufficient to allow the reaching of plasmamembrane, indicating that at Golgi level other quality control processes are present, such as ubiquitination. The expression of mutated α-subunit alone in cells deficient in ubiquitination processes (ts20 cells) lead to delivery of α-subunit at the plasma membrane, demonstrating that ubiquitination modulates the post-Golgi trafficking (Keller SH, 2001). The exposure of basic amino acids in an unassembled interface to the COP I machinery could sequester the subunits in the ER facilitating the assembly of additional subunits into the proper folded pentamer.

It has been demonstrated that also a transmembrane motif in α-subunit of muscle AChR regulates the trafficking of receptor: the mutation of this highly conserved motif, PL(Y/F)(F/Y)xxN, allowed surface expression of unassembled subunits. Conversely, insertion of the sequence to unrelated proteins that are normally transportated to the cell surface resulted in ER retention. This sequence is masked in assembled pentamer but is exposed on unassembled subunits in the ER, where it promotes proteasome degradation (Wang N, 2002).

A lot of information has been obtained from studies of other ion channel that belongs to the superfamily of Cys-loop receptor, the $GABA\text{-}_A$ receptor. It is a hetero-pentamer with

several possible stoichiometry that mediates fast inhibition of transmission at the synapse. The subunit expression and assembly are carefully regulated in the ER thanks to ER-resident chaperones, such as calnexin. The oligomerization of individual GABA-$_A$ R subunits into pentamers is fast but inefficient, less than 25% of translated proteins are assembled into heteromeric receptors and misfolded or unassembled subunits are degraded via proteasome. After their assembly, GABA-$_A$ Rs are trafficked to the Golgi apparatus and then to the plasma membrane segregated into vesicles. GABARAP (GABA-$_A$ receptor-associated protein) is a protein concentrated in the Golgi apparatus and in intracellular vesicles that interact with the intracellular domain of GABA-$_A$ R. This protein seems to play an important role in the intracellular transport of receptor, indeed its overexpression results in increased cell-surface GABA-$_A$ R expression, probably by increasing its trafficking. GABARAP interact directly with the protein NSF and when present together, they promote the forward trafficking from the Golgi apparatus. Also the protein PRIP (Phospholipase-C-related catalytically inactive proteins) was found to modulate GABA-$_A$ Rs by competitively inhibiting GABARAP binding. Furthermore, the protein PLIC1 was found to facilitate GABA-$_A$ Rs accumulation at the synapse by preventing the degradation of ubiquitylated receptors (Jacob TC, 2008), in a similar way as UBXD4 for nAChRs.

1.1.4.3 ER-ASSOCIATED DEGRADATION (ERAD) OF MEMBRANE PROTEINS

The quality control that ensure the fidelity of secreted and membrane protein folding and assembly in eukaryotic cells, comprises also the ER-associated degradation (ERAD) process. ERAD is a specialized function of the ubiquitin-proteasome systems (UPS), which prevents the accumulation of misfolded, extraneous, and potentially toxic proteins from the ER by degrading them in the cytosol. Misfolded and/or unassembled proteins in the ER are recognized and retro-translocated to the cytosol for degradation. During this process, ubiquitin is covalently and repeatedly attached to a substrate's lysine residues by components of an ATP-dependent, ubiquitin-conjugating enzyme cascade (E1, E2 and E3) that reside in or are recruited to the cytosolic face of the ER. The "poly-ubiquitin" chains attached to degradation-bound proteins are recognized by AAA-ATPases, such as p97/cdc48, whose binding action is sufficient to dislocate membrane proteins from the ER. Poly-ubiquitin chains are recognized by 19S cap subunits of the 30S-26S proteasome complex, which then degrades the substrate and recycles the ubiquitin.

It has been demonstrated that UPS regulates the degradation of nicotinic receptors (Christianson JC, 2004), like $\alpha 3$, $\beta 4$ and $\alpha 7$ and some studies (Rezvani K, 2007 and 2010) suggested a role of nicotine in UPS, where nicotine seems to reduce the proteasome activity, but more investigations are required.

1.2 NICOTINE REGULATION

nAChRs are target of nicotine, the most diffuse drug of abuse. Nicotine is an alkaloid imported in Europe by Columbus in 1492 and obtained by nightshade family of plants (Solanacee). Nicotine in plants presumably confers selective advantage on protection against herbivores, in a similar way as cocaine and morphine.

Chronic nicotine increases the number of nAChRs, a phenomenon called up-regulation. This is opposite to which happens usually, where the over-stimulation induced by agonists leads to a reduction in the number of receptors at the cell surface. Upregulation is not accompanied by an increase in AChRs subunit mRNA (Marks MJ, 1992; Huang LZ, 2007). In brain, chronic nicotine causes selective up-regulation of nAChRs in cortex, midbrain and hypothalamus, but not in thalamus or cerebellum. Within specific brain regions, there is selective up-regulation among cell types and between somato-dendritic versus axon terminal regions of individual neurons.

Nicotine is metabolized only by liver enzymes and has a half-life of 120 min, whereas ACh has a turnover rate of 10^4/sec, so it remains near receptors for less than 1ms: indeed acetylcholinesterase does not hydrolyze nicotine, so nicotine remains near receptors longer than ACh. Furthermore, nicotine permeates membrane readily, at least six membranes (in the lungs and capillary vessels within few seconds of inhalation) whereas ACh does not: so nicotine accumulates within cells. Finally, nicotine has stronger effect on neuronal than muscle-type nAChRs in comparison to ACh. These factors explain why nicotine is more effective than ACh in receptor desensitization.

Tobacco abuse lead to addiction: nicotine both activates and desensitizes nAChR of dopaminergic neurons in midbrain and while the pleasure effects associated with tobacco smoke involved mostly the mesolimbic dopaminergic reward system (Koob GF, 2010), the adversive effects of nicotine may involve medial habenula (Fowler CD, 2011). Nicotine intake leads to alterations in reward but also improved cognitive performance (called cognitive sensitization). Epidemiological studies have shown that smokers have a lower

incidence of Parkinson's disease and later it has been shown that nicotine has a protective effect on dopaminergic neurons, as well as on neurons of mice with ADFLE, a specific type of epilepsy.

The molecular mechanism by which nicotine upregulates nAChRs has been investigated both *in vitro* and *in vivo* and different mechanisms have been proposed. The rate of nicotine upregulation depends on cell type, nicotinic subtype and receptor stoichiometry:

1) Cell type: nicotine exposure decreases the functional response of $\alpha4\beta2$ and $\alpha7$ transfected in oocytes, whereas nicotine does not in stably transfected HEK293 cells (Buisson B, 2001) indicating that different host systems and/or their different protein repertoires can influence both the number and function of the expressed subtypes.

2) Nicotinic subtype: the most upregulated nAChR in brain is $\alpha4\beta2$ subtype, also the homomeric $\alpha7$ and various heteromeric $\alpha3\beta2$, $\alpha4\beta2$, $\alpha6\beta2$ and $\alpha3\beta4$ receptors are upregulated, but at different extent and at higher doses.

3) Receptor stoichiometry: the presence of $\beta3$ subunits in $\alpha6\beta2\beta3$ and $\alpha6\beta4\beta3$ subtypes promotes greater nicotine-induced up-regulation than that observed in the $\alpha6\beta2$ and $\alpha6\beta4$ subtypes (Broadbent S, 2006). This is not due to an increased affinity for nicotine, but probably to a direct effect of the $\beta3$ subunit on subtype assembly. Furthermore, nicotine seems to upregulate preferentially the high affinity $(\alpha4)_2(\beta2)_3$ subtype instead of the low affinity $(\alpha4)_3(\beta2)_2$ subtype.

1.2.1 MECHANISMS INVOLVED IN nACHR UP-REGULATION

Most of our knowledge of the up-regulation of nAChRs is based on studies on the $\alpha4\beta2$ subtype that, in transfected cells and neurons, is localised on the cell surface and in intracellular pools, where it co-localises with a number of ER markers. Surely, there is a general agreement concerning the fact that nicotine-induced upregulation is posttranscriptional effect since nicotine treatment does not alter subunit mRNA levels in rat and mouse brains.

Several models have been proposed to explain the different effects of nicotine on nAChR subtypes, but the mechanisms and cellular machinery required for nAChR up-regulation are still not completely understood.

1.2.1.1 INCREASE IN RECEPTOR TRANSPORT THROUGH THE SECRETORY PATHWAY TO PLASMAMEMBRANE

The Harkness and Millar's work (Harkness PC, 2002) shows that chronic exposure of nicotine upregulates $\alpha4\beta2$ nAChRs expressed in cultured cell lines. In the absence of nicotine $\alpha4\beta2$ nAChRs route inefficiently to plasma membrane: their number at the plasmamembrane increases only after chronic nicotine exposure. They showed that $\alpha4\beta2$ upregulation corresponds to an increase in the proportion of total receptor expressed on the cell surface (measured by comparing the number of pentameric receptor in permeabilzed cells and not-permeabilized cells), suggesting that nicotine increases the receptor trafficking.

It has been demonstrated that motifs in the M3-M4 loop of several nAChR subunits influence the targeting of receptors to subcellular compartments and therefore eventually promote or inhibit PM expression. The work of Lester's group (Srinivasan R, 2011) shows that the balance between ER export and retention motifs regulates receptor trafficking and indeed when the β2 M3-M4 loop sequence is modified to incorporate more LXM ER export motif, there is substantially increase in plasmamembrane localization of $\alpha4\beta2$ receptors. Nicotine, likely stabilising the receptors in the ER, increases their trafficking towards plasmamembrane.

1.2.1.2 INCREASE IN SUBUNIT ASSEMBLY

Using pulse-chase metabolic labelling of human nAChRs $\alpha4\beta2$ expressed in HEK293 cells, the authors (Sallette J, 2005) demostrate that nicotine acts intracellularly on early maturational steps. In the ER, the $\alpha4$ and $\beta2$ subunits rapidly hetero-oligomerize: these processes result in the generation of a mixed population of pentamer and of a heterogeneous higher molecular weight oligomer population that probably corresponds either to misfolded entities or to small aggregates formed from immature subunits. They found that only pentameric receptors exit from the ER, as observed for muscle-type nAChRs, and that the exit from the ER to medial Golgi compartment is a relatively slow process as compared to the maturation steps. Maturation is nearly completed after 30 min, whereas trafficking and complex oligosaccharide glycosylations require around 3 hr. Therefore, the authors showed that exit from the ER is the limiting step within the secretory pathway, pointing to an accumulation of protein within the ER (85% inside the cell and 15% at the cell surface). Thus, the $\alpha4\beta2$ receptor matures rather inefficiently, a process

that is partly overcome by the action of nicotine during the early steps of the intracellular maturational processes. Nicotine, therefore acting as a “maturational enhancer”, helps the assembly of $\alpha4\beta2$ receptor that otherwise is extremely inefficient.

These results were also confirmed by Lindstrom’s group, that demonstrated that nicotine up-regulation leads primarily to an increase in assembly of AChRs from large pools of unassembled subunits, and secondary lead to a 5-fold increase in the lifetime of AChRs in the surface membrane. Up-regulation does not require current flow through nAChRs at the surface membrane, indeed up-regulation occurs also in the presence of mecamylamine (a channel blocker) and with an $\alpha4$ mutant, that has lost nAChR function. Nicotine and the much less permeable quaternary amine cholinergic ligands can act as pharmacological chaperones within the ER to promote the assembly of AChRs (Kuryatov A, 2005).

1.2.1.3 INHIBITION OF PROTEASOMAL ACTIVITY

The authors (Rezvani K, 2007) show that nicotine acts as a partial proteasome inhibitor and this effect is due to partial decrease in catalytic activity rather than reduced expression level of proteasomal constituents. In particular, a 24 hr exposure to nicotine decreased proteasome-dependent degradation of the $\alpha7$ nAChR subunit. The nAChR antagonist mecamylamine was only partially able to block the effects of nicotine on the UPS, indicating that nAChR activation does not completely explain nicotine-induced inhibition of proteasomal catalytic activity.

1.2.1.4 ISOMERISATION OF SURFACE RECEPTORS INTO A MORE EASILY ACTIVATED HIGH AFFINITY CONFORMATION

Using HEK293 cells transfected with $\alpha4\beta2$ subunits, Vallejo YF (2005) found that nicotine exposure causes a fourfold to six-fold higher binding to $\alpha4\beta2$ receptors that does not correspond to any significant change in the number of surface receptors or change in the assembly, trafficking, or cell-surface turnover of the receptors. However, upregulation does alter the functional state of the receptor, slowing desensitization and enhancing sensitivity to acetylcholine. Based on these findings, the authors proposed an alternative mechanism to explain nicotine-induced upregulation in which nicotine exposure slowly stabilizes $\alpha4\beta2$ receptors in a high-affinity state that is more easily activated, thereby providing a memory for nicotine exposure.

1.3 NICOTINE AND LUNG CANCER

For decades it was generally believed that nAChRs only exist in the nervous system and at NMJ. However, recent research shows that nAChRs and their physiological agonist acetylcholine (ACh) are also expressed in mammalian cells, including cancer cells. They act as central regulators of a complex network of stimulatory and inhibitory neurotransmitters that govern the synthesis and release of growth, angiogenic and neurogenic factors in cancer cells and their microenvironment, and in distant organs.

It has also been shown that nicotine is not a direct carcinogen, but may be a tumor promoter. In animal studies, nicotine can inhibit apoptosis (Maneckjee R, 1990), resulting in impaired killing of cancer cells (Schuller HM, 1989). Nicotine also promotes angiogenesis in animals, an effect which could lead to greater tumor invasion and metastasis

Furthermore, recently large-scale genome-wide association studies have found a lot of single-nucleotide polymorphism in the gene cluster on the human chromosome 15 that codifies for $\alpha 3$, $\beta 4$ and $\alpha 5$ subunits. The polymorphisms in the gene cluster are associated with nicotine addiction (its age-dependent onset, number of cigarettes smoked per day) and lung cancer (Improgo MR, 2011). The major "risk allele" is rs16969968, located in the CHRNA5 codifying region, this polymorphism is located in the major cytoplasmic loop between TMD3-4 of the $\alpha 5$ subunit, and leads to changes an amino acid from aspartic acid to asparagine at position 398 (D398) and a decreased expression of $\alpha 5$ mRNA.

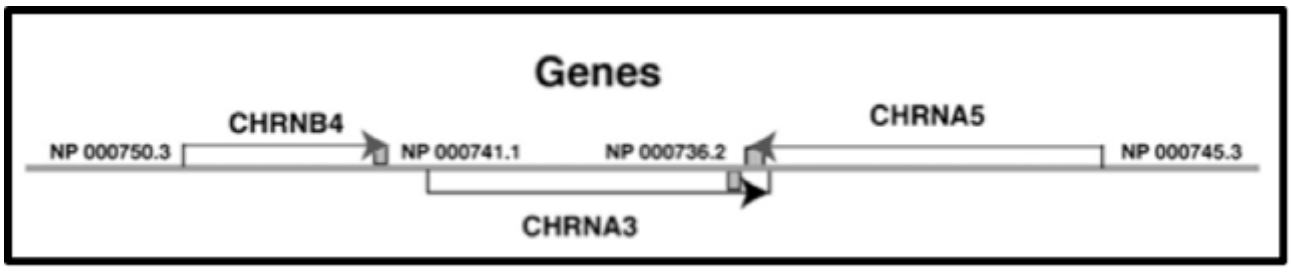

Fig. 7 The β4, α3, α5 cluster, the arrows indicate the sense of transcription

(from Hurst R, 2012)

Follow-up studies demonstrated that one copy of the risk allele confers a 1.3 fold increase in risk for developing nicotine addiction, where 2 copies confers 2-fold increase risk. This polymorphism was found in 14% of lung cancer cases in a European population, indicating that the chromosome 15 region is associated with both nicotine dependence and lung cancer (Saccone NL, 2009).

Another important polymorphism is rs1051730 in CHRNA3 gene. Further support for a direct role of $\alpha 3$ subunit in lung cancer is provided by a study showing that CHRNA3 gene is frequently hypermethylated and silenced in an aberrant way in lung cancer cells (Paliwal A, 2010). In addition, increased lung cancer risk in non-smokers support the idea that the polymorphisms have a direct effect on lung cancer and association with lung cancer persists even after accounting for cigarette consumption. The correlation between nAChR and lung cancer is also suggested by the fact that RT-PCR study has detected in lung and airways tissue mRNAs for the $\alpha 3$, $\beta 4$ and $\alpha 5$ subunits (Maus AD, 1998). Furthermore, lung cancers secrete ACh and utilize the same ACh signalling components as normal lung cells, but in these cells ACh acts as a growth factor that promotes cell proliferation. All these findings suggest a strong correlation among $\alpha 3\beta 4(\alpha 5)$ nAChR, nicotine and lung cancer, but the mechanism involved remain to be elucidated.

2 AIM OF STUDY

Neuronal nicotinic acetylcholine receptors (nAChRs) are a heterogeneous family of oligomeric ACh -gated cation channels found in the central and peripheral nervous systems. They are formed by the pentameric assembly of identical α subunits (α7, α8, α9, homomeric receptors) or combinations of α and β subunits (α2–α6 and β2–β4, heteromeric receptors). nAChR subtypes share a common basic structure but their pharmacological and functional properties arise from the wide range of different subunit combinations that makes up the distinctive subtypes involved in a number of physiological functions.

Once synthesized, the nicotinic subunits had to be assembled in pentameric receptors that could leave the ER and reach the plasma membrane. It has been demonstrated that the exit of nAChR from the ER membrane is a very inefficient event. At the level of the ER membrane, both the nicotinic and the well characterized muscle-type AChR are subjected to a very stringent quality control: the unincorporated subunits and misfolded pentamer are retro-translocated in the cytoplasm and degraded by the proteasome. The result of this stringent quality control is that the number of the receptor that could reach the plasma membrane is very few in compare with the total pool of receptor. It has been demonstrated that nicotine could increase the number of nAChRs, a phenomenon called up-regulation, without changing nAChR subunit mRNA levels, thus indicating that post-transcriptional mechanisms are responsible for this effect. nAChRs respond to chronic nicotine exposure in different ways depending on the receptor subtype, and this also occurs when receptors are expressed in cells other than neurons, indicating that the up-regulation is a property intrinsic to the receptor as opposed to a regulatory property specific to the neurons in which the receptors are normally found. Many studies share a common idea that nicotine acts at the level of ER membranes helping the nascent nAChR to fold and to assemble into pentamer as the intracellular chaperone do, with the final result to increase the number of nAChR at the plasmamembrane.

In the literature the large majority of papers focused on the α4β2 subtype, the most widespred nAChR in the brain. It has been demonstrated that the α4β2 subtype can exist in two alternate stoichiometries with two or three copies of the α4 subunit in the pentamer. Recently, studies based on imaging experiment using tagged fluorescent nicotinic subunits have revealed in the α and β subunits the presence of ER retention and export motif. The export motif (LFM) is recognized by Sec24D protein at the level of ER exit sites (ERES).

Nicotine stabilizes the receptors with the $(\alpha4)_2(\beta2)_3$ stoichiometry at the level of ER and subsequently at the level of ERES thus allowing them to leave the ER more efficiently. These data are consistent with electrophysiological data showing that exposure to nicotine increases the plasma membrane localization of the $\alpha4\beta2$ receptor with the $(\alpha4)_2(\beta2)_3$ stoichiometry.

Unfortunately, little is known about the effect of nicotine on other subtype, such as on the $\alpha3\beta4$ subtype. $\alpha3\beta4$ nAChR are highly expressed in the autonomic nervous system and in the adrenal medulla where they mediate acetylcholine-induced fast excitatory ganglionic transmission. In addition to their widespread expression in the autonomic nervous system, $\alpha3\beta4$ nAChRs are present in selected regions of the brain where they may influence the behavioural effects of nicotine and some manifestations of nicotine withdrawal. A series of linkage analyses, as well as candidate gene and genome-wide association studies, have recently shown that variants in the human $\alpha3\beta4\alpha5$ nAChR subunit gene cluster on chromosome 15q24-25.1 are involved in the risk of nicotine dependence, smoking and lung cancer. The $\alpha3$ and $\beta4$ genes are significantly over-expressed in the epithelial cells of small-cell lung carcinoma (SCLC), an aggressive form of lung cancer that is closely related to cigarette smoking and increasing evidence suggests that nicotine itself may directly contribute to carcinogenesis by inducing cell proliferation, transformation, apoptotic inhibition and angiogenesis through different intracellular signalling pathways. The up-regulation of nAChRs may provide a mechanism by which the effects of nicotine are potentiated in SCLC, thus contributing to its aggressiveness.

Understanding how $\alpha3\beta4$ subtype is regulated by nicotine in epithelial and neuronal cells is a critical step towards the designing of drugs that, by acting on $\alpha3\beta4$ nAChR, may affect cell proliferation and fine tune the cholinergic signalling that seems to play an important role in lung cancer. In this work, the effect of chronic nicotine exposure on the $\alpha3\beta4$ subtype was analysed using the human epithelial HeLa cells and human neuroblastoma SH-SY5Y cells, both of them transiently transfected with $\alpha3$ and $\beta4$ subunits. In these two cell lines the expression of the nicotinic subunits, the assembling of the pentameric receptor, its stability and its trafficking from the ER to plasma membrane and the effect of nicotine treatment were tested using biochemical, morphological and pharmacological approaches. Furthermore, since it has been shown that $\alpha3\beta4$ receptor can exist in two different stoichiometries depending on the host system, It is also analysed

whether chronic treatment with nicotine or nicotinic ligands can affect the stoichiometry of the expressed receptors and consequently the functional properties of the receptors expressed at the plasma membrane.

3 MATERIALS AND METHODS

3.1 CELL CULTURE AND TRANSFECTION

HeLa cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 1% glutamine, 1% penicillin, and streptomycin (GIBCO) in a 10% CO_2 incubator at 37 °C and SH-SY5Y were grown in RPMI (LONZA) supplemented with 10% heat-inactivated FBS, 1% glutamine, 1% penicillin, and streptomycin (GIBCO) in a 5% CO_2 incubator at 37 °C. HeLa were transfected by the Ca_2PO_4 method in DMEM supplemeted with chloroquine 100 μM (DMEM with chloroquine was removed after 5h) with the following plasmid concentration: 40 μg total for 100mm culture plate in a final volume of 1ml and 10 μg total for 35mm culture dish in a final volume of 250 μl . SH-SY5Y were transfected by Jet-PEI (Polyplus transfection) with the following plasmid concentration: 3 μg total for 100mm culture dish (ratio DNA:JetPEI 1:3, 3 μg DNA:18 μl JetPEI) in a final volume of 800 μl with NaCl2 150 mM and 1 μg total for 35mm culture dish(ratio DNA:JetPEI 1:3, 1 μg DNA:3 μl JetPEI) in a final volume of 200 μl with NaCl2 150 mM. All the cells were blocked or treated after 24h after transfection .

3.2 PLASMID CONSTRUCTIONS

human α3-pcDNA3 and human β4-pcDNA3 (kindly gift by Fucile's lab);

cytoplasmatic-GFP in the pN1eGFP (Clontech);

pCINeoHA-CD3Δ (kindly gift by Mariano Jennifer's lab);

FP-22 (kindly gift by Borgese's lab);

mRFP-KDEL (kindly gift by Snapp EL's lab).

To generate the ER-export motif mutant hβ4-pcDNA3, the mutation L345M was generated by PCR primers (forward primer, 5'-GCTGCCTACCTTCATGTTCATGAAGCGCCCTGGC CCCG-3' and reverse primer, 5'-CGGGGCCAGGGCGCTTCATGAACATGAAGGTAG GCAGC-3') with QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies).

To generate the nicotinic binding site mutant hα3-pcDNA3, the mutation W180A was generated by PCR primers (forward primer, 5'-CCATGAAGTTCGGTTCCGCGTCCTAC GATAAGGCG-3' and reverse primer, 5'-CGCCTTATCGTAGGACGCGGAACCGAACTT CATGG-3') with QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies).

3.3 TREATMENTS AND SAMPLE PREPARATION

Nicotinic ligands were added to the culture medium 24h after transfection: cells were treated with Nicotine (Sigma) at the concentration of 0.01, 0.1 and 1 mM. Cytisine was used at the concentration of 0.1 and 1 mM. CC4, hexamethonium and mecamylamine was used at 1 mM. To block protein synthesis cell were treated with cycloheximide (Sigma) at the concentration of 50 μg/ml. To block proteasome activity cells were treated with MG132 (Calbiochem) at 10 μM. After treatments, cells were lysated in 1% SDS buffer, boiled 3 minutes and protein contenent was measured using the BCA protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

3.4 BIOCHEMICAL ANALYSES.

SDS-PAGE-immunoblotting. SDS-PAGE and blotting were carried out by standard procedures. Blots were incubated for 2 hours with the primary antibody (Ab) diluted in Tris-saline, 5% milk, 0.1% Tween.

After removal of primary Ab and washing for three times, blots were incubated with peroxidase-conjugated secondary antibodies (Sigma, final concentration used 1:150000) for 1 hours, the blots were developed with ECL-Plus or ECL-Ultra (GE Healthcare) reagents. The films were digitized, and band intensities were determined with Image J software (National Institutes of Health) after calibration with the optical density calibration step table (Stouffer Graphics Arts).

Quantitative InfraRed Western Blots detection with Odyssey CLx: the blots were incubated with the secondary antibody IRDye 800CW Goat Anti.Rabbit IgG (M-Medical, final

concentration used 1:15000) and band intensities were determined with Image Sudio software (M-Medical).

3.5 BINDING TO CELL MEMBRANES

The cells were collected, rinsed twice with PBS (phosphate buffer saline) containing 2 mM PMSF (phenylmethylsulfonylfluoride), homogenised in an excess of phosphate buffer saline and 2 mM PMFS using a Potter homogeniser, and then washed twice by centrifugation for 60 min at 30,000 x g before final suspension in the same buffer containing 2 mM PMSF. To determine the number of receptors present after treatment with different concentrations of nicotine, cytisine, mecamylamine, hexamethonium and CC4, membrane homogenates were incubated with a saturating concentration of ^{3}H-Epi (1 nM) in the presence (non-specific binding) or absence (total binding) of 250 nM cold Epi. At the end of the incubation, the samples were filtered on a GFC filter soaked in 0.5% polyethylenimine, washed with 15 ml of buffer (Na phosphate, 10 mM, pH 7.4; NaCl, 50 mM) and counted in a β-counter. To calculate the Ki (apparent displacing constant) for each drug, aliquots of the membranes of control or drug-treated cells were incubated with increasing drug concentrations (1 nM to 250 mM for nicotine and cytisine; 0.03 nM to 1.4 mM for CC4) for 30 min at room temperature, followed by overnight incubation with a final concentration of 0.15 nM ^{3}H-Epi. Protein content of the membranes, 2% Triton X-100 extracts was measured using the BCA protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

All the binding assays were performed by Dott. Francesco Pistillo (lab C. Gotti)

3.6 PRIMARY ANTIBODY

The human α3 and β4 subunit-specific polyclonal antibodies (Abs) used were produced in rabbit against peptides derived from the intra-cytoplasmic loop regions of human α3 (TRPTSNEGNAQKPRPLYGAELSNLNC) and β4 (GPDSSPARAFPPSKSCVTKPEATATS PP) and affinity purified as previously described (Zoli M, 2002). Both of them are used at the final concentration of 5 μg/ml in WB and 1:100 in IF.

Rabbit IgG anti-GFP (ab290; Abcam) used at the final concentration of 1: 8000

Rabbit IgG anti HA (Y-11, Tebu-bio) used at the final concentration of 1: 200

Mouse IgG ANTI-UBIQUITIN FK1 (PW 8805; Biomol) used at the final concentration of 1: 1000

3.7 SUCROSE GRADIENT CENTRIFUGATION

In order to analyse the subunit assembly of the $\alpha 3\beta 4$ receptors, membranes of transfected cells were washed by centrifugation for three times with PBS containing 2 mM PMSF. Triton X-100 at the final concentration of 2% was added to the membranes, which were extracted for 2 h at 4°C. The extracts were then centrifuged for 1.5 h at 60,000g and the solubilised receptors analysed on the 5-20% sucrose gradients to separate the assembled nAChRs from the unassembled subunits. As internal sedimentation standard 2% Triton extract from *Torpedo californica* electric organ was also prepared. Linear 5-20% sucrose gradients in phosphate buffered saline plus 1 mM PMFS and 0.1% Triton X-100 were prepared using a Buckler gradient maker (Fort Lee, NJ, USA) and stored for 4 h at 4°C before use. The volume of each gradient was 12 ml. 500 μL of 2% Triton X-100 extracts obtained from Torpedo electric organ was labeled by incubation with 6 nM ^{125}I α–Bgtx and 500 μL of HeLa or SH-SY5Y 2% Triton X-100 extracts was prepared from cells grown in control condition or 24 hours incubation with 1 mM nicotine. The extracts were loaded onto the gradients and centrifuged for 14 hours at 40,000 rpm in a Beckman SW41. Fractions of 0.5 ml were collected from the top of the gradient and directly counted on a γ-counter (in the case of the Torpedo gradients), or added to the affinity-purified anti-$\alpha 3$ or anti-$\beta 4$ Abs bound to microwells and processed as previously described (Gotti C, 2005).

Affinity-purified anti-$\alpha 3$ or anti-$\beta 4$ Abs (10 μg/ml in 50 mM phosphate buffer, pH 7.5) were bound to microwells (Maxi-Sorp, Nunc) by overnight incubation at 4°C. The next day, the wells were washed in order to remove excess unbound Abs. Then, 100-200 μL aliquots of the gradient fractions that had been diluted 1: 2 with a buffer (TRIS-HCl, 50 mM, pH 7; NaCl, 150 mM ; KCl, 5 mM ; $MgCl_2$, 1 mM ; $CaCl_2$, 2.5 mM ;BSA, 2 mg/ml; and 0.05% Tween 20) were added to the wells and incubated overnight at 4ºC. After this incubation, the wells were washed and immobilized receptors were quantified using 1nM ^{3}H-epi to label the binding sites. The wells were then washed seven times with ice-cold PBS containing 0.05% Tween 20, and the bound radioactivity was recovered by incubation with

200 µl of 2N NaOH for two hours. The bound radioactivity was then determined by beta counting.

All the sucrose gradient analysis were performed by Dott. Francesco Pistillo (lab C. Gotti)

3.8 BIOTINYLATION ASSAY

Cells were labeled for 30 min at 4°C with EZ-link NHS-SS-biotin 0,3 mg/ml (Pierce) to biotinylate surface proteins (in PBS plus $CaCl_2$ 0,1 mM and $MgCl_2$ 1mM at pH 8). Free biotin was quenched with glicine (50 mM in PBS plus $CaCl_2$ 0,1 mM and $MgCl_2$ 1mM at pH 8) for 10 minutes at 4°C.

Cells were lysed in lysis buffer (50 mM Tris, pH 6.8, 1% SDS) by boiling for 3 min. Equal amounts of total protein were incubated with streptavidin beads to capture biotinylated protein O.N. at RT . After washing in lysis buffer, biotinylated proteins were eluted from streptavidin beads by boiling in sample buffer (20 mM Tris pH 6.8, b-mercaptoethanol 1%, glicerol 8,5%, SDS 1%) for 3 minutes.

3.9 IMMUNOFLUORESCENCE

Cells grown on coverslips were fixed with 4% paraformaldeyde and processed for immunofluorescence using Alexa 488 or 568 (Invitrogen) at the final concentration of 1:500 (488nm) and 1:250 (568nm), rispectively. Immunostained preparations were mounted in Mowiol (Sigma-Aldrich).

Wide-field imaging was done with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany), using the 63x PlanNeofluar lense. Illustrations were prepared with Adobe Photoshop software. Z-stack images were taken with Axiovert 200M Microscope (Zeiss) equipped with Spinning Disk confocal system LCI MLS (Perkin Elmer) using the 63x PlanApo lens with the use of the 50 mW solid state diode laser 488/561.

3.10 STATISTICAL ANALYSIS

Data from binding studies and western blotting were expressed as mean ± SEM (Standard error mean) and analyzed by one-way analysis of variance (ANOVA) followed by *post-hoc* Bonferroni test. The accepted level of significance was $P<0.05$. All statistical analyses were done with software Prism, version 5 (GraphPad, San Diego, California).

4 RESULTS

4.1 ANTIBODIES CHARACTERISATION

To study the effect of nicotine on the α3β4 nicotinic receptor, human epithelial HeLa cells and human neuroblastoma SH-SY5Y cells were transiently transfected with the α3 and β4 subunits for 24h. Cell lysates of HeLa (Fig.11-A) or SH-SY5Y (Fig.11-B) cells transfected or not with the α3 and β4 subunit, as indicated, were loaded on SDS-PAGE. After western blotting (WB), membranes were incubated with the anti-α3 (left panel) or anti-β4 (right panel) antibodies. It is indicated with an arrow the nicotinic subunit, with asterisks non-specific bands.

4.2 CELL LOCALISATION OF α3β4 nACHR

To characterize the cellular localisation of nAChR, HeLa and SH-SY5Y cells were transfected with α3 and β4 subunits and also with fluorescent ER-marker RFP-KDEL (Fig. 12, panel a-c; d-f) and the fluorescent plasma membrane marker FP-22 (Fig. 12, panel g-i). 24h after transfection, the cells were fixed, permeabilised and immunostained with anti-α3 antibody, followed by coniugated secondary antibody. In HeLa cells the receptors are localized in the ER, as demonstrated by their co-localisation with the ER marker RFP-KDEL (panel a-c). In SH-SY5Y, a more physiological system, the receptors are localized both in the ER (panel d-f), as demonstrated by their co-localisation with the ER marker RFP-KDEL, and at the plasma membrane (panel g-i), as demonstrated by their co-localisation with the plasma membrane marker FP-22.

4.3 THE α3β4 NACHRS ARE PENTAMER IN THE CELL

As known from literature, only the full assembled pentameric receptor can leave the ER: in order to assess whether the receptor cannot leave the ER because is not correctly assembled, the size of the transfected receptors was analysed by means of sucrose gradient sedimentation analysis both in HeLa and SH-SY5Y (Fig 13, A-B). After transfection, the cells were solubilised with 2% Triton X-100 and the extract was loaded on a 5-20% sucrose gradient. After centrifugation, the receptors present within each fraction

were analysed by WB for α3 and β4 subunit (lower panel) and after being captured using anti-α3 (black circle) and anti-β4 (grey squares) Ab-coated wells, they were quantified by ^{3}H-epibatidine binding (upper panel). In previuos studies of C. Gotti's lab, they characterize the ^{3}H-epibatidine binding on the α3β4 subtype demonstrating that epibatidine binds only pentameric receptor.

Both the binding of ^{3}H-epibatidine to immunoimmobilised receptors and the WB analysis showed that the detergent solubilised α3β4 receptors sediment as a single species between the T. californica AChR monomers (9.5S) and dimers (13S), indicated by arrows. Fig 13 A-B shows a single peak, (corresponding to fractions 12-15) which indicates that α3β4 receptors have a uniform pentameric size. The α3 and β4 subunits can assemble to form pentamers in HeLa (A) and SH-SY5Y (B) cells but the receptors cannot leave the ER and reach the plasmamembrane. In both cells the most of receptor is retained in the ER, even if, surely, SH-SY5Y cells shows also a plasma membrane staining (Fig 12, g-i)

4.4 CHRONIC NICOTINE UP-REGULATES α3β4 NACHRS

To analyse the effect of chronic nicotine treatment on the α3β4 subtype, HeLa (Fig 14, A) and SH-SY5Y (Fig 14, B), transiently transfected with the nicotinic subunits, were incubated with the indicated concentration of nicotine for 24h. Nicotine binds to various nicotinic receptor subtypes, although with different affinity: in preliminary studies competition binding experiments were used to investigate the affinity of nicotine for the human α3β4 transfected subtype, and we determined an affinity (Ki) of 300 nM. Given this relatively low affinity, we chose to treat transfected HeLa and SH-SY5Y cells with concentrations 0.01, 0.1 or 1 mM of nicotine for 24h. WB (upper panel) showed that the amount of both α and β subunits increased in a dose-dependent manner in both cell lines and the ratio of the increase in α3 and β4 subunits with a higher increase in β4 subunit. This increase is progressive and statistical significant, as demonstrated by quantification of 3 different experiments (one-way ANOVA test, Bonferroni test, *P<0,05, **P<0,01, ***P<0,001) in Hela (A) and 5 experiments in SH-SY5Y (B).

The dose-dependent increase in subunit receptor protein was paralleled by an in increase in ^{3}H-epibatidine binding (Fig. 4 A,B lower panel), with an half maximal effect (EC_{50}) of 14,8±4 μM in HeLa cells and 16,5±2 μM (mean ± SE,n=3) in SH-SY5Y cells. The

quantification of epibatidine correspond to 6 different experiments (one-way ANOVA test, Bonferroni test *P<0,05, **P<0,01, ***P<0,001). Analysis of ^{3}H-epibatidine binding to membrane obtained from transfected HeLa and SH-SY5Y cells, determined a Kd of respectively 0,443 ±0,09 nM, n=3 and 0,350±0,08 nM, n=3 and did not revealed difference in affinity between control and nicotine treated cells with a Kd of respectively of 0,548 ± 0,09 nM, n= 3 (HeLa) and 0,485 ± 0,09 n=3 nM (SH-SY5Y). These binding studies only showed that in the case of nicotine treated cells there is an increase in B_{max} indicating that the number of receptors was double.

Moreover, analysis by sucrose gradient sedimentation analysis of control and 1 mM nicotine treated HeLa cells confirmed that the number of pentameric receptors was quantitatively increased (Fig 15, A) but is not qualitatively altered by chronic nicotine (Fig 15, B).

4.5 α3β4 RECEPTOR IS QUICKLY DEGRADED BY PROTEASOME

To investigate and characterize the mechanism by which nicotine up-regulates α3β4 receptor, the stability of the transfected receptor subunits was first analysed. 24h after transfection, HeLa cells were incubated for 8h in the presence of 50 μg/ml of cycloheximide to block protein translation. Western blot (Fig 16, A) and ^{3}H-epibatidine binding (Fig 16, B) analyses reveal that within 8h nearly 40% of the receptors are degraded (lane 2 in compare to lane 1). This decrease is statistical significant, as demonstrated by quantification of 6 experiments for WB analyses and 6 for Binding analyses (Unpaired T Test *P<0,05, **P<0,01). The degradation of the nicotinic receptor is mainly due to the proteasome since it is prevented by exposing cells to 10 μM of proteasome inhibitor MG132 (lane 3).

Also immunofluorescence images (Fig 17 A, panel a-b) confirm that 8h exposure with cycloheximide strongly reduced the receptor signal, and this decrease is prevented with proteasome inhibitor MG132 (Fig 17 A, panel c). On the opposite side, the presence of nicotine during the cycloheximide incubation did not affect proteasome subunit degradation, as demonstrated by IF analysis (Fig 17 A, panel d) and WB analysis with anti-β4 antibody (Fig 17 B, lane 3), in which was quantitatively identical to that observed in the presence of cycloheximide alone (fig 17 A-d, B, lane 3). This result indicates that

α3β4 receptor is degraded via proteasome and that nicotine do not prevent protein degradation of pre-synthesised receptor.

4.6 NICOTINE PREVENTS THE PROTEASOMAL DEGRADATION OF THE NEWLY SYNTHESIZED α3β4 NICOTINIC RECEPTOR

Since nicotine does not act on pre-synthesised receptors, it has been investigated if nicotine acts on newly-synthesized receptors. In order to create a pool of receptors synthesised in the presence of nicotine, 24 h after transfection cells were incubated overnight (O.N) in the absence or presence of nicotine (Fig 18, lanes 1,2 respectively) and then for 8h (lanes 4 and 6) whit or without (lanes 3 and 5) the presence of the proteasome inhibitor MG132. The subunit content of the six samples where then analysed. As shown in the WB analysis (Fig 18, A) and in the quantification panel (Fig 18, B), O.N incubation with nicotine up-regulates α3β4 receptor (lane 1 in compare to lane 2), as expected. In the absence of nicotine, the proteasome inhibitor prevents the receptor degradation (lane 3 in compare to lane 4); in the presence of nicotine, it does not alter the amount of the receptor (lanes 5-6), and its effect is not synergistic with that of nicotine (lane 3-4 in compare to lane 5-6). These findings indicate that the presence of nicotine during or immediately after the nicotinic subunits synthesis makes the receptor less prone to be degraded via proteasome.

4.7 THE PROTEASOME ACTIVITY IS NOT IMPAIRED BY THE PRESENCE OF NICOTINE

Recently, it has been reported that 1) ubiquitination regulates the degradation and membrane trafficking of synaptic proteins, 2) concentrations of nicotine achieved by smokers reduce proteasomal activity, induce the accumulation of ubiquitinated synaptic proteins and increase total ubiquitinated protein levels. In order to exclude direct impairment of the proteasome activity by nicotine, in HeLa and SH-SY5Y cells the amount of ubiquitinated proteins were measured in presence and absence of nicotine (Fig 19, A). Incubation of cells with cycloheximide plus 1mM nicotine for 3h (lanes 3-6) did not increase the amount of ubiquitinated proteins in compare to control with only cycloheximide (lanes 1-4), whereas, as expected, treatment with cycloheximide plus

MG132 for 3h lead to the accumulation of ubiquitinated proteins in both cell lines (lanes 2-5).

Moreover, the degradation of the CD3Δ proteasome substrate was analysed in the presence or absence of nicotine (Fig 19, B). CD3Δ is a well characterised substrate of proteasome (Ballar P, 2011). It is a subunit of the T cell antigen receptor (TCR) and when expressed in the absence of other subunits, is degraded quickly via proteasome (Yang M, 1998). 24h after transfection of CD3Δ-HA and cytoplasmatic GFP (as a control of transfection efficiency), translation was blocked with cycloheximide alone (lane 1), cycloheximide plus the proteasome inhibitor MG132 (lane 2) or with different concentration of nicotine (lane 3-5) for 3h. CD3Δ-HA was revealed in WB using anti-HA antibody and the signal quantified (Fig 19, C): as expected, degradation of the substrate was prevented only in the presence of MG132 (lane 2), whereas none of the nicotine concentrations indicated had any effect.

The lack of accumulation of ubiquitinated proteins and the lack of an effect of nicotine on the degradation of CD3Δ excludes in our cell system any possible direct effect of nicotine on proteoseome activity.

4.8 NICOTINE FAVOURS THE EXIT OF THE RECEPTOR FROM THE ER MEMBRANE AND ITS ARRIVAL ON THE PLASMA MEMBRANE

Under physiological conditions the receptors that contribute to the cell function and excitability are those that reside at the cell surface, so in order to discriminate between cell surface and intracellular receptor, due to the fact that ^{3}H-epibatidine diffuse into the cells and binds both pentameric intracellular and plasmamembrane receptors, IF approach and biotinylation of surface receptors were used.

HeLa cells were transfected with α3 and β4 subunits and the plasma membrane marker FP-22. After 24h of transfection (Fig 20, panel a), cycloheximide was added for 8h (panel b), in this way the pre-synthesided receptors were degraded and new-synthesised receptors were assembled in presence (panel d) or not (panel c) of 1mM nicotine for 22h. Cells were fixed and analysed by IF with anti-β4 antibody. Only when nicotine is present during receptor synthesis (panel d) a plasma membrane staining is detectable, as

demonstrated by the co-localization with the plasma membrane marker FP22 (panel e-g), indicating that more pentameric receptors can leave the ER membrane and reach the plasma membrane. The increase of receptor trafficking was obtained also with low concentrations of nicotine (Fig 20, B): the number of cells with staining for plasma membrane (measured as percentage to total cells) obtained measuring the co-localisation of $\alpha 3\beta 4$ nAChRs with the plasma membrane marker FP-22 in cells fixed after treatment with 8h of CHX and then 22h with nicotine or not, increases in progressive manner with higher concentrations of nicotine (from 0.01 mM to 1 mM).

To determine the amount of $\alpha 3\beta 4$ receptors that reaches the plasma membrane, a cell-surface biotinylation assay was performed (Fig.8) applying the same experimental scheme of previous IF analyses, but in biotinylation assay Hela cells were transfected with $\alpha 3$, $\beta 4$ subunits and cytoplasmatic GFP (as a control of transfection efficiency and goodness of biotynilation assay, i.e. not biotinylation of intracellular protein).

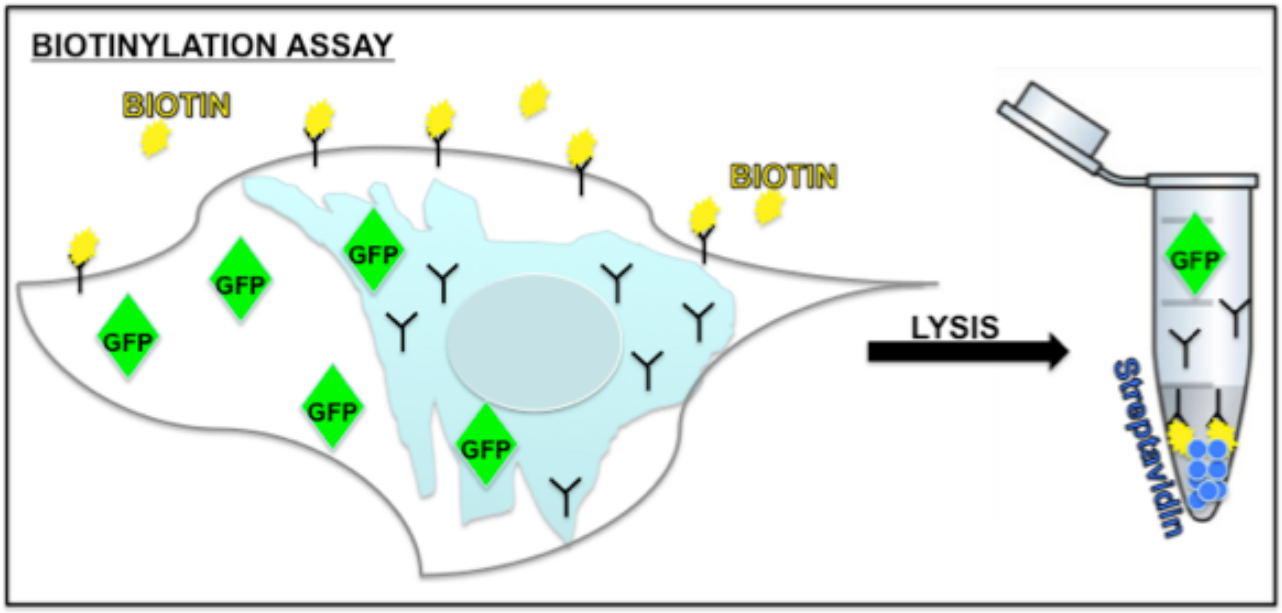

Fig. 8 Experimental scheme

After treatments, cells were incubated with cell-impermeable biotin (0,3 mg/ml)in PBS for 20 minutes at 4°C: all plasma membrane proteins were linked to biotin. After it, free biotin was quenched with 50 mM glycine for 10 minutes. Cells were lysated in 1% SDS buffer and boiled for 3 minutes. The same amount of proteins (measured with BCA assay) were attached O.N. at RT to streptavidin beads, that allow to recovery biotinylated plasma membrane proteins. After centrifugation, the Not-bound proteins (NB), including the intracellular GFP, were recovered in supernatant, the bound proteins (B) were collected

after boiled streptavidin beads for 3 minutes to detached biotinylated proteins. The nAChRs were revealed with anti-β4 antibody and the protein GFP with anti-GFP antibody: when nicotine is present, the amount of receptor doubles (Fig 21, input 1 and 2). If nicotine is present during the receptor synthesis, a nearly three folds increase of surface receptor was observable (lane 1B in compare to lane 2B). This effect is quantified (fig 21, left panel): in control sample, treated for 8h with cycloheximide and then for 22h without nicotine (1), only the 2,5% of total receptors is localised at the plasma membrane in comparison with 7,5% of sample exposed for 8h with cicloheximide and then for 22h with 1mM nicotine (2). GFP, as expected for intracellular proteins, is not biotinylated (Fig 21, A). So, the presence of nicotine during receptor synthesis increases almost three folds the bound fraction that represents the surface receptors, although the mature α3β4 that reach the cell surface is a small proportion of the total receptor pool.

4.9 THE BLOCKING OF PROTEASOME ACTIVITY HAS NOT EFFECT ON α3β4 TRAFFICKING

The positive effect of nicotine on the receptor trafficking is not merely due to the higher amount of receptor in the ER that escape proteasome degradation, because incubation with the proteasome inhibitor MG132 does not lead to the same effect (Fig 22, B). Incubation with the proteasome inhibitor MG132 for 8h increases the amount of nicotinic subunits in transfected HeLa cells: it is demonstrated by IF (Fig 22, A), ^{3}H-epibatidine binding receptors (B) and WB analysis (C, quantification panel on the right). Most of these subunits are assembled to form pentamers (Fig 23, A), as shown by the ^{3}H-epibatidine binding of the sucrose gradient fractions, but the resulting receptors can't leave the ER membrane, as demonstrated by the lack of co-localization with the plasma membrane marker FP22 (Fig 23, B) In conclusion, even if the incubation with MG132 prevents the proteasome degradation of the α3 and β4 subunits (their amount double) and the pentamers are correctly assembled, the receptor can't leave the ER.

4.10 EXPORT SEQUENCE IS NECESSARY AND SUFFICIENT FOR α3β4 EXIT

The mechanism by which nicotinic receptors leave the ER is poorly understood. Some data indicate that nicotinic receptors are recruited by Sec 24 proteins at the level of ER exit sites (Srinivasan P, 2011 and Mancias JD, 2008). The presence of "retention" and "export" motifs in the sequences of α or β subunits and the stoichiometry of the assembled pentamers seem to be crucial for receptor export. It has been reported that h-β2 has one ER retention motif and one ER export motif (Lester HA, 2011), whereas we found that the human β4 subunit has no "retention" sequence (RRQR) but has an "export" motif (LFM) in position 345-347 in the cytosolic loop between the transmembrane domains M3 and M4.

In order to abolish this export motif, we mutated the Leu in position 345 to Met (as indicated in the Fig.9) and the cells were then transfected with the mutated β4* subunit together with the wild type α3 subunit, for which no ER export/retention motif has been described so far.

Fig. 9 H-β4 sequence with ER export motif (LFM) mutated into MFM

24h after transfection, the cells were incubated for 8h with cycloheximide to allow the degradation of the pre-synthesised receptors, and then incubated 22h in the presence or absence of nicotine before being fixed and immunostained with the anti-α3 antibody. Fig 24 A shows the percentage of cells with α3 subunit plasma membrane staining. The deletion of the export motif led to the almost complete retention of α3β4* receptors at the level of ER membrane in the absence and presence of nicotine. In order to exclude a possible effect of the mutation on the pharmacological properties of the receptors we performed saturation binding experiments on WT and mutated α3β4* receptors under control conditions and after chronic nicotine treatment were performed (Fig. 24, B): ^{3}H-epibatidine had the same affinity for the WT and α3β4* (0,443 ±0,09 and 0,37±0,07 nM respectively) and both receptors were equally up-regulated by nicotine. These

experiments clearly indicate that nicotine is able to up-regulate the α3β4* receptor, but the lack of the export motif blocks the nicotine-induced increase in receptor traffic to the plasma membrane. In order to obtain a positive effect on nicotinic receptor trafficking the presence of the export motive is needed. Altogether these results clearly demonstrated that the increase in surface α3β4 receptors observed after nicotine exposure is caused by a decrease in receptor degradation and increase trafficking to the plasma membrane.

4.11 PERMEABLE COMPETITIVE LIGANDS RESEMBLE THE BEHAVIOUR OF NICOTINE

To analyse the mechanism by which nicotine has an effect on nicotinic receptor degradation and trafficking, HeLa cells (Fig 26, A) and SH-SY5Y (Fig 26, B) after transfection, were incubated with cytisine, CC4 or hexametonium (Fig.10). Cytisine and CC4 are permeable competitive nicotinic ligands (Fig.25), whereas hexamethonium is not permeable nicotinic ligand. After 24h of transfection, the results were analysed by WB (Fig. 26, upper panel) with anti-α3 and β4 antibodies and by ^{3}H-epibatidine binding analyses (Fig 26, lower panel).

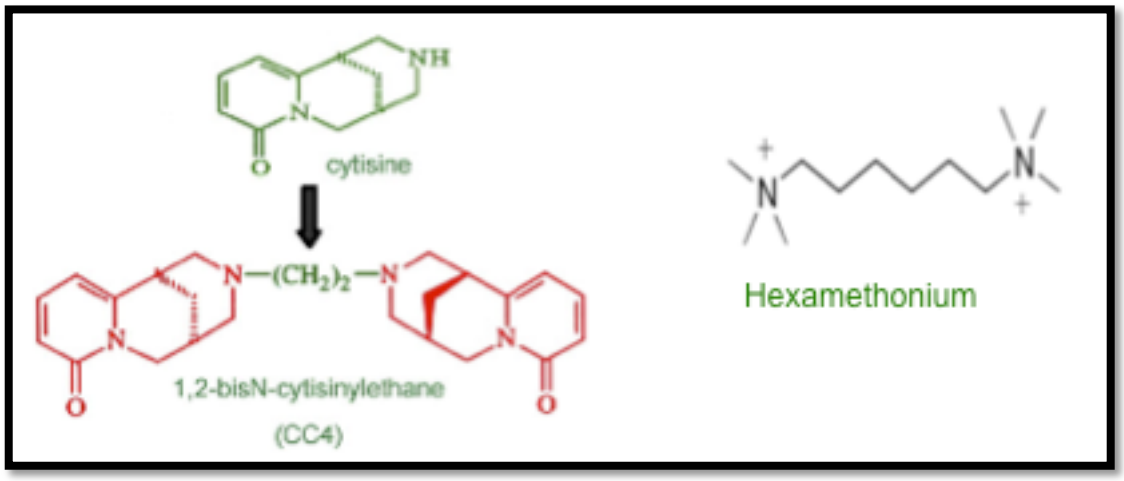

Fig. 10 Chemical structure of nicotinic ligands cytisine, CC4 and hexamethonium

Only the permeable ligands, regardless they are agonist (cytisine) or very partial agonist CC4 (Sala ME, 2012), up-regulate the α3β4 nicotinic receptor (lanes 2-3-4). The impermeable antagonist hexametonium does not increase the number of α3 and β4 subunits (upper panel, lane 5) and the pentamers assembled, measured by ^{3}H-epibatidine

binding experiments (lower panel, lane 5). In order to up-regulate the receptor, the ligand must bind the pentamers at the level of the ER membrane, thus preventing the retrotranslocation of the receptor in the cytosol and its subsequent proteosomal degradation. Moreover, like nicotine, the permeable ligand CC4 promoted the exit of receptors from the ER and their arrival at the plasma membrane (Fig 27, A), as demonstrated by the co-localisation with the plasma membrane marker FP-22 (Fig 27, B). To facilitate the exit from ER the ligand must act intracellularly.

4.12 PERMEABLE NON-COMPETITIVE LIGAND DOESN'T UP-REGULATE $\alpha 3\beta 4$ NACHR

As nicotine, cytisine and CC4 are all competitive ligands (Fig 25) that bind the same site of nicotine at the interface between the α and β subunit. To verify if the permeable non competitive antagonist, which binds to nicotinic receptors in the lumen of the channel rather than at the orthosteric site, has the same effect of permeable competitive ligands, mecamylamine was tested. Chronic treatment with mecamylamine did not up regulate $\alpha 3\beta 4$ in Hela cells, as demonstrated by WB for $\alpha 3$ and $\beta 4$ subunits (Fig 28 A, lane 3) and by ^{3}H-epibatidine binding (Fig. 28 B, lane 3).

These results show that, like nicotine, other permeable and competitive nicotinic ligands can up-regulate $\alpha 3\beta 4$ nicotinic receptors and favour their exit from the ER membrane and arrival at the plasma membrane. Moreover these data suggest that occupancy of the binding site, but not ion channel activity, are required for receptor up-regulation.

4.13 NICOTINE INDUCES AN $(\alpha 3)_2(\beta 4)_3$ STOICHIOMETRY

Recently it has been demonstrated that the major subtype express in brain, the nicotinic $\alpha 4\beta 2$ subtype, is upregulated by nicotine via a stochiometry- dependent mechanism: in this model nicotine stabiles the $(\alpha 4)2(\beta 4)3$ stoichiometry in the ER. Like other neuronal nicotinic receptors, also the $\alpha 3\beta 4$ subtype can exist in two different stochiometry depending on whether is expressed in oocyte or mammalian cell lines (Krashia P, 2010). So, in order to understand whether the stochiometry of the receptor can influence the

traffic and/or the effect of nicotine, the stoichiometry of α3β4 receptors was analysed both in control and nicotine treated HeLa cells.

To this end, in preliminary experiments increasing concentration of cell lysates (obtained from HeLa cells transfected with an equal ratio of α3 and β4 cDNAs) was analysed by WB using primary anti α3 and β4 antibodies and infrared-conjugated secondary antibodies, in particular the ratio of the infrared fluorescence signal of β4/α3 subunit of control and nicotine treated cells was analysed. After fixing the ratio between β4/α3 subunits of control cells as 1, the ratio in nicotine treated cells was derived. As shown in Fig 29, after nicotine treatment there is an increase of the β4/α3 ratio (A: 1,39±0,03, n=3), indicating that in the receptor there are more β subunits in comparison to untreated sample. The same result was obtained in cells (B) incubated with nicotine for 22h after cycloeximide exposure (1,37±0,01, n=4). This means that nicotine, present during synthesis, induces the assembly of receptors that have the unique stoichiometry of α3:β4= 2:3. Similar results was obtained in HeLa cells treated with CC4 after cycloeximide exposure (mean of the ratio± SE was 1,292±0,004, n=4) and in HeLa cells transfected with the mutated b4* subunit (mean of the ratio± SE was 1,424±0,01, n=3). However α3β4* is up-regulated and has a stoichiometry with α3:β4= 2:3, it cannot leave the ER.

These results clearly indicate that in the presence of nicotine or CC4, the receptor is less prone to proteasome degradation and can more easily exit from the ER, probably because of the presence of a higher number (3 instead of 2) of export motif (LFM) that allow a more efficient recruitment at the ER exit sites.

4.14 THE MUTATION OF NICOTINIC BINDING SITE ON THE α3 SUBUNIT ACTIVATE CONSTITUTIVELY THE RECEPTOR

To confirm the importance of the nicotinic binding site for the effect of nicotine and other competitive permeable nicotinic ligands, we mutated the tryptophan (W) to alanine (A) in position 180 on the α3 subunit, using a site-directed mutagenesis kit. The tryptophan (W) in this position it has been demonstrated being very important for the binding of nicotinic ligands (Xiu X, 2009).

H-α3 161-IDVTYFPFDYQNCTMKFGS**W**SYDKAKIDLVLIGSSMNLKD-200

H-α3 161-IDVTYFPFDYQNCTMKFGS**A**SYDKAKIDLVLIGSSMNLKD-200

HeLa cells transiently transfected with α3∗β4 subunits for 24h were analysed by IF with α3 antibody (Fig.30, A): even in the absence of nicotine, in comparison with the wt receptor, the α3∗β4 receptors leave easily the ER and reach the plasma membrane very efficiently: more than the 80% of cells show a plasma membrane staining for α3∗β4 receptor, as shown in the quantification panel (B). The α3∗β4 receptor exit efficiently from the ER, but, as expected after site-directed mutagenesis, is no more sensitive to nicotine up-regulation, as demonstrated in WB analyses of the α3 and β4 subunits (C) and in ^{3}H-epibatidine binding analysis (D). This result strongly confirmed the importance on the LBD for nicotine up-regulation, but why this mutation facilitates the receptor exit from the ER to plasmamembrane remains to be elucidated.

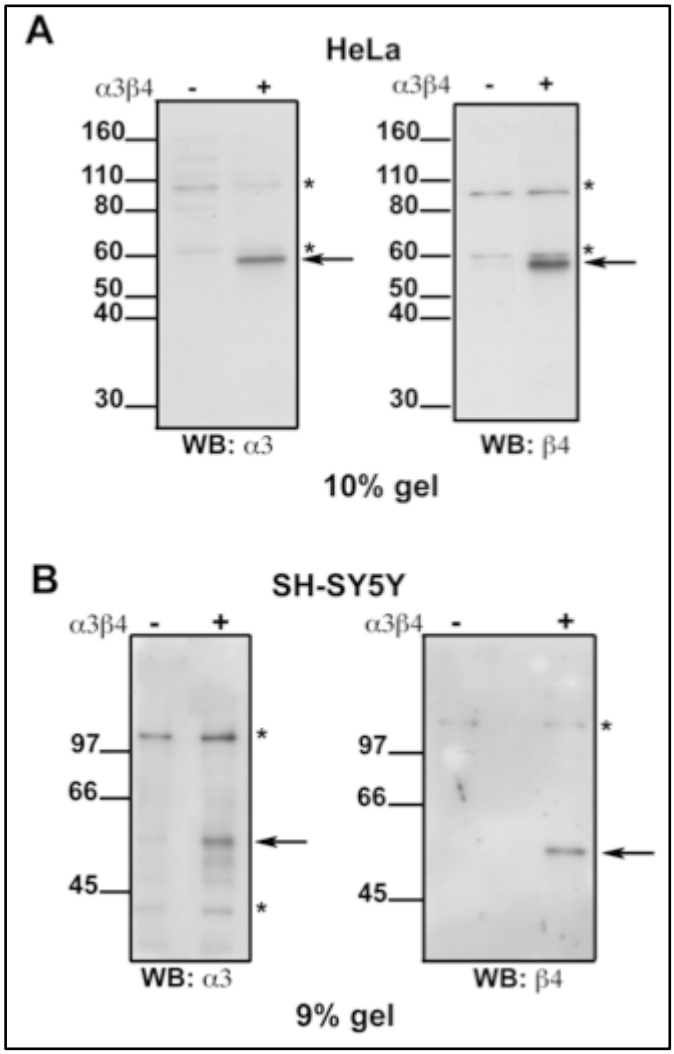

Fig. 11 Characterisation the anti-human α3 and anti-human β4 antibodies

Cell lysates of HeLa (A) or SH-SY5Y (B) cells, transfected or not with α3 and β4 subunit cDNAs as indicated, were loaded on SDS-PAGE. After Western blotting, membranes were incubated with the anti-α3 (left panel) or anti-β4 antibodies (right panel). The nicotinic subunit is indicated by an arrow, the asterisks indicate non-specific bands.

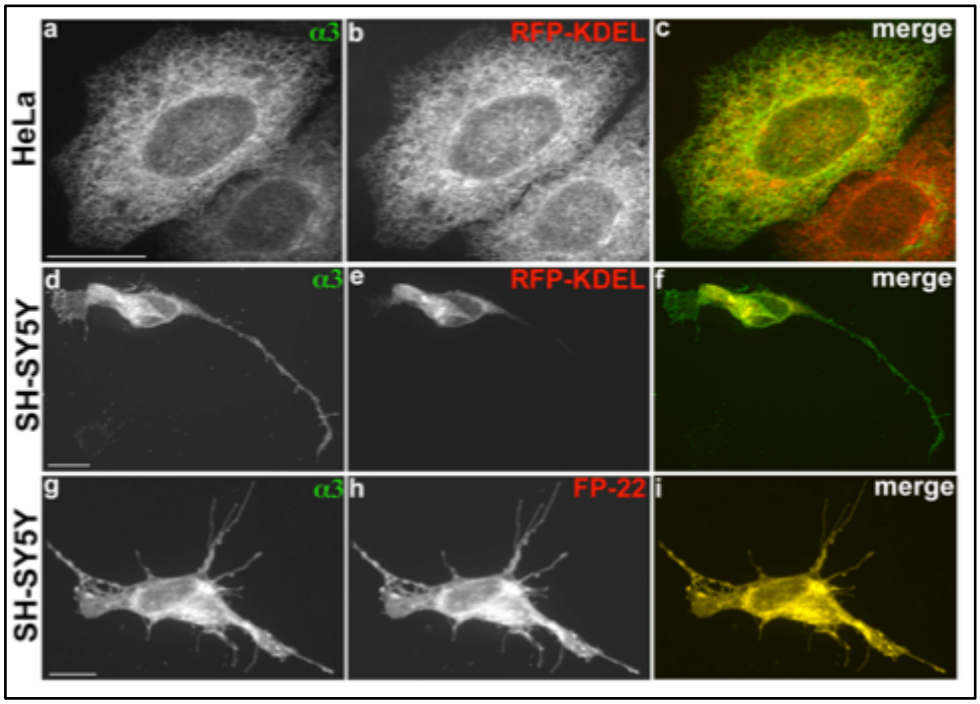

Fig. 12 Expression of nicotinic receptors in cells.

HeLa (a-c) or SH-SY5Y cells (d-f, g-i) were transfected with α3 and β4 nicotinic subunit cDNAs and the cDNA of the ER protein RFP-KDEL (a-c and d-f) or the plasma membrane fluorescent protein FP22 (g-i). After 24h transfection, the cells were fixed, permeabilised and immunostained with antibody against the α3-antibody, followed by coniugated secondary antibodies. Confocal images showed immunostaining for the α3 subunit (a,d.g), the fluorescence of the ER (b,e) or PM marker (h), and the merge (c,f,i).

Scale bar= 10 μm.

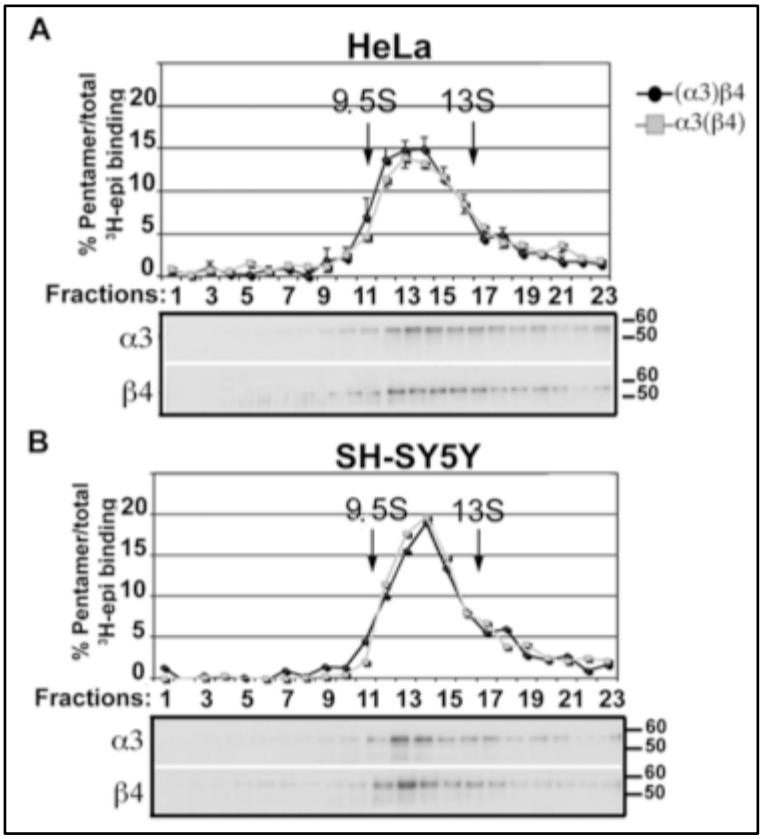

Fig. 13 Analysis of α3β4 receptors expressed in HeLa and SH-SY5Y cells.

Upper panel: sucrose gradient analysis. 2% Triton X-100 extracts (500 ul) obtained from HeLa **(A)** and SH-SY5Y **(B)** cells transfected with the α3β4 subtype were loaded onto a 5-20% sucrose gradient in phosphate buffer saline pH 7.5, 0.1% Triton X-100 and 1 mM PMFS, and centrifuged for 14 hours at 40000 rpm in a Beckman rotor at 4°C. The fractions were collected, added to anti-α3 or β4 antibodies bound to microwells, left for 24 hours, and then assayed for ^{3}H-epibatidine binding as described in Materials and Methods. As a standard, ^{125}I-αBungarotoxin labelled torpedo AChRs were subjected to sucrose gradient centrifugation in parallel, and the radioactivity of the fractions was determined by means of γ-counting; the peaks of the monomer (9.5S) and dimer (13S) of pentameric Torpedo AChR are shown.

Lower panel: Western blot analysis of the sucrose gradient fractions. The fractions obtained from the gradients were prepared as described in Materials and Methods. The proteins were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, probed with 5 μg/ml of the primary antibodies, and then incubated with the secondary antibody (anti-rabbit conjugated to peroxidase, dilution 1:40000). The bound Abs were revealed by a chemiluminescent substrate (Pierce, USA)

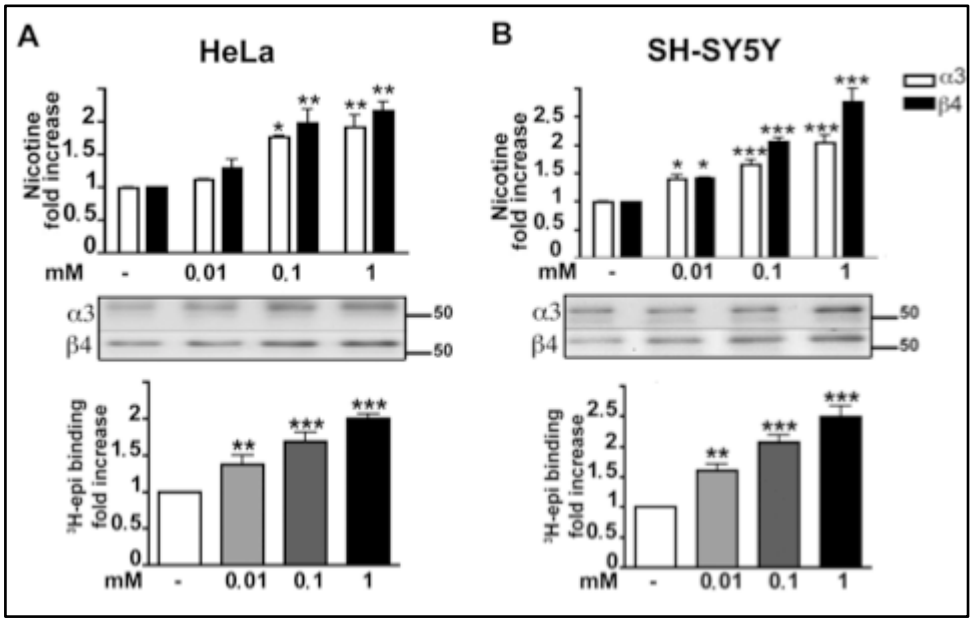

Fig. 14 Chronic treatment with nicotine increases the number of nicotinic subunits and the number of assembled pentamers.

(A) Upper : Western blot analysis of cell lysates. After transfection with α3 and β4 nicotinic subunit cDNAs, HeLa cells were incubated for 24h with the indicated concentrations of nicotine, lysed and the extracts were analyzed by means of Western blotting with the α3 and β4 antibodies. The result of five experiments with the α3 (white columns) or β4 (black columns) are shown in the quantification panel. A representative experiment of cell lysates probed with the α3 and β4 antibody is shown under the quantification panel. **Lower**: ^{3}H-epibatidine binding to membrane homogenates of cells expressing $\alpha 3\beta 4$ receptors. The cells were grown in the presence of the indicated concentration of nicotine for 24 hours before ^{3}H-epibatidine binding was measured. The concentration of ^{3}H-epibatidine used when measuring binding site density was 2 nM. The results are expressed as the fold increase over control samples grown in parallel in the absence of nicotine. Mean values and standard error of the mean (S.E.M.) from six independent measurements. One-way ANOVA with Bonferroni 's post multiple comparison test. *P<0.05; **P<0.001; ***P<0.0001

(B) As in **(A)** except that SH-SY5Y cells were transfected and the Western blot results are the mean values and ± S.E.M of six experiments. The lower panel shows the results of six of ^{3}H-epibatidine binding experiments.One-way ANOVA, Bonferroni multiple comparison test. *P<0.05; **P<0.001; ***P<0.0001

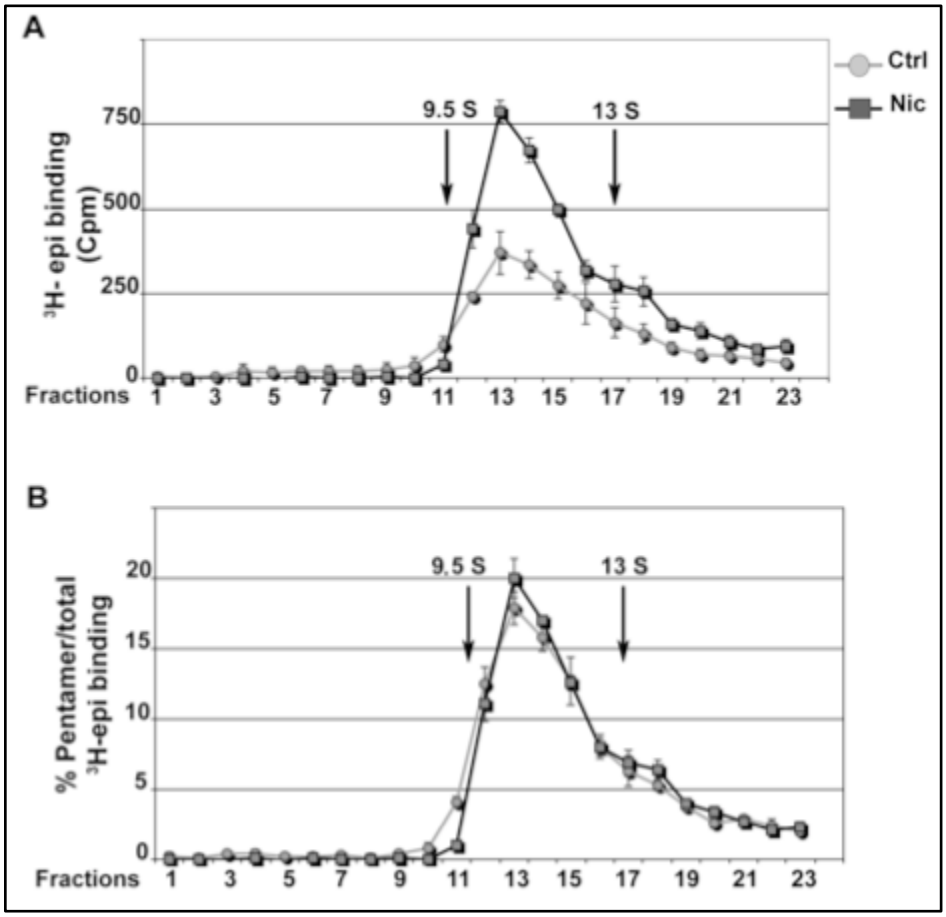

Fig. 15 Nicotine increases the number of pentameric α3β4 receptors

(A) Specific ^{3}H-epibatidine binding to fractions obtained from sucrose gradient sedimentation. 2% Triton X-100 extracts (500 µl) obtained from HeLa cells transfected with the α3β4 subunits and treated for 24 hours with control medium or 1 mM nicotine were loaded onto a 5-20% sucrose gradient. The fractions were collected, added to anti-β4 antibodies bound to microwells, left for 24 hours, and then assayed for ^{3}H-epibatidine binding. The arrows indicate the position of the ^{125}I-αBungarotoxin labelled Torpedo monomer (9.5S) and dimer (13S).

(B) The percentage of pentameric over total ^{3}H-epibatidine labeled receptors in the gradient fractions of control and 1 mM nicotine treated cells .

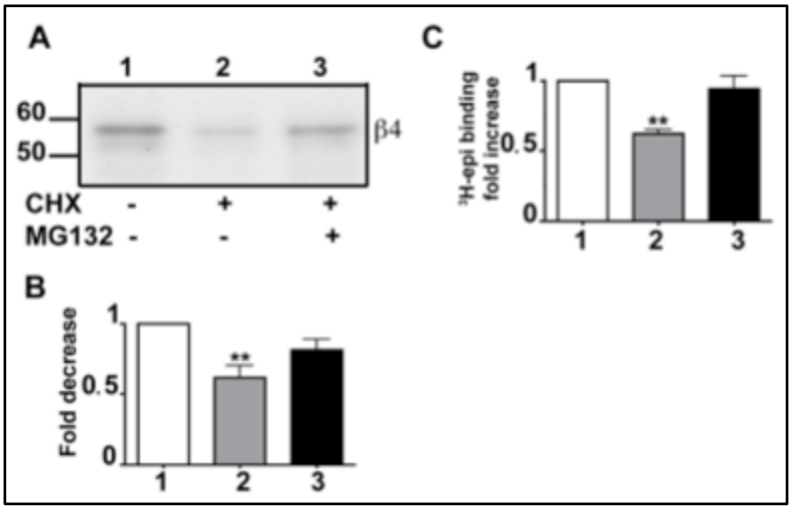

Fig. 16 Nicotinic α3β4 receptors are quickly degraded by proteasome.

HeLa cells transfected with α3 and β4 nicotinic subunit cDNAs were incubated with 50 ug/ml cycloheximide (CHX) (lane 2 and column 2), or CHX plus 10 uM of the proteasome inhibitor MG132 (lane 3 and column 3). After incubation the cells were lysed and their subunit content was analyzed by Western blotting **(A)** and the quantified **(B)** six independent (columns1-2) and two independent experiments (column 3) are shown. The pentamers in the cell homogenates were quantified by ^{3}H-epibatidine binding **(C)** in five independent experiments. The value of ^{3}H-epibatine binding obtained in the absence of CHX and MG132 was set to 1.

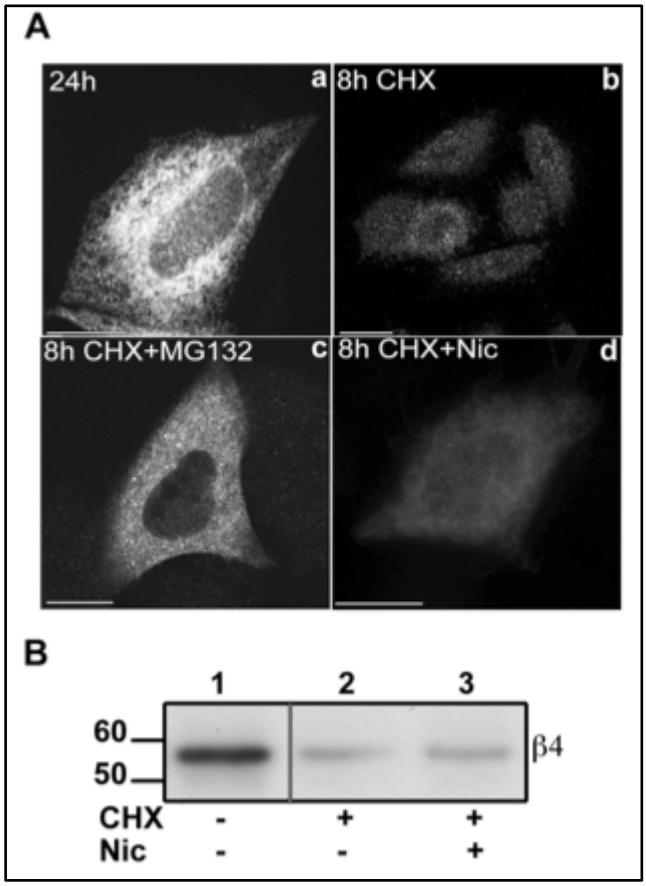

Fig. 17 Nicotine cannot act on pre-synthesised receptors.

HeLa cells after 24h transfection with α3 and β4 nicotinic subunit cDNAs (A, image a and B, lane 1), were incubated with CHX alone (**A,** image b and **B**, lane 2), CHX plus MG132 (**A**, image c), CHX plus 1 mM nicotine (**A**, image d and **B**, lane 3). After 8h of incubation, the cells were either fixed and immunostained with anti-α3 antibody **(A)**, or lysed and analyzed by Western blotting with anti β4 antibody **(B)**.

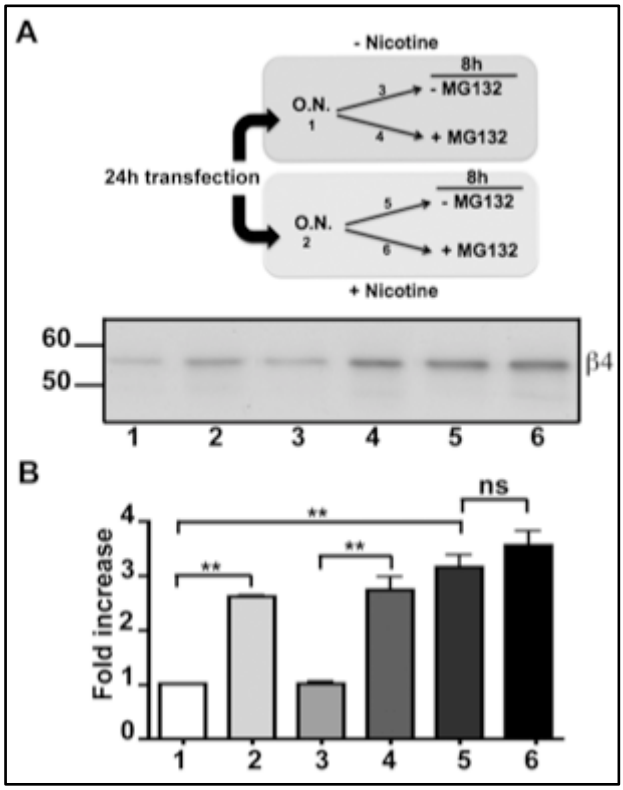

Fig. 18 α3β4 receptors synthesized in the presence of nicotine are less prone to proteasome degradation.

(A) Twenty four hours after transfection the cells were incubated O.N. in the presence (2) or absence (1) of 1 mM nicotine, as shown. One petri dish of cells was lysed for each condition and parallel petri dishes were incubated for an additional 8h in the absence (3,5) or presence (4,6) of the proteasome inhibitor MG132 before being lysed. The amount of nicotinic subunits present in each extract was analyzed by Western blotting using anti-β4 antibody. The quantification **(B)** shows the results of three independent experiments. The optical density obtained after O.N. incubation in the absence of nicotine was set to 1.

One-way ANOVA, Bonferroni's multiple comparison test. *P<0,05; **P<0,001; ***P<0,0001

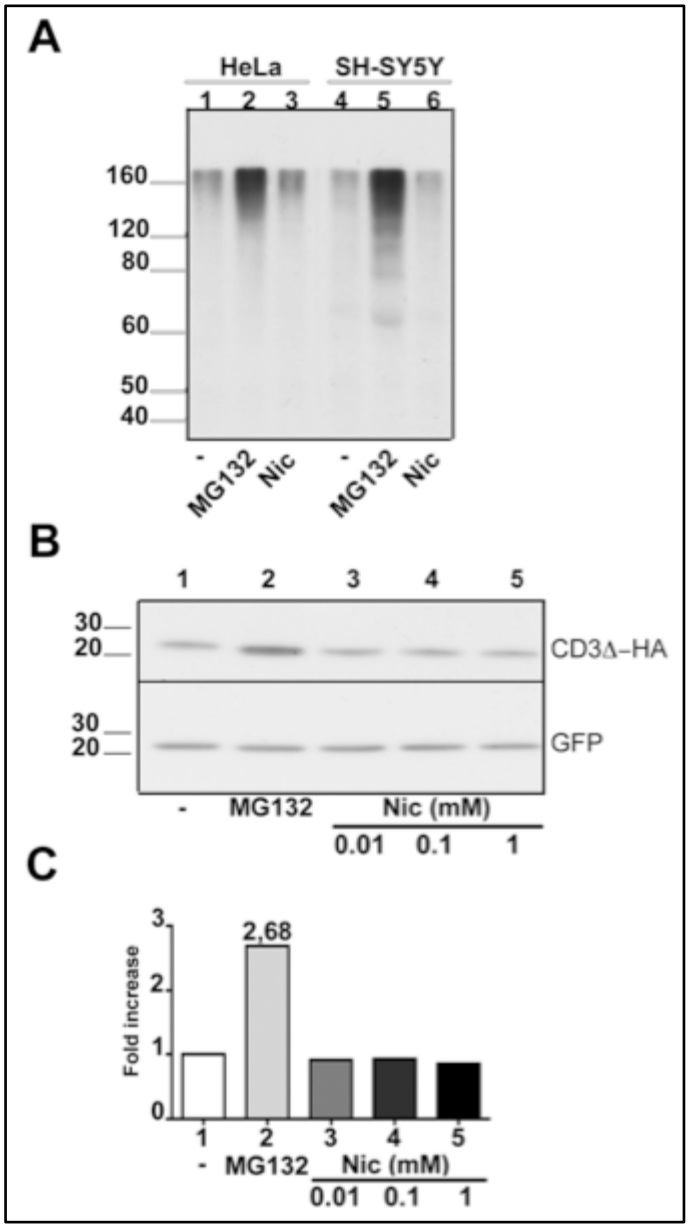

Fig. 19 Nicotine does not impair proteasome activity.

(A) Hela (lanes 1-3) and SH-SY5Y cells (lanes 4-6) cells were incubated with CHX 50 ug/ml for 3h in the absence (lanes 1, 4) or in the presence of 10 μM MG132 (lanes 2,5) or 1 mM nicotine (lanes 3,6). The cell lysates were then run on SDS-PAGE, and blotted after which the membranes were incubated with anti-ubiquitin antibody. **(B)** The degradation of the proteasome substrate CD3Δ is not affected by the presence of nicotine. HeLa cells were transfected with HA−tagged CD3Δ and soluble GFP (as internal control). After 24h the cells were incubated for 3h with CHX 50 μg/ml and 10 μM of the proteasome inhibitor MG132 or the indicated concentration of nicotine. The cell lysates were then run on SDS-PAGE, blotted and the proteins were revealed using anti-HA and anti-GFP antibodies. **(C)** Quantification of the amount of CD3Δ present under each condition.

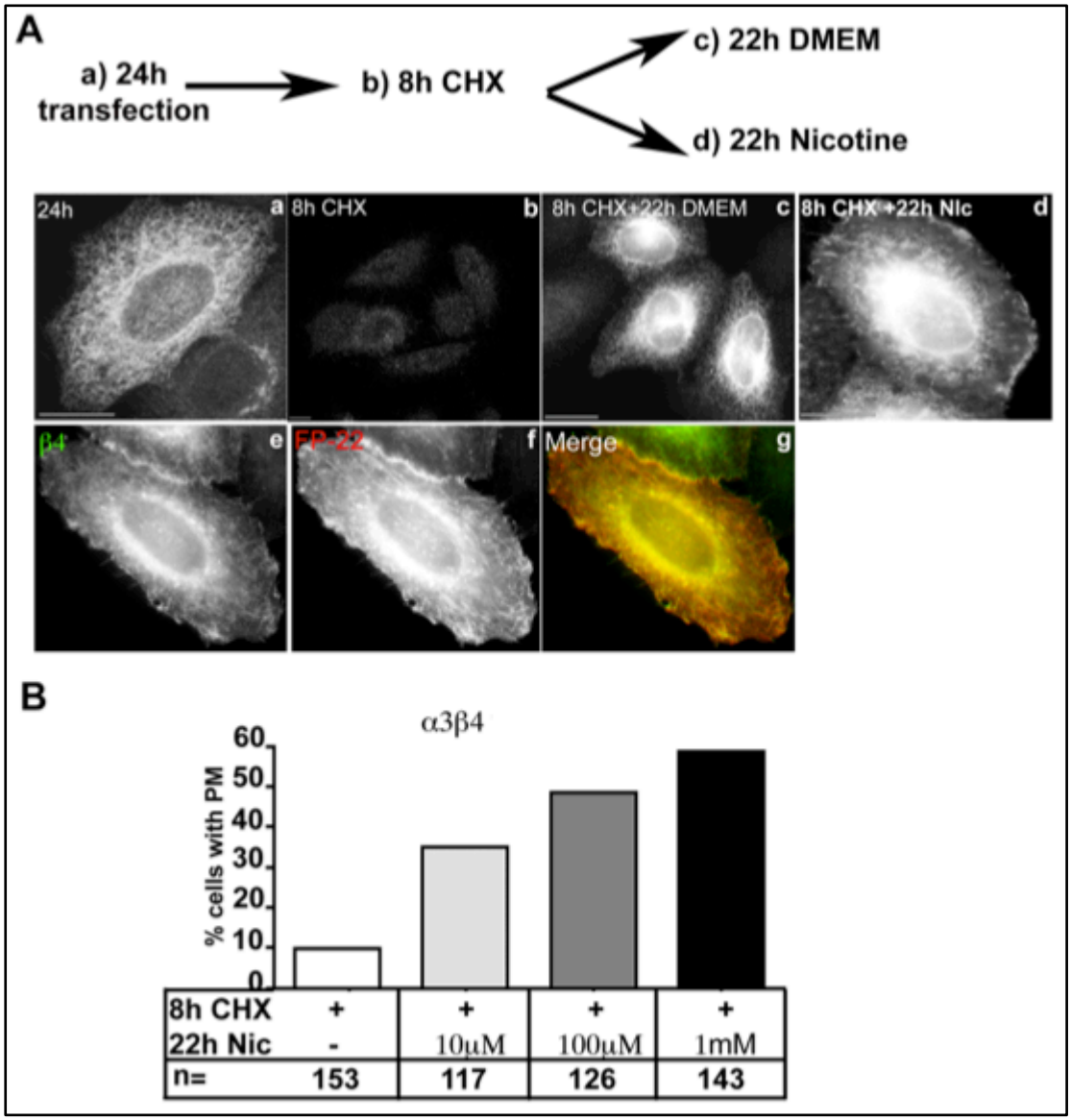

Fig. 20 Nicotine facilitates the receptor arrival at the PM.

(A) HeLa cells transiently transfected with nicotinic subunit cDNAs (a) were treated for 8h with CHX (b) and then incubated for 22h in the presence (d) or absence of 1 mM nicotine (c). They were then fixed, permeabilised and immunostained with the anti β4 antibody (a-d). HeLa cells transfected with the α3 and β4 nicotinic subunit cDNA and the cDNA of the fluorescent plasmamembrane marker FP-22 were incubated for 8h with CHX and then for 22h with nicotine. The confocal images show the staining of the β4 antibody (e), the fluorescence of the marker (f) and the merge (g). Scale bar= 10 μm

(B) After transfection with α3 and β4 subunit cDNAs, cells were incubated for 8 h with CHX and then for 22 h with the indicated concentration of nicotine. They were then fixed, permeabilized and immunostained with anti-a3 antibody, and the number of cells with PM staining was counted.

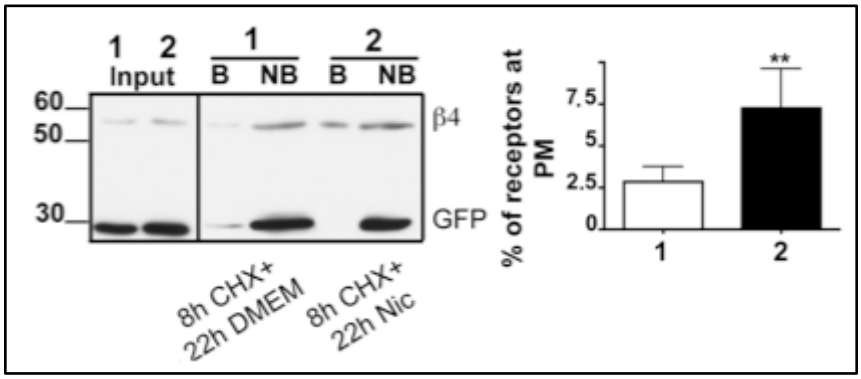

Fig. 21 Plasma membrane biotinylation assay.

HeLa cells, transfected with the nicotinic α3 and β4 subunit cDNAs plus soluble GFP cDNA were treated for 8h with CHX and then for 22h in the absence (sample 1) or in the presence (sample 2) of 1 mM nicotine. Cells were then incubated with EZ-link NHS-SS-biotin for 20 min at 4°C, after which an excess of glycine was added for 10 min to quench free biotin, and the cells were then lysed. A fraction of the cell lysate was directly analyzed on SDS-PAGE followed by Western blotting (input), while the remaining samples were incubated with streptavidin beads followed by centrifugation to recover the bound (B) and non-bound (NB) fraction. After blotting, nitrocellulose membranes were stained with anti-β4 and anti-GFP antibodies. The input samples correspond to ¼ of non bound and 1/16 of bound samples. The bar graph on the right shows the percentage of β4 subunit recovered by streptavidin beads (n=5) under control condition (1) or after nicotine treatment (2).

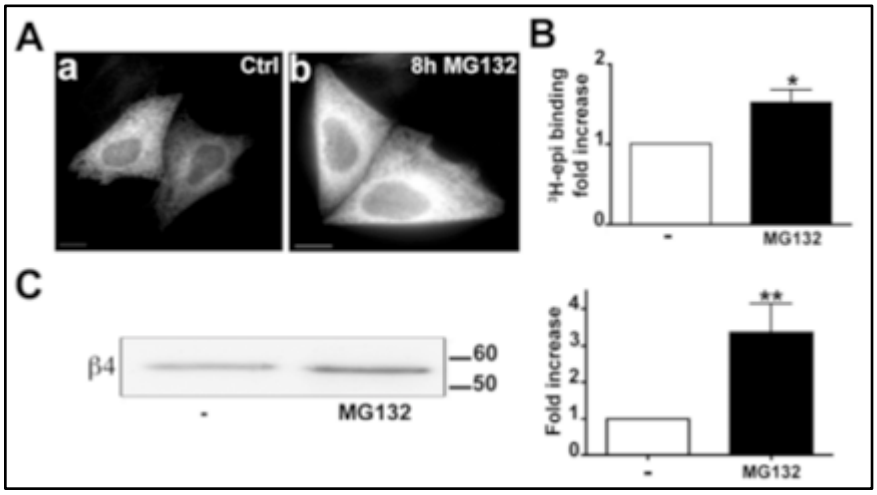

Fig. 22 Inhibition of the proteasome increases the number of α3β4 receptors

24h after transfection with the nicotinic subunits cDNAs, HeLa cells were incubated or not for 8h in the presence of the proteasome inhibitor MG132 at a concentration of 10 μM. The cells were then fixed, permeabilysed and immunostained with anti-α3 antibody **(A),** analysed by ^{3}H-epibatidine binding **(B)** or lysed **(C). (C)** The lysed cells were analysed by Western blotting using an anti-β4 antibody (left panel). The right panel shows the quantification of three experiments, the amount of β4 subunit revealed in the absence of MG132 was set to 1.

Unpaired t test. *P<0,05; **P<0,001

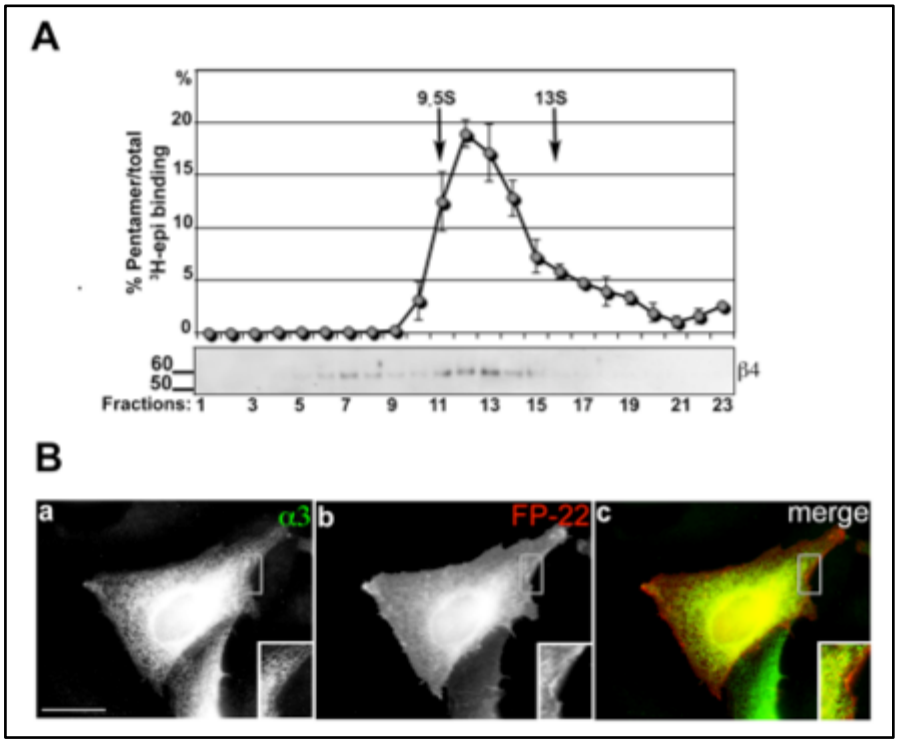

Fig. 23 Inhibition of the proteasome does not promote their exit from the ER.

(A) ^{3}H-epibatidine binding (upper panel) and Western blotting analysis (lower panel) of the fractions obtained from sucrose gradient sedimentation of 2% Triton X-100 extract obtained from cells treated for 8h with 10 μM MG132.

(B) HeLa cells were transfected with α3 and β4 subunit cDNAs and the cDNA of the fluorescent plasmamembrane marker FP-22. 24h after transfection, the cells were incubated for 8 h with 10 μM MG132 after which they were fixed, permeabilized and immunostained with the anti-α3 antibody. The confocal images show the staining obtained using the anti-a3 antibody (a), the fluorescence of FP-22 (b) and the merge (c). (Scale bar=10 μm). The insets show a 3X enlargements.

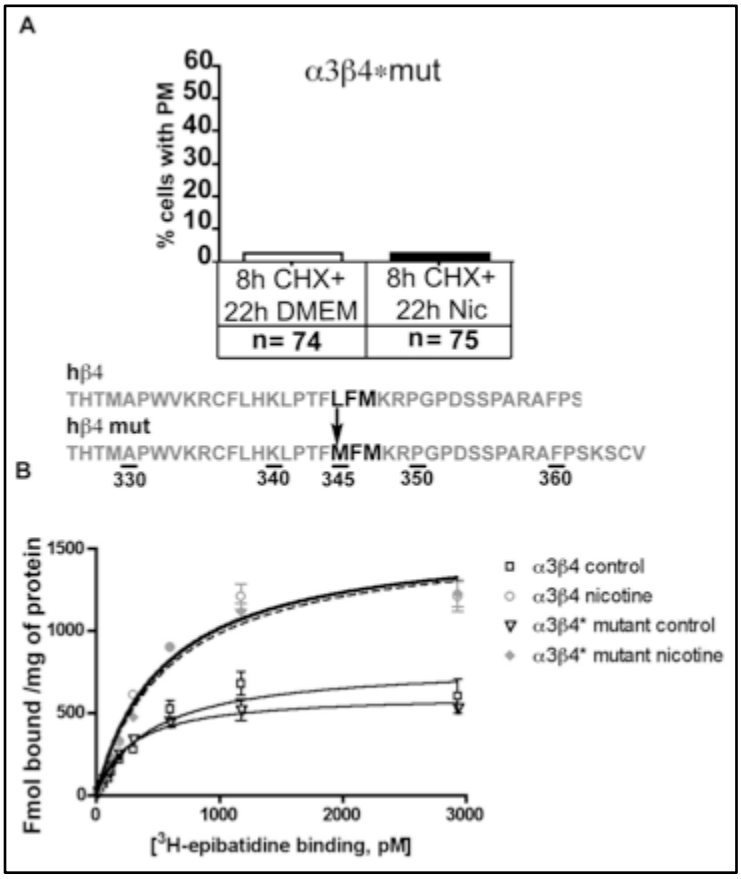

Fig. 24 The LFM export motif, is necessary for the receptor to leave the ER membrane.

(A) HeLa cells were transfected with wild type α3 cDNA and cDNA of the β4* subunit with a point mutation that disrupts the ER export motif LFM (the aminoacid mutated is shown below). Cells were incubated for 8 h with CHX and then for 22 h with 1mM nicotine. They were then fixed, permeabilized and immunostained with anti-α3 antibody, and the number of cells with PM staining was counted.

(B) Pharmacological characterization of HeLa cells transfected with WT α3β4 or mutant α3β4* receptors. Saturation curve of specific ^{3}H-epibatidine binding to membrane homogenates of control and nicotine-treated HeLa cells. The binding curves are from a representative experiment in which ligand concentrations were tested in triplicate and the data are expressed as fmol bound of ^{3}H-epibatidine receptors /mg of protein. The data from three-four separate experiments were analysed by means of nonlinear regression analysis using Prism 4.0 (GraphPad, San Diego, CA).

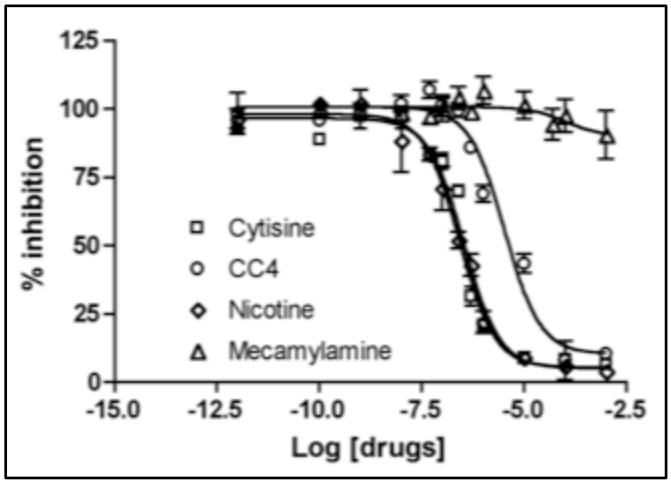

Fig. 25 Inhibition of ^{3}H-epibatidine binding to the α3β4 subtype by increasing concentration of CC4, cytisine, nicotine and mecamylamine.

The HeLa cell homogenates were incubated for 5 min with the indicated concentrations of compounds after which the ^{3}H-epibatidine (0.25 nM) was added and the samples were incubated overnight at 4°C. In each experiment, each dilution of the drug was tested in triplicate. All of the data are expressed as the percentage of specific ^{3}H-epibatidine binding obtained in the absence of drugs.

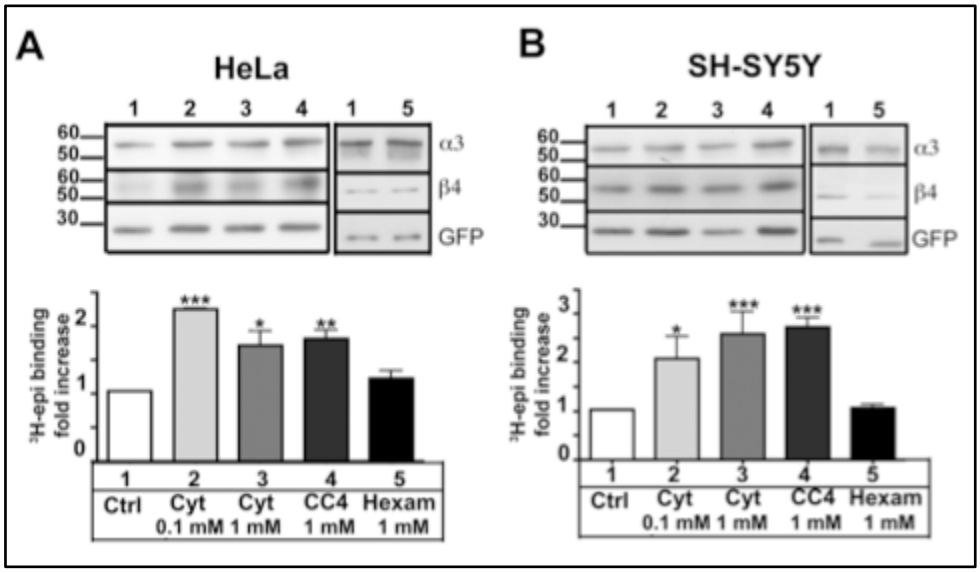

Fig. 26 Competitive nicotinic ligands up-regulation.

(A) Upper panel: after transfection with cDNAs coding for α3, β4 subunit and soluble GFP, HeLa cells were incubated for 24h in the absence (lane 1) or presence of the agonist cytisine (lanes 2 and 3), the very partial agonist CC4 (lane 4) or the non permeable antagonist hexametonium (lane 5). The cell lysates were than analyzed on SDS-PAGE and the subunits were revealed using anti-α3 and anti-β4 antibodies. The GFP signal was used to normalize transfection efficiency. **Lower panel**: ^{3}H-epibatidine binding to membrane homogenates obtained from cells expressing α3β4 receptors treated with the indicated concentrations of cytisine (lanes 2 and 3), CC4 (lane 4) or hexametonium (lane 5). The results obtained from three independent experiments, are expressed as the mean values ± S.E.M. of the fold increase over the control sample grown in parallel in the absence of drugs.

(B) Upregulation of nicotinic receptors in SH-SY5Y cells. The same experiment as in **(A)** of the figure except that SH-5YSY cells were transfected. The results of the binding assay are the mean values ± S.E.M of the three-four independent experiments.

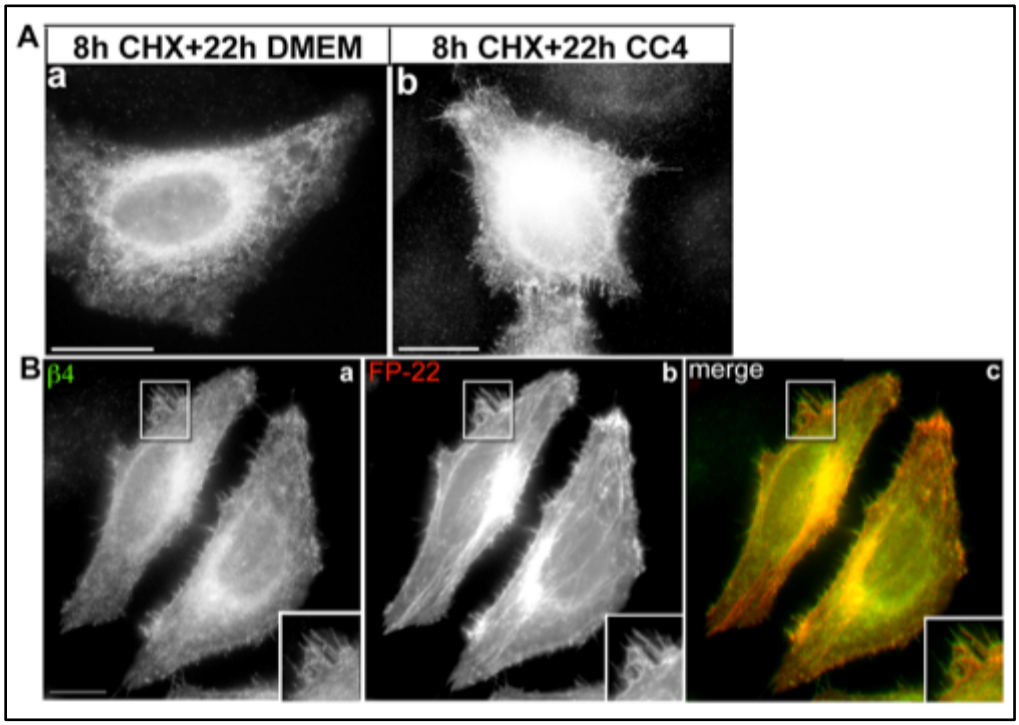

Fig. 27 The permeable competitive ligand CC4 favors the arrival of the receptor at the plasmamembrane.

(A) HeLa cells transfected with α3 and β4 subunit cDNAs were incubated for 8h with CHX and then for 22h in the absence (a) or presence (b) of the permeable competitive partial agonist CC4 at the concentration of 1 mM. The cells were then fixed, permeabilized and immunostained with the anti-α3 antibody (bar=10 μm).

(B) HeLa cells were transfected with α3 and β4 subunit cDNAs and with FP-22 cDNA. Twenty-four hours after transfection the cells were incubated for 8h with CHX, and then for 22h with 1 mM CC4. They were then fixed, permeabilised and immunostained with the anti-β4 antibody. The confocal images show the staining with anti- β4 antibody (A), the fluorescence of the FP-22 (b) and the merge (c). Scale bar =10 μm. The insets show 3X enlargements.

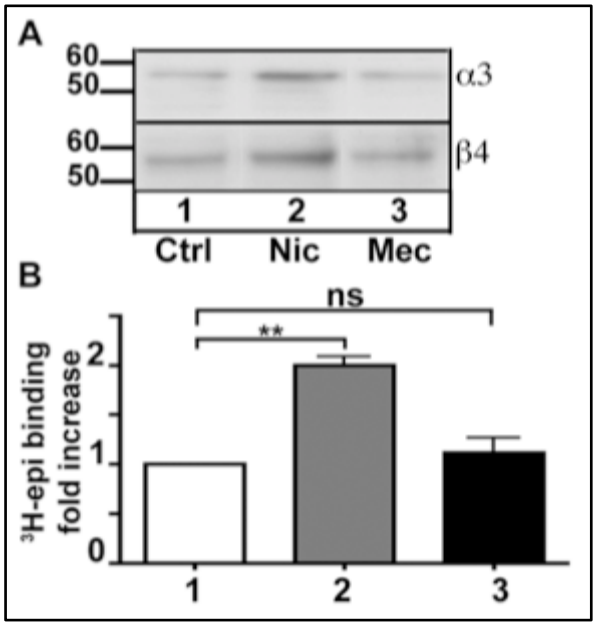

Fig. 28 Mecamylamine does not up-regulate $\alpha 3\beta 4$ receptors.

(A) HeLa cells transfected with α3 and β4 subunit cDNAs were incubated for 24h in the absence (lane 1) presence of 1 mM nicotine (lane 2) or 1 mM mecamylamine (lane 3). The cell lysates were then loaded on SDS-PAge and, after Western blotting, incubated with anti-α3 and anti- β4 antibodies.

(B) ^{3}H-epibatidine binding to membrane homogenates obtained from cells expressing α3β4 receptors treated with nicotine (lane 2) or mecamylamine (lane 3). The results of the binding assay are the mean values ± S.E.M of three independent experiments.

One-way ANOVA, Bonferroni's multiple comparison test. *P<0.05; **P<0.001; ***P<0.0001

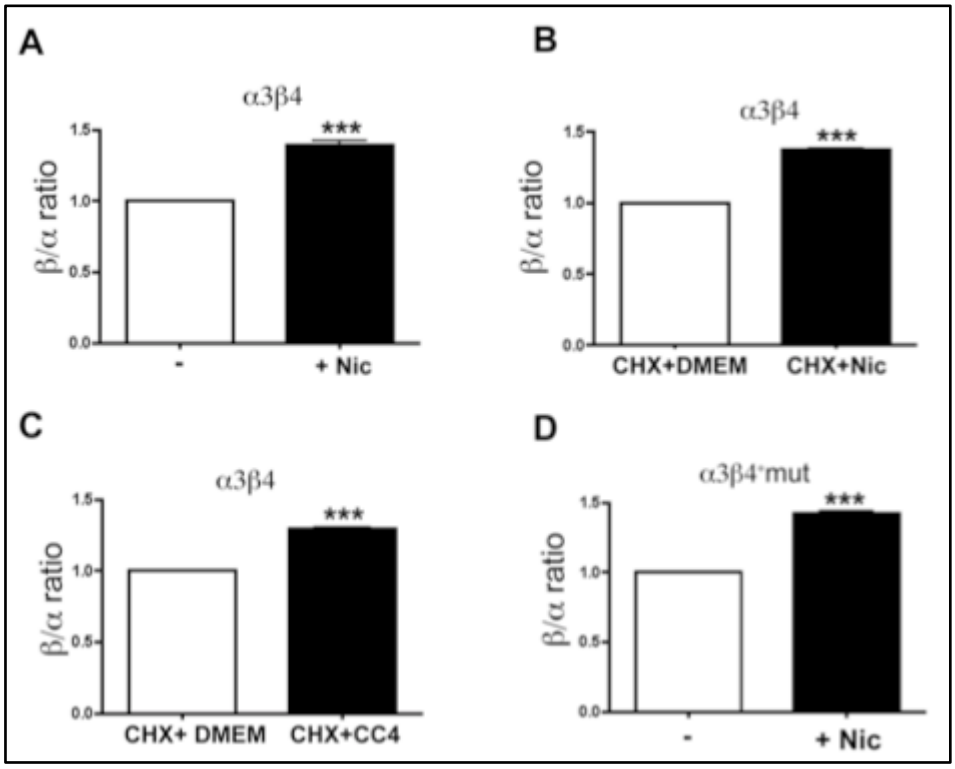

Fig. 29 Nicotine and CC4 promote the formation of pentameric receptors with 2α and 3β subunits.

(A, D) HeLa cells were transfected with cDNA coding for wild type α3 subunit and either for wild type β4 subunit **(A)** or for mutant *β4 subunit with the mutated export sequence (β4*mut, **D**). Twenty four hours after transfection, the cells were incubated for 24h with or without the 1 mM nicotine (**A, D**) or for 8h with CHX plus 22h with 1 mM of nicotine **(B)** or 1 mM CC4 **(C).** The cell lysates were run on SDS-PAGE and, after Western blotting, the membranes were incubated with anti-α3 or β4 antibody followed by incubation with an anti-rabbit infrared-conjugated secondary antibodies (IR). The IR signal was revealed using a Lycor Odyssey CLx and quantified using the Image Studio software. The mean β4/α3 signal ratio it is shown, having set the ratio obtained in the absence of nicotine to 1 (n=4 for A and n=3 for D). **(B and C)** We performed the same experiment as in A and D except that here cells, after transfection with the nicotinic wild type subunits, were incubated for 8h with CHX and then for 22h with or without 1 mM nicotine (B, n=3) or with or without 1 mM CC4 (C, n=4)

Paired T test. ***P<0,0001

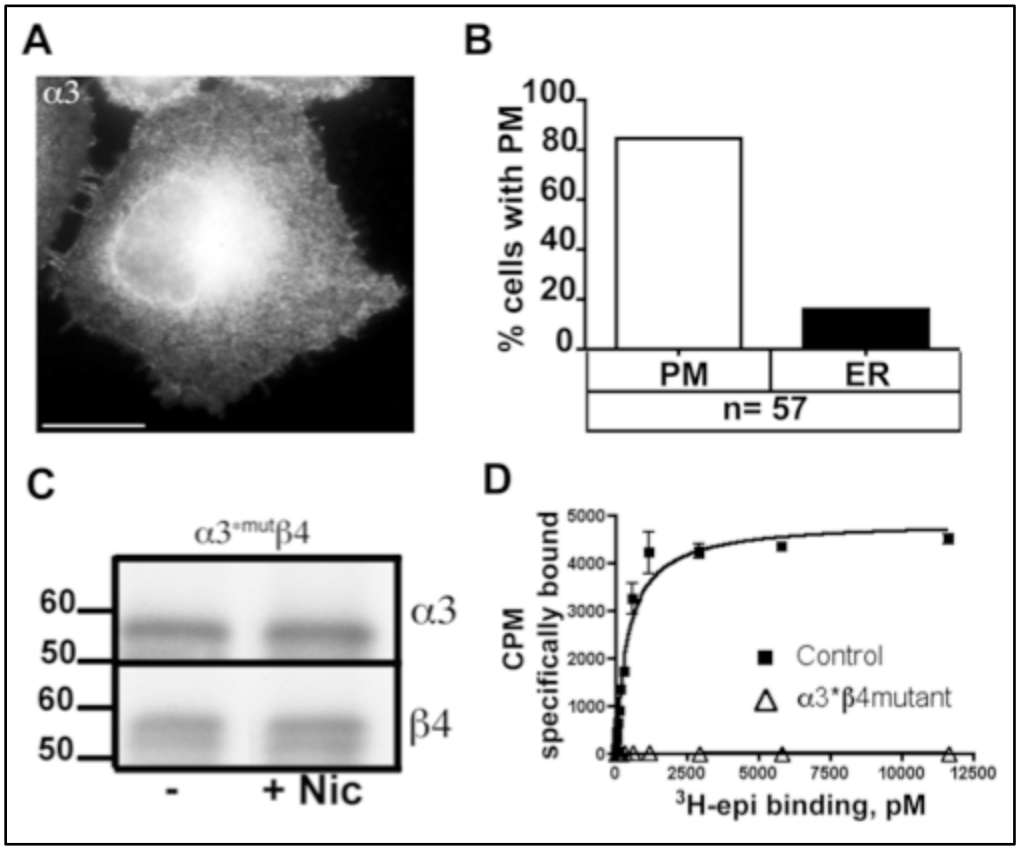

Fig. 30 α3*mutβ4 shows increases in plasma membrane trafficking

(A) HeLa cells transfected with α3* and β4 cDNAs subunits, after 24h, cell are permeabilesed and immunostained for a3 antibody and the number of cells with PM and ER staining was counted **(B).** After 24h transfection HeLa cells were treated with 1mM of nicotine (lane 2) or not (lane 1), lysated and analysed by WB with anti α3 and β4 antibodies **(C)** or analysed by ^{3}H-epi binding **(D).**

5 DISCUSSION

Based on the results previously showed, we proposed that chronic nicotine up-regulates the $\alpha3\beta4$ nAChR, a subtype that is rare in brain but the predominant subtype in the autonomic nervous system and that recently has been implicated in the risk of nicotine dependence, smoking, and lung cancer.

The principal findings of this study demonstrates that $\alpha3\beta4$ up-regulation is due to nicotine acting at two different levels: 1) its presence during $\alpha3\beta4$ receptor synthesis leads to a $\alpha3_{(2)}\beta4_{(3)}$ stoichiometry that is no longer sensitive to proteasome degradation and this increases the number of pentameric receptors localised in the ER; 2) nicotine increases the exocytic traffic of the $\alpha3_{(2)}\beta4_{(3)}$ receptors probably because a higher number of $\beta4$ subunit export motif are present in this receptor stoichiometry, as we demonstrated that the unique export motif (L349M) present in the $\beta4$ subunit is necessary for the receptor recruitment at the ERES (ER exit sites). However our results clearly indicate that the two effects of nicotine are distinct. Indeed, the increase in ER receptors per se, as in the case of treatment with the proteasome inhibitor MG132, does not lead to increase in plasma membrane receptors. Furthermore we found that the same double effect of nicotine on $\alpha3\beta4$ degradation and trafficking towards the plasma membrane is obtained with other nicotinic agonists and/or antagonists providing that they are cell permeable and, like nicotine, bind the orthosteric binding site, favoring the assembly of $\alpha3\beta4$ receptors with $\alpha3_{(2)}\beta4_{(3)}$ stoichiometry.

Ligand-dependent upregulation of the $\alpha3\beta4$ nAChR with the $\alpha3_{(2)}\beta4_{(3)}$ stoichiometry

Fluorescence colocalisation imaging experiments with an ER marker (RFP-KDEL) show that, as already shown for many other ligand gated heteromultimeric receptors, the receptors are mainly localised in the ER (Fig.12). Although they are correctly assembled (Fig.13), they poorly localize on PM of both HeLa and SH-SY5Y cells (Fig. 12)

After twenty-four hours of nicotine exposure the total $\alpha3$ and $\beta4$ subunit level is increased two fold as well as the number of pentameric receptors labeled with ^{3}H-epibatidine (Fig 14-15). Furthermore, immunofluoresce colocalisation studies with a PM marker (FP-22) and biotinylation experiments show that there is also an increase in PM receptors (Fig.20-21).

Unlike to what previously reported for the $\alpha3\beta2$ and $\alpha4\beta2$ subtypes (in which nicotine

increases receptor assembly and maturation (Sallette J, 2004 and 2005), blocking *de novo* synthesis with translational inhibitor cycloheximide decreased the level of assembled receptors (Fig.16), and the presence of nicotine did not counteract this effect (Fig.17). Taken together, these data indicate that nicotine does not have a major effect on receptor assembly probably because the $\alpha3\beta4$ subtype assembled very efficiently. Sallette J. (2004). has found that $\alpha3\beta2$ expression in HEK293 cells is much lower than that of $\alpha3\beta4$ in the absence of nicotine, but almost identical after nicotine expression. The difference in up-regulation between $\alpha3\beta4$ and $\alpha3\beta2$ is due to different levels of expression under control conditions. Similar results have been obtained by Wang F. (1998) who found that the main effect of nicotine on $\alpha3\beta2$ is an increase in receptor maturation because there was a 12-fold up-regulation in the absence of protein synthesis during a period (2–3 h) in which the receptors do not undergo any significant degradation. This demonstrated that nicotine converted pre-existing $\alpha3\beta2$ pools of immature subunits/oligomers into high affinity receptors.

At steady-state level, nicotine up-regulation is quantitatively similar to that obtained by blocking the proteasome (Fig.22) and the two effects are not synergistic. The mechanisms involved in the upregulation of $\alpha3\beta4$ by nicotine are different from those involved in MG132 induced up-regulation (Fig.18) because MG132, by blocking the proteasome, leads to the accumulation of nAChR subunits and this, in turn, increases receptor assembly. Moreover in the presence of MG132 there is an increase in ubiquitinated proteins, whereas nicotine, in the conditions measured in this study, does not affect protein ubiquitination (Fig.19) or proteasome activity, as demonstrated by the lack of effect of increasing concentrations of nicotine on the degradation of the proteasome substrate protein CD3Δ.

Concerning the possible mechanisms involved in the decreased receptor degradation as our quantitative analysis of the ratio of $\beta4{:}\alpha3$ subunits in control condition and after nicotine exposure clearly states that the presence of nicotine during receptor synthesis and assembly induces an $\alpha3_{(2)}\beta4_{(3)}$ stoichiometry that prevents the receptor degradation by proteasome (Fig.29). The precise mechanism by which the receptor with this stoichiometry is less prone to be degraded needs to be investigated.

This effect of nicotine on subunit degradation has also been reported by Govind AP.(2012) who found that the presence of nicotine decreases the degradation rate of newly synthesized subunits, largely $\beta2$, leading to increased assembly of $\alpha4\beta2$ receptors and this

is responsible for the second long lasting mechanism that participates in upregulating of the $\alpha4\beta2$ subtype. In line with our data, Govind AP. (2012) also found that nicotine treatment has a differential effects on $\alpha4$ and $\beta2$ subunit levels compared with the proteosomal block suggesting that nicotine was not directly inhibiting proteosomal activity. This latter result is in contrast to what previously published by Revzani K (2007) who found a direct effect of nicotine on proteasome activity.

In our cell systems, the protein synthesis inhibition had no effect on receptor assembly maybe because this subtype is very efficiently assembled. This is also suggested by the analysis of the fractions obtained after sucrose gradient sedimentation where, both by western blots analysis and ^{3}H epibatidine binding, we never detected intermediate such as free subunits or dimers (Fig.13).

The $\alpha3\beta4$ subtype is also up-regulated after chronic incubation with the cell permeable agonist cytisine and the competitive partial agonist/antagonist CC4, but not after chronic exposure to the cell impermeable $\alpha3\beta4$ antagonist hexamethonium or the cell permeable antagonist mecamylamine(Fig.26-28). These findings indicate that the site of drug action is intracellular and only permeable competitive compounds that bind to the α/β interface lead to upregulation. Moreover we also determined that also in the case of CC4, chronic treatment induce the $\alpha3_{(2)}\beta4_{(3)}$ receptor stoichiometry (Fig.27) and an increase in PM $\alpha3\beta4$ receptors (Fig.29). The importance of the LBD is also strongly confirmed by the mutation on $\alpha3$ subunit, mutation that completely abolish the effect of nicotine (Fig.30).

We don't know the mechanism trough which binding of ligands to the ACh binding site induce the $\alpha3_{(2)}\beta4_{(3)}$ stoichiometry, we can only speculate that binding of the ligands at the interface between $\alpha3$ and $\beta4$ subunits may promote conformation changes in very short lived assembly intermediate that further promote the assembly of mature AChRs with the $\alpha3_{(2)}\beta4_{(3)}$ stoichiometry.

As no difference, both in the level of upregulation as well as subtype stoichiometry, was determined between the agonist nicotine and the very partial agonist /antagonist CC4, we believe that, as described by Gao F. (2005) in the case of acetylcholine with the $\alpha4\beta2$, the orthosteric nicotinic ligands by entering the binding site bring together conserved tryptophan residues from adjacent subunits. This might be a “molecular glue” effect shared by orthosteric ligands, which would help to stabilize the association of $\alpha3$ and $\beta4$ subunit

and favoring the $\alpha 3_{(2)}\beta 4_{(3)}$ stoichiometry.

Surface $\alpha 3\beta 4$ receptor up-regulation requires exocytic traffic

Our examination of $\alpha 3\beta 4$ nAChR trafficking under control condition showed that HeLa cells have no surface-expressed nAChRs and SH-SY5Y cells have very few (Fig.12). Chronic exposure to nicotine is sufficient to induce surface receptor up-regulation, for which exocytic traffic is essential (Fig.20-21). Deletion of the unique export motif (L349M) present in the intracellular loop between TMD3-4of the $\beta 4$ subunit, leads to the formation of $\alpha 3\beta 4$*mut receptors that are expressed at the same level as wt $\alpha 3\beta 4$ receptors, identically up-regulated by nicotine but which cannot reach the surface (Fig.24). It is the first time that it has been clearly demonstrated that blocking the recruitment at the ER exit site by Sec24D (through the export motif LFM) is sufficient to completely abolish the ER exit of the receptor. Indeed this unique effect is due to the presence in the $\beta 4$ subunit of a single ER export motif while for instance in human $\beta 2$ subunit the situation is more complex because in the same subunit are present both an ER retention and an ER export motif that compete each other. These data are consistent with previous studies of $\alpha 4\beta 2$ subtype where it has been shown that the limiting step of nicotine induced up regulation is the ER upregulation and the exocytic traffic (Darsow T, 2005).

Furthermore, unlike what has been previously reported in the case of muscle type receptor, (in which blocking ER-associated degradation leads to increased ER receptor assembly and surface expression) our experiments using MG132 showed that during proteasome inhibition, subunits accumulated in the ER are capable of assembling into pentameric receptors but this does not lead to an up-regulation of surface receptors (Fig.22-23). This is another evidence that underline that the two effects of nicotine on degradation and trafficking are independent. Upregulation of intracellular receptor (as in the case of MG132 treatment) is not sufficient to drive the export and, as shown here for $\alpha 3\beta 4$ receptors, only nicotine and orthosteric nicotinic ligands can induce in the up-regulated receptors a competent conformation for their transport to the PM.

Finally, we would like to point out a possible pathophysiological significance of this work. It has been shown that the $\alpha 3\beta 4$ subunits are expressed in non-neural cells, including bronchiolar and epithelial cells, and lung cell lines, and a lot of evidence indicates that these receptors play crucial roles in signal transduction underlying tumour initiation or

growth (Eglenton AB, 2008;Improgo MR, 2010). ACh stimulates cell proliferation by acting as an autocrine growth factor in small cell lung carcinoma (SCLC), a phenomenon that can be blocked by the non-specific nAChR antagonist, mecamylamine (Schuller HM, 2009). Recently it has been shown that in the airway surface liquid of human primary bronchial epithelial cultures exposed to whole cigarette smoke, nicotine concentration of approximately 30 μM is present. As this concentration is in the range of the EC50 that we have determined for the up regulation of the α3β4 receptors, both in neuronal and epithelial cells we believe that nicotine by increasing the expression of the α3β4 receptors, may potentiate the proliferative and pro-survival effects of nicotine on tumor cells.

In conclusion, due to the increasing importance of the α3β4 subtype in relation with lung cancer and nicotine addiction, further studies are required to better understand 1) the mechanism by which the nicotine-induced stoichiometry makes the α3β4 subtype less prone to degradation (maybe masking some not-identified ER retention motifs) and more prone to exit (maybe changing the physical conformation of the receptor revealing ER export motifs, such as those identified by us, that facilitates the recognition from ER export machinery in the ER exit site) 2) why the mutation of the LBD on the α subunit, even if impaired the response of receptor to nicotine, greatly increases receptor trafficking to plasmamembrane.

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