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Neuronal nicotinic acetylcholine receptors: functional properties in lung cancer cell lines and response to tobacco-specific nitrosamines

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A mi familia

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CHAPTER 1

“General introduction”

NICOTINIC ACETYLCHOLINE RECEPTORS

Structure, stoichiometry and ligand binding site

The nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels which belong to a gene superfamily including the $GABA_A$, glycine and 5-hydroxy tryptamine (5-HT) receptors (Paterson and Nordberg, 2000). These are known as Cys-loop receptors, as all of them have a conserved sequence containing a pair of cysteines separated by 13 residues and linked by a disulfide bridge. nAChRs can be divided into two groups: muscle receptors, which are found at the skeletal neuromuscular junction, and neuronal receptors, which are found throughout the peripheral and central nervous system (Hogg et al., 2003). Both muscle and neuronal nicotinic receptors have a pentameric structure consisting of five subunits around a central pore selective for cation. There are multiple nAChR subunits, whose genes have been cloned (Paterson and Nordberg, 2000). The subunit composition of fetal muscle nAChRs is $(\alpha)_2\beta\gamma\delta$ and in the adult γ is replaced by ε to give an $(\alpha)_2\beta\varepsilon\delta$ composition. In contrast, the neuronal nAChRs can be found as homopentamers or heteropentamers of subunits α and β (Fig. 1), which exhibit distinct kinetics, ion permeability and pharmacological profile (Gotti and Clementi, 2004).

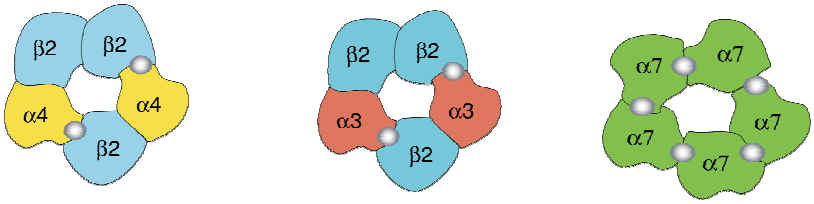

Fig. 1 Homopentameric and heteropentameric receptors
(Hogg and Bertrand, 2004)

To date, nine α subunits (α2-α10) and three β subunits (β2-β4) have been described; these are differently expressed throughout the nervous system (Colquhoun et al, 2003).

The α7, α8, α9 subunits prevalently form homopentamers, whereas α2-α4, β2 and β4 subunits usually form $(\alpha)_2(\beta)_3$ functional channels (Anand et al, 1991). The β3, α5 and α6 subunits do not form functional receptors when expressed alone or with a single type of α or β subunit. They are thought to associate with heteromeric receptors *in vivo*, and modulate their properties (McGehee and Role, 1995; Ramirez-Latorre et al, 1996).

Other stoichiometric combinations of subunits are possible, such as $(\alpha)_1(\beta)_4$, $(\alpha)_3(\beta)_2$ and $(\alpha)_4(\beta)_1$. When tested in heterologous expression systems, they turn out to be functional but show a lower affinity for the agonists compared to $(\alpha)_2(\beta)_3$ (Lòpez-Hernàndez et al, 2004).

Subunit	Gene	Chromosome
α2	CHRNA2	8p
α3	CHRNA3	15q
α4	CHRNA4	20q
α5	CHRNA5	15q
α6	CHRNA6	8p
α7	CHRNA7	15q
α9	CHRNA9	8
α10	CHRNA10	11p
β2	CHRNB2	1q
β3	CHRNB3	8p
β4	CHRNB4	15q

Table 1: Locations of the genes coding for nAChR subunits in humans (Colquhoun et al, 2003)

Each nAChR subunit consists of an extracellular N-terminal domain which partakes in the formation of the ligand-binding domain, four hydrophobic membrane spanning domains (M1-M4) and an extracellular C-terminal domain. The intracellular loop between M3 and M4 contains consensus sequences for phosphorylation sites (Fig. 2; Paterson and Nordberg, 2000). The N-terminal domain of the α subunits comprises most of the binding site. In particular, it contains three loops A, B, C, which are involved in ACh binding. The adjacent subunit comprises three loops D, E, F with an accessory role in ligand binding. The M2 segments of the five subunits align the pore and the residues located along the entire length of M2 and the N-terminal end of M1 form the ion conduction pore (Hogg et al, 2003). Some of these residues are involved in ion selectivity, permeability and channel

gating. The electrostatic profile of the channel pore has been investigated by site-specific mutagenesis experiments. The pore comprises a region of putative negative charges, formed by a ring of glutamic acid residues, which favors cation entry (Pascual and Karlin, 1998).

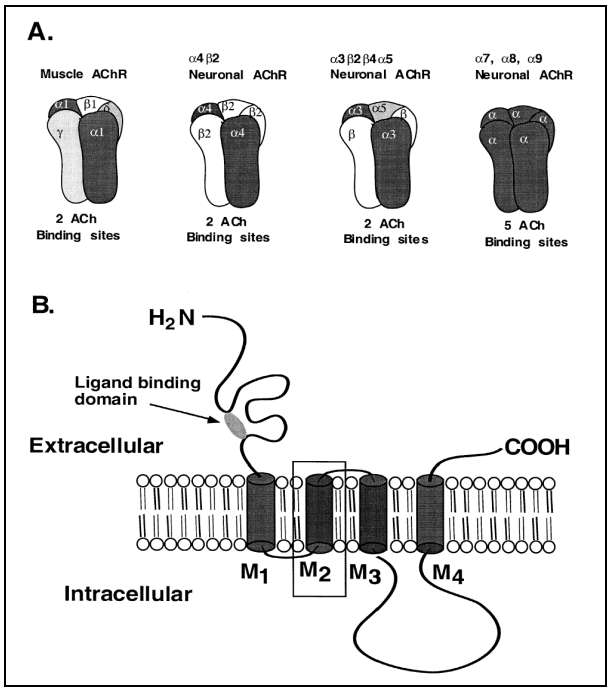

Fig. 2 Nicotinic receptor structure. A. Pentameric structure, subunit stoichiometry and number of binding sites of muscle and neuronal nicotinic receptors. B. Nicotinic receptor sequence. Highlighted in the box is transmembrane segment M2 which is thought to form the lining of the ion channel (Paterson and Nordberg, 2000)

In order to define how ligands bind to the receptor and the signal for channel activation or gating is transduced, ligands were studied through ligand-binding and functional assays in combination with site-directed mutagenesis (Albuquerque et al., 2009). When a ligand such as nicotine binds, it does it in a hydrophobic pocket formed at the interface between the α subunits and their adjacent subunits (Corringer et al, 2000; Itier and Bertrand, 2001). Most of the binding pocket is formed by a loop in the α subunit that contains a Cys-Cys pair at its apex. This loop extends like an interlocking finger around the face of the adjacent subunit. In addition to the Cys-Cys pair, other residues required for ligand binding are predominantly hydrophobic aromatic amino acids. The identity of the hydrophobic residues present in the loop determines ligand affinity, whereas the other residues determine ligand selectivity. (Albuquerque et al, 2009). Heteromeric nAChRs with $(\alpha)_2(\beta)_3$ subunit stoichiometry are thought to have two ACh-binding sites. A total of five ACh-binding sites is present in homomeric receptors. Two sites must be occupied by ACh in order to induce significant channel activation (Hogg et al, 2003).

Ligand binding is converted by the receptor structure into channel opening within microseconds, suggesting that the entire protein structure is well tuned to convey rapid conformational change (Sine and Engel, 2006).

The activation gate (the domain whose conformational change opens the pore) is probably located in the central region of the M2 domain (Wilson and Karlin, 1998). The channel region that obstructs the pore in the desensitized state is also found in the M2 domain closer to the

extracellular mouth of the pore (Wilson and Karlin, 2001). Changes in the M1 and M2 domains are implicated in coupling the ACh-binding site and the channel activation gate (Akabas and Karlin, 1995). In order to open the channel to ion flow, ligand binding induces rotation of the extracellular domain, and this is translated into rotation of the M2 helices. This process involves the transient removal of hydrophobic barrier residues from the pore, an increase of the pore diameter, and the movement of hydrophilic residues into the channel to support ion flow (Albuquerque et al, 2009).

nAChRs have often been presented as prototypes of allosteric membrane proteins (Changeux, 1990; Itier e Bertrand, 2001). These proteins can exist in different states and undergo spontaneous conformational transitions (Rush et al, 2002). At rest, the equilibrium between conformational states favours the closed state. Exposure to agonists stabilizes, on a microsecond-to-millisecond timescale, the receptor in the open state (activation), with a low affinity for ACh (1-10 μM). In the presence of agonist, the channel can also assume one of two desensitized closed states, I and D, which are refractory to activation on a millisecond-minute timescale, but exhibit high affinity for ACh (10 nM-1 μM) (Galzi and Changeux, 1995). Transition from one state to another depends upon both the presence of a ligand and the isomerization coefficient. Binding of a molecule at a site distinct from the agonist-binding site may interact with the isomerization coefficient. Molecules which reduce the isomerization coefficient are termed positive allosteric effectors, whereas compounds that increase the isomerization coefficient are termed negative allosteric effectors (Buisson and Bertrand, 1998).

Pharmacology of nAChRs

The multiple possible combinations of nAChR subunits produce a multitude of nicotinic receptors with distinct pharmacological and physiological profiles.

The studies which characterize nAChRs are based on binding assays with nicotinic radioligands in different brain areas and have demonstrated that there are at least two main pharmacological classes of putative nAChRs in the nervous system. The first type consists of receptor molecules which bind ^{3}H agonists with nM affinity but not αBungarotoxin (αBgtx), and the other that bind the agonist with μM affinity and αBgtx with nM affinity. The first group contains the α2-α6 and β2-β4 subunits; thus, only heteromeric receptors are formed. The second group contains homomeric or heteromeric receptors made up of α7, α8 or α9, α10 subunits (Patterson and Nordberg, 2000; Gotti and Clementi, 2004).

Agonists

Besides the main pharmacological agonist Ach, the list of nicotinic agonists is long and consists of natural compounds (choline, nicotine, cystisine, lobeline, epibatidine, anabaseine) and synthetic compounds (tetramethylammonium (TMA), 1,1-dimethyl-4-phenylpiperazinium (DMPP) and carbachol) (Karlin, 2002; Colquhoun et al., 2003; Dani and Bertrand 2007).

ACh: acetylcholine is an ester of acetic acid and choline. The EC50 value for homomeric receptors is approximately between 80- 150 μM. In heteromeric receptors, such α4β2, the acetylcholine concentration-response relation shows a high- and a low-affinity component because two receptor subpopulations exist with different subunit stoichiometry that determine distinct affinity profiles. The EC50 values for the α4β2 subtype are around 0.7-1.6 μM for ACh high affinity receptors and 68-74 μM for low affinity receptors.

Nicotine: is an alkaloid. Nicotine is a non-selective nAChR agonist interacting with the different neuronal receptor subtypes with various affinities, from 1 to 130 μM. Applications of nicotine provokes first the stabilization of the receptor in a high-affinity open state followed by a progressive stabilization of a closed desensitized state. Long exposure to a low concentration of nicotine favors receptor desensitization (Catassi et al, 2008).

Choline: it is a useful agonist for α7 receptors but it is ineffective or a very poor partial agonist on α3β4 and α4β2 - type receptors.

Cytisine: it is a potent and efficacious agonist on heteromers containing the β4 subunits but it is only a partial agonist on β2 containing receptors.

DMPP: it is a nonselective agonist but it is very potent on heteromeric receptors. It becomes a full agonist following exposure to ivermectin.

Antagonists

Functional studies usually report antagonist IC_{50} values. Furthermore IC_{50} experiments do not tell us anything about the mechanism of action of the antagonist, if is a competitive antagonist or open channel block. Much potentially valuable information on the binding site is lacking. This is true for functional studies and for binding studies. Binding assays for neuronal nicotinic antagonist use displacement of a labeled agonist by the antagonist.
Below I will describe the antagonists that are most useful for receptor classification (Colquhoun et al., 2003).

α Bgtx: It is a peptide of 74 amino acids. It belongs to the family of α-neurotoxins. These toxins, present in the venoms of elapid and hydrophid snakes, are high-affinity competitive inhibitors of ACh in striated muscle (Karlin, 2002).
This toxin is often used at concentrations between 10 and 100 nM so as to specifically target α7 subtype receptors. The block is nearly irreversible. The α9 and α9/α10 homomeric receptors are also sensitive to nanomolar concentrations of this antagonist.

κ-Bgtx: It is a peptide of 66 amino acids. It is a competitive blocker of neuronal receptors and it is particularly potent on α3β2 receptors.

Methyllicaconitine (MLA): It is an alkaloid derived from *Delphinium brownii*, which competitive by blocks α7 receptors at low nanomolar

concentration (2-5 nM). It also inhibits heteromeric receptors but at considerably higher concentration.

Strychnine: It is a good competitive antagonist of the α7 and α9/α10 homomeric receptors at submicromolar concentrations.

Dihydro-β-Erythroidine (DHβE): It is an alkaloid obtained from the seeds of several species of the genus *Erythina*. It is a competitive antagonist for some nAChR subtypes. It is effective at submicromolar concentrations on the α4β2 and α4β4 receptors, whereas it is a poor antagonist of α3β4 and α7 receptors.

αConotoxins: There are different types of these toxins. αConotoxins ImI are effective antagonists of homomeric receptors. αConotoxins MII are selective for rat α3β2 and other heteromeric and homomeric combinations of rat neuronal subunits. Rat homomeric α7 receptors are resistant to this toxin.

Mecamylamine: It is not selective for the different receptor types and it is effective at low micromolar concentrations. At concentrations higher than 1μM, it is an open channel blocker on recombinant α4β2. This antagonist gives rise to a persistent block as it is trapped in the channel.

(+)-Tubocurarine: it is a natural alkaloid obtained from the bark of the South American plant *Chondrodendron tomentosum*. It blocks nAChRs at micromolar concentration.

Ionic permeability

The nAChR are selective, to monovalent and divalent cations. Nicotinic receptors' activity causes depolarization, and the calcium permeability plays the usual important physiological role .

All nicotinic receptor subtypes are calcium permeable, the most permeable are the homomeric receptor α7 and α9 (Colquhoun et al, 2003). The relative permeability of calcium to sodium estimated from permeability ratios is ~ 0.1 for muscle, ~ 2.0 for heteromeric neuronal and ≥ 10 for homomeric nAChRs (Dani and Bertrand, 2007).

Calcium has multiple effects on nicotinic receptors. Not only is it to some extent permeant, but extrecellular Ca^{2+} also modulates the agonist response of nAChRs. The nicotinic receptors are progressively potentiated by $[Ca^{2+}]_o$, up to the physiological concentration (Mulle et al, 1992; Vernino et al, 1992) , whereas higher $[Ca^{2+}]_o$ produces channel block (Buisson et al, 1996). This issue is further discussed in chapter 3.

Localization and distribution in the central nervous system (CNS)

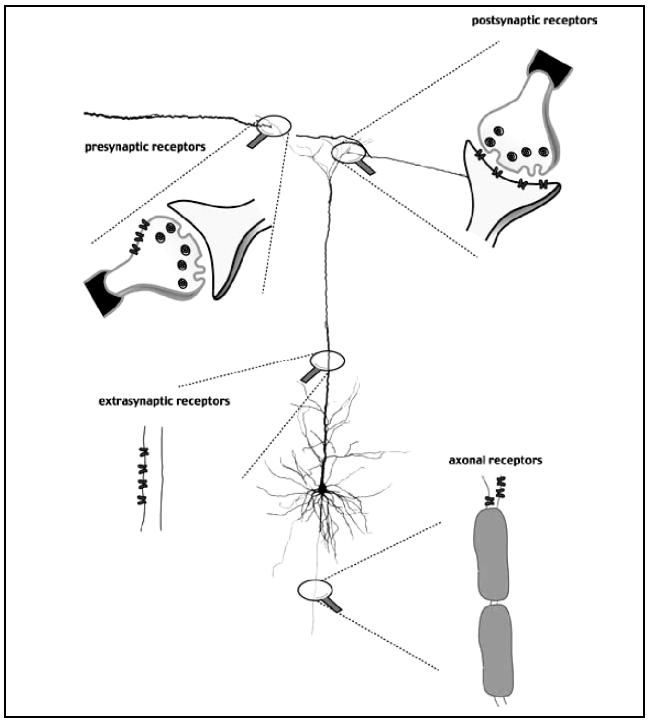

Fig. 3 Putative location of nAChR on a central neuron
(Hogg et al, 2003)

Neuronal nAChRs are present in a variety of CNS regions. They are found on cell bodies or dendrites, where they may mediate direct postsynaptic effects, or on axon terminals, where they may play a role in modulating neurotransmitter release. nAChRs are also observed in extrasynaptic locations (Fig. 3; Hogg et al, 2003).

Presynaptic nAChRs

Modulation of neurotransmitter release by presynaptic nAChRs is probably the prevalent nicotinic role in the central nervous system. Activation of presynaptic nAChRs potentiates the release of many different neurotransmitters (Paterson and Nordberg, 2000). Nicotinic agonists enhance, and nicotinic antagonists often diminish, the release of ACh, dopamine, norepinephrine, and serotonin, as well as glutamate and GABA, in different brain regions (Dani and Bertrand, 2007).

The activity of presynaptic nAChRs can initiate intracellular calcium signals that enhance neurotransmitter release in different ways (McGehee and Role, 1995; Role e Berg, 1996; Wonnacott, 1997; Gray et al, 1996). Nicotinic receptors mediate a small calcium influx (Seguela et al, 1993; Castro et al, 1995; Vernino et al, 1994) which can trigger *calcium-induced calcium release* from intracellular stores (Sharma et al, 2003). In addition, nAChR activity produces a depolarization which can activate voltage-gated calcium channels in the presynaptic terminal (Tredway et al, 1999). The overall effect is that presynaptic nAChR activity elevates intraterminal calcium, with ensuing stimulation of neurotransmitter release.

Postsynaptic nAChRs

nAChRs present on cell bodies and dendrites of central neurons mediate fast nicotinic synaptic transmission (Hogg et al, 2003). Although fast nicotinic dependent depolarization drives neurotransmission of neuromuscular junctions and autonomic ganglion synapses, only rare cases of fast nicotinic transmission have been reported in the mammalian brain.

The hippocampus, a cerebral structure involved in learning and memory, contains a high amount of nAChRs (Fabian-Fine et al, 2001). Most of them are located on GABAergic interneurons, but some are also present in pyramidal neurons (Ji et al, 2001). Hippocampal nAChRs can modulate the induction of synaptic plasticity and may thus, at least in part, explain the effect of nicotinic agonists on learning and memory (Ji et al, 2001).

Evidences of postsynaptic roles of nAChR have also been obtained in the developing visual cortex (Lecchi et al, 2005) and hypothalamus (Hatton and Yang, 2002).

In the spinal cord, the VII and XII nuclei express heteromeric nAChRs of the heteromeric type, whereas the X nucleus contains the α7 subunit. nAChRs are also present in Renshaw cells, a subpopulation of spinal glycinergic interneurons. Activation of these neurons by motoneuron axon collateral results in recurrent inhibition (Curtis and Ryall, 1966).

Because cholinergic neurons in the brain are usually loosely distributed and often sparsely innervate broad areas, it is experimentally difficult to stimulate a large number of these neurons

and to record from the precise location of their innervation. It is likely that fast nicotinic transmission is present at low densities in more neuronal areas than has been reported until now. Overall, however, evidence suggests that direct, fast nicotinic transmission is not a major excitatory mechanism in the mammalian brain (Dani and Bertrand, 2007).

Extrasynaptic nAChRs

Nicotinic receptors are also distributed to preterminal, axonal, dendritic, and somatic locations (Lena et al, 1993; Zarei et al, 1999) Preterminal nAChRs located before the presynaptic terminal bouton indirectly affect neurotransmitter release by activating voltage-gated channels and, potentially, initiating action potentials (Alkondon et al, 1997; Lena et al, 1993; Albuquerque et al, 2000). The evidence for preterminal nAChR is strongest at some GABAergic synapses, where they can produce local membrane depolarization, thereby activating voltage-gated Ca^{2+} channels and consequently GABA release. Alternatively, activation of nonsynaptic nAChRs can alter the membrane's impedance, thereby altering the space constant of the cellular membrane. These factors influence the spread and efficiency of synaptic inputs. Strategically located nAChRs may enable an action potential to invade only a portion of the axonal or dendritic arbor by locally inactivating some voltage-dependent channels. Nonsynaptic nAChRs could also contribute to control the threshold for action potential (Dani and Bertrand, 2007).

Distribution of nicotinic receptor subunits in the human brain

In comparison to muscarinic receptors, neuronal nAChRs are expressed in relatively low density in the human brain. Their pattern of distribution is relatively homogenous and is not restricted to brain cholinergic pathways. Nicotinic receptors are present in a variety of brain structures, in particular the thalamus, cortex and the striatum. This distribution of receptors determined by immunocytochemistry in rodents is consistent with the distribution of receptors described in the human brain by PET (Positron Emission Tomography) and *in situ* hybridization. $\alpha 4\beta 2$ nAChR constitutes the predominant subtype present in the brain. (Paterson and Nordberg, 2000; Gotti et al, 2006).

β2: its distribution is fairly homogeneous. β2 mRNA shows a strong signal in the insular cortex, the granular layer of the dentate gyrus, the CA2/3 region of the hippocampus, cortex and cerebellum.

α3: its mRNA is most abundant in the thalamus**.** It is present in low to moderate amounts in most cortical regions and hippocampus. In the cortex, α3 mRNA is most predominantly expressed in pyramidal neuron layers III-IV.

α2: Its mRNA expression is very high in the thalamus, in the cingular, temporal, parietal and occipital cortex, whereas its expression is moderate in the hippocampus.

α4: The expression of α4 mRNA is higher in the temporal cortex and cerebellum. Its distribution in the neocortex is more widespread than the distribution of α3, but both are associated with pyramidal neurons.

α7: The reticular nucleus of the thalamus, the lateral and medial geniculate bodies and the horizontal limb of the diagonal band of Broca are regions with high levels of α7 mRNA. In the frontal cortex, α7 mRNA is high in layers II and III, moderate in layers V and VI and low in layers I and IV.

α5 β3 β4: These subunits can be detected in the spinal cord, medulla oblongata, cerebellum, mesencephalon, subcortical forebrain and neocortex.

The pattern of nAChR expression over time suggests that these receptors play a particular role during brain development because their concentration is high during the stage of synapse formation (Mansvelder and Role, 2006). nAChRs are of great relevance in two critical periods for brain life: early pre- and perinatal circuit formation, and cell degeneration during aging.

Brain region	*β2*	*β3*	*β4*	*α3*	*α4*	*α5*	*α7*
Prefrontal Cortex	+	+	+	++	+	+	++
Motor Cortex	+	+	+	++	+	+	+++
Entorhinal Cortex	+	+	+	++	+	+	+
Cingular Cortex	+	+	+	+	+	+	
Temporal cortex	+	+	+	+	+(+)	+	
T. Dorsomedial	+	+	+	+++			++
T. Lateroposterior		+	+++			+	
T. Reticular		+	++			+(+)	
T. ventro-posterolateral	+	+	+	+++		+	
Geniculate bodies		+	+			++	
Hippocampus	+(+)			+			++
Dentate gyrus	+(+)			+			++
Caudate putamen	+(+)	+	+			+	++
Cerebellum	+	+	+	+	+(+)	+	

Table 2: Distribution of nicotinic receptor subunit mRNA in the human brain. T=Thalamus (Paterson and Nordberg, 2000)

Nicotinic receptors' functions in the brain

The Ach released from the cholinergic fibers ascending from the pons and basal forebrain activates nAChRs and muscarinic receptors to enhance the level of arousal and attention. Cholinergic stimulus is generally high during waking and increases at the transition between slow-wave and rapid-eye-movement sleep.

Specific effects of nicotine include EEG desynchronisation, producing a shift in the direction of higher frequency, increased cerebral blood flow and increased cerebral glucose utilization through stimulation of nAChRs in the basal forebrain (Paterson and Nordberg, 2000). These nicotine effects can be explained as a consequence of the interaction of nicotine with the presynaptic nAChRs. The activation of nicotinic receptors induces the release of a number of neurotransmitters including Ach, GABA, NA, DA, 5-HT and glutamate, many of which are known to play a role in mediating/modulating a number of behavioural tasks (Paterson and Nordberg, 2000; Alkondon et al, 2000; Mesulam, 2004; Dani and Bertrand, 2007).

Nicotine and nicotine agonists have cognitive and memory - enhancing properties in animals and humans, while antagonists such as mecamylamine impair memory function. The hippocampus, in conjunction with the cerebral cortex, is particularly implicated in learning and memory formation. Cholinergic projections from the septum innervate hippocampal inhibitory GABAergic interneurons and principal glutamatergic cells, and intrinsic cholinergic interneurons have also been described within the hippocampus.

Potentiation of glutamate-mediated synaptic transmission at

thalamocortical synapses in rats occurs via α7 receptors. At prefrontal cortical pyramidal cells, nAChR activation potentiates excitatory synaptic transmission mediated by NMDA but not AMPA receptors. NMDA receptors participate in long-term changes in synaptic efficacy that are proposed to underlie memory formation, and therefore nAChR-mediated modulation of glutamate release could potentially contribute to plasticity at hippocampal and cortical synapses (Jones et al, 1999).

In conclusion, nAChRs play an important role in cognition and memory, but the specific subtypes involved are unclear.

nAChRs pathology in the brain

Schizophrenia: It is a chronically deteriorating psychosis which begins in late adolescence or early adulthood and involves hallucinations, disturbances of thought and self-awareness. Abnormalities in dopaminergic synaptic transmission have been observed and excessive release of dopamine may cause overactivity of synapses in the mesolimbic system (Paterson and Nordberg, 2000; Hogg et al., 2003).

The possible involvement of nAChRs in schizophrenia is suggested by the high prevalence of smoking among schizophrenic patients, the fact that neuroleptic neuronal side effects are fewer among smokers and the fact that there is a positive correlation between smoking and negative symptoms (Poirier et al., 2002). Postmortem studies have shown that the brain of schizophrenic smokers had reduced numbers of nicotinic receptors especially α7, compared to control smokers (Hogg et al., 2003).

However, it is unclear whether the alterations in nAChRs observed in schizophrenic patients are cause or effect of the disease.

Parkinson's disease: It is a neurodegenerative pathology characterized by motor dysfunction resulting in muscular rigidity, tremors and difficulty in initiating and sustaining movement. Patients show reduced dopamine levels in the striatum and this has been shown to be caused by degeneration of neurons in the *substantia nigra* (Hogg et al., 2003). In Parkinson's disease there is a loss of cholinergic cells in the basal forebrain, accompanied by a significant reduction in the number of high affinity nicotine binding sites in the brain. The down regulation of the nAChR was closely associated with primary histopathology changes in patients (Paterson and Nordberg, 2000; Hogg et al., 2003). The physiological meaning of these observations is still debated.

Alzheimer's disease: It is a neurodegenerative condition that affects almost 10% of individuals over the age of 65 and is characterized by a progressive loss of short-term memory and higher cognitive functions. (Paterson and Nordberg, 2000; Hogg et al., 2003).
Postmortem brains of patients display two different neuropathological features: intracellular neurofibrillary tangles and extracellular neuritic senile plaques (Paterson and Nordberg, 2000).
A severe neurochemical abnormality associated with this pathology is the loss of cholinergic innervation of the cerebral cortex and hippocampus (Paterson and Nordberg; 2000; Hogg et al., 2003; Dani and Bertrand, 2007).

Tourette's syndrome: It is a neuropsychiatric disorder with unknown etiology which starts in childhood. It is characterized by persistent motor and verbal tics and is commonly associated with aggression, hyperactivity, obsessive-compulsive behavior, phobias and anxiety (Paterson and Nordberg; 2000; Hogg et al., 2003).

The administration of nicotine significantly improves the motor disorder and other symptoms (Sanberg et al., 1997). Whether nAChRs are directly involved in this syndrome is unknown, but the positive effects of nicotine suggests that nAChRs may play a role in symptom manifestation (Gotti and Clementi, 2004).

Epilepsy: This disease affects 0,5-1% of the world's population and approximately 5% of the people experience at least one seizure in their lifetime (Combi et al, 2004). A seizure is a sudden alteration of electrical activity in the brain of sufficient magnitude to alter motor or sensory function, behavior or consciousness. An epileptic syndrome is a constellation of recurrent seizures, EEG patterns, family histories and age-specific characteristics sufficient to produce a reproducible and recognizable seizure pattern with predictable outcome (Freeman, 1995).

Seizures lasting from seconds to minutes occur repetitively or in isolation, and they can be focal or spread across the entire brain causing motor, sensory, or cognitive disturbances. Although its basic manifestation starts at single neuron level, epilepsy is fundamentally a circuit phenomenon, and seizures are only possible because the brain

is organized in a series of interconnected neuronal networks (Steinlein, 2004).

Epilepsies are classified according to whether the source of the seizure within the brain is localized (partial or focal onset seizures) or distributed (generalized seizures). Partial seizures are further divided depending on the extent to which consciousness is affected. If consciousness is unaffected, then it is denominated a simple partial seizure; otherwise, it is called a complex partial (psychomotor) seizure. A partial seizure may spread throughout the brain, a process known as secondary generalization. Generalized seizures are classified according to the effect on the body, but all generalized seizures involve loss of consciousness. They include absence (petit mal), myoclonic, clonic, tonic, tonic-clonic (grand mal) and atonic seizures.

The causes of sporadic or recurrent seizures are numerous, including acquired structural brain damage, altered metabolic states and inborn brain malformation. However, about 1% of all epileptic patients develop recurrent unprovoked seizures for no obvious reason and without any other neurological abnormalities. These epileptic forms are named idiopathic epilepsies, and they are increasingly recognized as being caused by genetic alteration, especially mutation of genes coding for ion channels. Nicotinic receptor alterations have a causative role in some forms of sleep-related epilepsy, such as autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). This is the only neuronal pathology for which a causative role of nAChR alteration has been demonstrated.

ADNFLE: It is an idiopathic partial epilepsy characterized by clusters of nocturnal seizures which occur mostly during the non-REM sleep. ADNFLE has an average penetrance around 70-80%. The seizures start in middle childhood and usually persist throughout adult life. Seizures can be preceded by an aura and can start with vocalization. The motor features are described as trashing hyperkinetic activity or tonic stiffening with superimposed clonic jerking. Secondary generalization with loss of consciousness can occur, but most patients remain conscious throughout the seizure. The attacks are usually brief, lasting 30-60 seconds and often relatively well tolerated, since they occur only during the night. Most of the affected individuals are otherwise neurologically and intellectually normal (Combi et al., 2004; Steinlein, 2004; Sutor and Zolles, 2001; Marini and Guerrini, 2007).

Mutations in genes encoding different nicotinic subunits are linked with ADNFLE.

Four mutations in CHRNA4 have been described:

- a substitution of a serine with a phenylalanine at position 248 [S248F] (Steinlein et al., 1995).
- a substitution of a serine with a leucine at position 252 [S252L] (Hirose et al., 1999)
- a substitution of a isoleucine with a threonine at position 265 [T265I] (Leniger et al., 2003).
- an insertion of three nucleotides at position 766 [766ins3] (Steinlein et al., 1997).

Five mutations in CHRNB2 have been found:

- a substitution of a valine with leucine at position 287 [V287L] (De Fusco et al., 2000).
- a substitution of a valine with methionine at position 287 [V287M] (Phillips et al., 2001).
- a substitution of a isoleucine with methionine at position 312 [I312M] (Bertrand et al., 2005).
- a substitution of a leucine with a valine at position 301 [L301V] (Hoda et al., 2008).
- a substitution of a valine with an alanine at position 308 [V308A] (Hoda et al., 2008).

One mutation in CHRNA2 has also been described, consisting in a substitution of an isoleucine with an asparigine at position 279 [I279N] (Aridon et al., 2006).

All these ADNFLE related mutations, excepted β2I312M and α2I279N, are located within or close to the second transmembrane region (M2) of the nAChR subunit. The M2 region builds the wall of the ion channel, thus it seems that only mutations that have a direct effect on the ion pore are able to cause ADNFLE. The ADNFLE related mutations cause an increase in Ach sensitivity of the nicotinic receptors. It can be therefore concluded that a gain of function in the most widely distributed nAChR subtype in brain is the origin of neuronal network dysfunction that causes the epileptic seizures in ADNFLE patients (Bertrand et al, 2002).

The antiepileptic commonly administered to ADNFLE patients is carbamazepine (CBZ). However, about one third of the patients are

resistant to therapy (Oldani et al., 1998). Oxcarbazepine has recently been developed through structural variation of CBZ, to avoid some serious side effects caused by CBZ metabolites (Schmidt D. and Elger C.E., 2004). Both antiepileptics can modulate ligand-gated channels, including nAChRs, and appear to be particularly effective for treatment of nocturnal frontal lobe epilepsy (Di Resta et al., 2010).

Non-neuronal localization of nAChRs

Acetylcholine (ACh) is synthesized by pratically all living cells and can play a role in the interactions of non-neuronal cells with the environment. Choline and acetyl-CoA are the precursors of Ach and are present in nearly all cells. Acetyl-CoA is the major product of carbohydrate, protein and lipid catabolism in aerobic organisms. Choline originates from the intracellular breakdown of choline-containing phospholipids or from the uptake of extracellular choline via low- or high-affinity choline transporters. Evidence for ACh synthesis is not only provided by positive anti-ChAT immunoreactivity; but ChAT enzyme activity and/or ACh content have also been determined in the majority of the human cells (Wessler and Kirkpatrick, 2008)

ACh is involved in cell-to-cell communications in various non-neuronal tissues and controls important cell functions such as proliferation, adhesion, migration, secretion, survival and apoptosis in an autocrine and paracrine manner (Gotti and Clementi, 2004). The nAChRs are thought to regulate these vital functions through Ca^{2+}-dependent mechanisms (Chernyavsky et al, 2004).

Muscle

mRNAs coding for the α4, α5, α7, β4 and β2 subunits have been found in vertebrate adult muscle. α7 is highly expressed in mammalian muscle during development and the perinatal period, and decreases in adult life. The function of nAChRs in this tissue is not known, but nAChRs are likely to play a role in the control of various

metabolic and trophic functions (Gotti and Clementi, 2004).

Immune cells

Nicotinic binding sites are present in B-lymphocytes and in circulating and thymic T lymphocytes, and their density increases during aging. Ligand binding and RT-PCR have shown that human T lymphocytes and lymphocyte cell lines express the α3, α4, α7, β2 and β4 receptor subunits. The receptor subtypes most likely to be expressed are thus α3β4, α4β2 and α7 (Gotti and Clementi, 2004).

It has been demonstrated that ACh can modify immune response, for example ACh is involved in the induction of $CD4^+$ T-cell maturation. ACh modulates the activity of immune cell via auto- and paracrine loops (Wessler and Kirkpatrick, 2008).

Functional nAChRs containing α4 or α7 subunits are also present on B cells, where they stimulate growth and decrease antibody production.

Circulating phagocytic cells also express nAChRs. Human macrophages present α7 receptors and their activation by nicotine reduces the release of TNFα, interleukins 1 and 6 induced by the endotoxin polysaccharide (Gotti and Clementi, 2004).

Skin

Human epidermal keratinocytes express nAChRs which have the biophysical and pharmacological properties of α3-containing subtypes. Furthermore, the presence of α3, α7, α9, β2 and β4 subunits in these cells has been demonstrated by means of antibody binding, RT-PCR experiments and Ca^{2+} influx. The α3 subunit is

more expressed in small cells, localized in membranes forming cell junctions, and the α7 in large differentiated cells (Gotti and Clementi, 2004).

Lateral migration of eukaryotic cells is central to many important biological processes, such as embryogenesis, angiogenesis, metastasis and inflammation. Activation of nAChRs has been shown to affect chemotaxis and chemokinesis through Ca^{2+} dependent mechanisms (Chernyavsky et al, 2004; Chernyavsky et al, 2009).

Lung cells

Different nAChR subtypes are expressed in lung cells. α7, α3 and α5 nAChR subunits are present in bronchial epithelial cells, α4 in alveolar epithelial cells and α7, α4 and β2 in neuroepithelial bodies. Various nAChRs are also present in pulmonary neuroendocrine cells and in the human small-cell carcinoma cell lines. The presence of these receptors in lungs is important since the nicotine contained cigarette smoke reaches lung cells at high concentrations and may play a role in stimulating the growth of small-cell lung carcinoma (Gotti and Clementi, 2004).

Vascular tissue

The vascular system contains a number of nicotinic subunits in endothelial cells (β2, β4, α3, α5, α7 and α10) and vascular smooth muscle (α2, α3, α5, α4, α7, α10). Depending on the tissue localization of the vessels, the smooth muscle cells selectively express the nAChR subtypes. α5 and α3 are widely distributed among arteries but are not present in intrapulmonary or kidney vessels. α4 is not

present in muscle, kidney or lung small arteries. α7 is widespread but lacking in renal circulation (Gotti and Clementi, 2004).

Nicotine is a potent stimulus of angiogenesis and cell proliferation, this suggests that nAChRs play an important role in the regulation of these mechanisms (Gotti and Clementi, 2004).

Brain endothelial cells express α5, α7, β2 and β3 nicotinic subunits. Nicotine alters the permeability of the blood-brain barrier, and can be mediated by a decrease of the β2 and α7 subunits expression (Gotti and Clementi, 2004).

Astrocytes

α7, α4, α3, β3 and β4 nAChR subunits are all expressed in astrocytes. The role that these receptors play in astrocytes' life or astrocyte-neuron relationships is unknown, but they are thought to have a function in synaptic activity (Gotti and Clementi, 2004).

Non-neuronal pathology

All components of the system, synthesis, storage, release, inactivation, expression and function of the different nAChRs can be affected as a key pathogenetic event or secondary to the disease state (Wessler and Kirkpatrick, 2008).

Chronic inflammation may upregulate ACh synthesis. For example, substantially enhanced levels of ACh have been detected within the skin in atopic dermatitis. Enhanced levels of ACh have been associated with pruritus (Grando et al., 2006). A downregulation of ChAT has been found in the colon epithelium of patients with

ulcerative colitis. ACh release by the vagal nerve, as well as non-neuronal ACh, can induce an anti-inflammatory effect via α7 nAChRs (Wessler and Kirkpatrick, 2008).

ACh content is reduced in blood cells, leukocytes, as well as in bronchi of patients with cystic fibrosis, despite a somewhat enhanced ChAT activity. ChAT, a widely expressed transport and regulator protein, may be linked in a functional way, presumably to regulate the storage and transport of non-neuronal ACh within a cell. In cystic fibrosis, storage may become impaired and in consequence cells contain less ACh. In the airways of cystic fibrosis patients, this cholinergic dysfunction causes alteration in ion and water movements. On endothelial cells, α7 nAChRs are upregulated by hypoxia or ischemia. Nicotine promotes the growth of atherosclerotic plaques and potentiates endothelial monocyte interactions and the incorporation of endothelial progenitor cells into newly established vessels. (Wessler and Kirkpatrick, 2008).

However, the widest evidence about the pathological implications of alterations in the nAChR function in non-neuronal tissues concerns neoplasie, as is discussed in the following paragraphs.

Lung cancer

Lung cancer is the leading cause of cancer-related deaths for both men and women worldwide. In terms of incidence, lung cancer is second only to prostate cancer in men and breast cancer in women (Improgo et al., 2010). Lung cancer remains relatively untreatable, despite intense current clinical efforts (Gordon et al, 2009).

Cancer cells often carry somatic mutations of tumor-related genes, although other modifications, such as gene amplification or inactivation possibly caused by epigenetic mechanism, are also often observed. Cancer is a multistep process. Early steps comprise alterations of a relatively small number of genes implicated in cell proliferation, apoptosis and differentiation. The growing tumor mass then stimulates angiogenesis, in order to sustain itself. At later stages, phenotypic features are selected in order to enable cells to invade and colonize neighbouring or even distant tissue (Arcangeli et al., 2009).

The World Health Organization (WHO) made a classification list of three main forms of preinvasive neoplastic lesions in lung:

Squamous dysplasia and carcinoma *in situ*: usually observed in smokers and frequently correlated with the number of cigarettes smoked. The lesions include mucosal abnormalities that accompany squamous cell carcinoma such as basal cell hyperplasia, squamous metaplasia, dysplasia and carcinoma in situ. Dysplasia and carcinoma *in situ* are preinvasive, reversible, and may regress with the cessation of smoking. Chronic irritation and stimulation results in hyperplasia and multipotent progenitor basal cells residing in the respiratory

epithelium. Basal cells can also differentiate toward the squamous phenotype, an adaptation favoring survival and protection in a harsh environment. Normally there are no squamous cells in the airways. Persistent stimulus cause cellular damage, resulting in squamous dysplasia and carcinoma *in situ* (Gordon et al., 2009).

Atypical adenomatus hyperplasia: It is localized proliferation of mildly to moderately atypical cells lining alveoli, and sometimes respiratory bronchioles, resulting in a focal lesion in peripheral alveolated lung usually less than 5 mm in diameter. Atypical adenomatus hyperplasia consists of peripheral lesions, found in centriacinar regions close to terminal and respiratory bronchioles, that arise from bronchioloalveolar epithelium (Gordon et al., 2009).

Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DOPNECH): Lesions are not visible; as they progress to carcinoid tumorlets and tumors they appear as small, gray-white nodules. Microscopically, DOPNECH lesions are seen as a widespread proliferation of pulmonary neuroendocrine cells with patterns that include individual cells, small groups, or nest. Proliferation is centered in the bronchial or bronchiolar epithelium. Aggregates of neuroedocrine cells greater than 5 mm are regarded as typical carcinoid tumors (Gordon et al., 2009).

Patients with preinvasive lesions are ideal candidates for chemopreventive therapy. Chemoprevention for lung carcinoma involves exploring the mechanism of action of agents with suspected

antineoplastic properties in murine models and human cell lines, as well as identifying molecules in human preinvasive and invasive lung lesions that can be specifically targeted to prevent growth progression. An example is vitamin D, which is considered as a protective molecule because it regulates some cellular processes involved in tumorigenesis and metastasis (Gordon et al., 2009).

The two major histopathological types of lung cancer are small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC can be subdivided into adenocarcinoma, squamous cells, bronchioalveolar and large cell lung carcinoma (Improgo et al., 2010).

SCLC

Small cell lung cancer accounts for approximately 15 % of new cases of lung cancer deaths each year. Recent evidence suggests that women of all ages are more likely to present SCLC than men, and that younger women are more likely to present SCLC than older women.

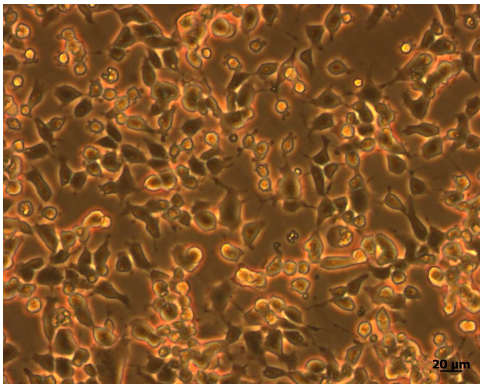

Fig. 4 SCLC U2020 cells

Histologically, the tumor cells are small, round to ovoid or spindle - shaped, with scant cytoplasm (fig. 4). The mitotic count is high and cells grow in clusters which exhibit neither glandular nor squamous organization. Neuroendocrine and neuronal differentiation results in the expression of dopa decarboxylase calcitonin, neuron-specific enolase, chromogranin A and other molecules (Sher et al., 2008).

The most important cause of SCLC is cigarette smoking, accounting for approximately 95% of cases. In the pathogenesis of SCLC are implicated autocrine growth loops, proto-oncogenes and tumor-supressor genes. Several chromosome and oncogene abnormalities have been identified in fresh SCLC tissues and cell lines, such as deletions on the short arm of chromosome 3 found in more than 95% of cases of SCLC. At the basis of carcinogenesis, processes are implicated that mediate proliferation, antiapoptosis, angiogenesis and metastasis. In SCLC, the presence of multiple neuropeptides and polypeptides with their specific receptors promotes the growth of SCLC via the establishment of autocrine growth loops (Sher et al., 2008). Different pathways are involved in the pathogenesis of this tumor, but the best characterized signal trasduction involved is the ras-raf-MAPK pathway, which mediates proliferation, cell cycle regulation, cell migration and angiogenesis. Dysregulation of the apoptotic process has been implicated in both tumorigenesis and therapeutic resistance. BCL2 is an important regulator of apoptosis and is overexpressed in most patients with SCLC. BCL2 confers resistance to treatment with cytotoxic chemotherapy, radiotherapy and monoclonal antibodies (Sher et al., 2008).

The most common symptoms caused by this tumor are dyspnea,

persistent cough and hemoptysism. Metastatic disease can produce pain, headache, malaise, seizures, fatigue, anorexia, and weight loss. Common targets of metastasis include bone, liver, lymph node, the central nervous system, adrenal glands, subcutaneous tissue and pleura. Combined modality treatment with chemotherapy and concurrent radiotherapy is the current standard of treatment (Sher et al., 2008).

NSCLC

It accounts for more than 85% of all lung cancer cases. The three major histological NSCLC types are adenocarcinoma, squamous cell carcinoma and large cell carcinoma, with a predominance of adenocarcinoma (Schuller, 2007).

Contrary to SCLC, NSCLC does not display c-myc amplification. By contrast, activating point mutations in k-ras is common, as well as inactivating mutations in p53 (Schuller, 2007).

Several biomarkers have emerged as prognostic and predictive markers for NSCLC, such as epidermal growth factor receptor (EGFR) and k-ras oncogene. A prognostic biomarker is a biomolecule which indicates patient survival regardless of the treatment received.

EGFR is a transmembrane receptor which, upon binding onto its ligand, activates pathways that control multiple cellular functions, including proliferation and survival. EGFR is detectable in approximately 80% to 85% of patients with NSCLC. The most common mutations in EGFR are exon 19 deletion and exon 21 mutation.

K-ras is a GTP-binding protein and is involved in G-protein-coupled receptor signaling. When mutated, it is costitutively active and, able to transform immortalized cells, thus promoting cell proliferation and survival (Ettinger et al., 2010).

Common symptoms of this cancer are cough, dyspnea, chest pain and weight loss. Symptomatic patients are more likely to have chronic obstructive pulmonary disease (COPD).

The three modalities commonly used to treat patients with NSCLC are surgery, radiation therapy and chemotherapy. These treatment modalities can be used either alone or in combination, depending on the disease status (Ettinger et al., 2010).

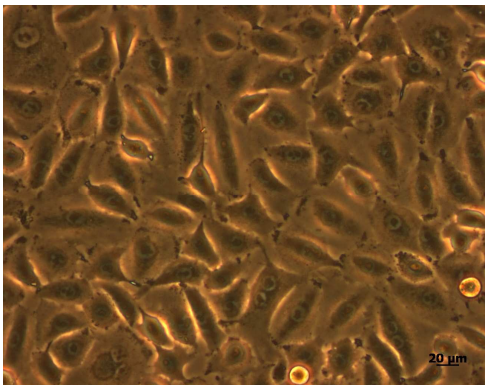

Fig. 5 NSCLC A549 cells

Polmonary Adenocarcinoma (PAC): It is a kind of NSCLC which originates in the lining of the lung from Clara cells and ciliated cells. Compared to other types of lung cancer, adenocarcinoma is more likely to be contained in one area. If it is appropriately located, it may

respond to treatment better than other lung cancers. It is the most common form of lung cancer. It is generally found in smokers, although it is the most common type of lung cancer in nonsmokers. It is also the most common form of lung cancer in women and people younger than 45.

Squamous cells carcinoma (SCC): SCC usually begins in the bronchial tubes (large airways) in the central part of the lungs. SCC is thought to arise from the pseudostratified epithelial lining compounds of basal cells, ciliate cells, and mucous cells of large airways (Schuller, 2007). Because of its position, SCC often causes symptoms earlier than other forms of lung cancer. Obstruction of the airway can lead to infections such as pneumonia, or collapse of part of a lung. Individuals with squamous cell carcinoma are also more likely to experience an elevated calcium level (hypercalcemia) which may result in muscle weakness and cramps.

Large cell lung carcinoma (LCC): Large cell lung cancer is thus named because the abnormal cells appear large under the microscope. LCC often begins in the central part of the lung. Of the non-small cell lung cancers, this type is usually discovered at a later stage. Large cell lung cancers tend to grow quickly and spread. Cancer may spread into nearby lymph nodes and into the chest wall. It can also spread to more distant organs, even when the tumor in the lung is relatively small.

Nicotinic acetylcholine receptors in lung cancer

More than 1 billion people worldwide smoke tobacco. Smokers have twenty levels greater risk of developing lung cancer than non-smokers and increased risk of many other tumor types. Tobacco smoke deposits hundreds of chemicals in the airways and lungs, among which more than 60 mutagens are contained that bind and chemically modify DNA. Each carcinogen-associated mutation represents the consequence of three processes: chemical modification of a purine by a mutagen, failure to repair the lesion by genome surveillance pathways and incorrect nucleotide incorporation opposite the distorted base during DNA replication (Pleasance et al., 2010).

Expression of nAChRs is seen in both SCLC and NSCLC. Expression on nicotinic receptors in lung cancer derives from the expression of nAChRs in normal lung cells. Normal bronchial epithelial cells express nAChRs as part of a cholinergic autocrine loop in which all proteins needed for cholinergic signaling are present, including the ACh-synthesizing enzyme choline transferase (ChAT), vesicular ACh transporter (VAChT), ACh hydrolyzing enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), high-affinity choline transporters CHT1, nAChRs and muscarinic ACh receptors (Song et al., 2008).

The nicotinic subunit expression changes between normal lung cells and tumor cells. Data obtained by Lam et al. (2007) show statistically significant differences in the expression levels of CHRNA4 and CHRNB4 between NSCLC tumor and normal lung tissues as well as CHRNA6 and CHRNB3 between smokers and nonsmokers. The

expression level of CHRNA4 was found to be lower in NSCLC tumors compared to normal lung tissues. A modest elevation of CHRNB4 subunit in NSCLC compared with normal tissues was also detected. The genes CHRNA5, CHRNA7, CHRNA9 and CHRNB2 showed elevated levels of expression in NSCLC cell lines and this suggest that they are involved in cellular processes in these cancer cells. CHRNA6 and CHRNB3 subunit genes showed lower expression in NSCLCs from smokers compared with nonsmokers; this could imply desensitization with chronic exposure to tobacco smoke (Lam et al., 2007). Studies in normal neuroendocrine (NE) cells and neuroendocrine tumors shown that the β2 subunit of nicotinic receptors is never expressed in normal NE cells of lungs and very rarely expressed in NE tumors. In contrast, the α7 subunit is constantly found in NE cell in normal lungs. In tumors, its expression is significantly higher (Sartelet et al., 2008). However, nAChR expression is different among the diverse types of lung cancer, and this is understandable since lung tumors derive from different lung cells types that also have different patterns of receptor expression (Song et al., 2008).

Changes in cholinergic signaling in tumors are not limited to nAChR expression but large changes in ACh synthesis and degradation are also present. Data obtained from real-time PCR, performed to characterize the cholinergic genes, show that ChAT was significantly increased SCC compared to normal tissue, whereas the levels of CHT1 and VAChT were not significantly changed. The levels of mRNAs which encode proteins that serve to limit cholinergic signaling, such as AChE, BChE and Lynx1 (an allosteric modulator of

nAChRs), were all significantly less in tumors compared with controls (Song et al., 2008).

Therefore, lung cancer expresses an intrinsic cholinergic signaling system, so that exogenous nicotine and endogenous ACh can stimulate tumor growth. The cholinergic system in tumors is upregulated at multiple levels. This upregulation, combined with smoking, provides not only a proliferative stimulus but also a pathway to target for new therapeutic approaches to lung cancer.

Non-neuronal nAChRs also regulate other cell functions, by acting as central regulators of a complex network of stimulatory and inhibitory neurotransmitters. These molecules govern the synthesis and release of growth, angiogenic and neurogenic factors in cancer cells and their microenvironment, as well as in distant organs. In addition, nAChRs stimulate intracellular signaling pathways in a cell-type specific manner (Schuller, 2009).

nAChRs and cell proliferation: Nicotine can stimulate the proliferation of various normal and cancer cells. The role of nAChRs in cell proliferation is shown by the ability of nicotinic receptor antagonists to reverse the proliferative effect of nicotine.

Treatment of several SCLC and NSCLC cell lines with nicotine induces proliferation in a receptor - dependent manner. The effects of nicotine are mediated by growth factors. For example, nicotine induces transactivation of EGFR through an increase in intracellular Ca^{2+} and stimulation of L-type voltage-sensitive Ca^{2+} channels (Egleton et al., 2008).

The $\alpha 7$ nAChR appear to be particularly implicated in mediating the

proliferative effect of nicotine.

One characteristic shared by tobacco-related and chronic lung diseases is the altered content and composition of the lung connective tissue. In particular, there is an increase of expression and deposition of fibronectin. This is an extracellular matrix glycoprotein which can modulate many cellular functions such as cell adhesion, migration, chemotaxis, proliferation, differentiation and apoptosis. Fibronectin is expressed in several cancer cells, and the adhesion of lung carcinoma cells to fibronectin enhances tumorigenicity and provides resistance to apoptosis induced by chemotherapeutic agents. Nicotine binds to the α7 nicotinic receptor and stimulates lung carcinoma cell growth through activation of extracellular signal-regulated kinase (ERK) and phosphoinositide3-kinase (P13-K)/mTOR signaling pathways which lead to increased fibronectin expression (Zheng et al., 2007).

In SCLC, α7 nAChRs stimulate cell proliferation *in vitro* through activation of protein kinase C (PKC), serine/threonine kinase RAF1, the mitogen activated kinase ERK1 and ERK2, and FOS, JUN and MYC transcription factors (Fig. 6; Schuller, 2009).

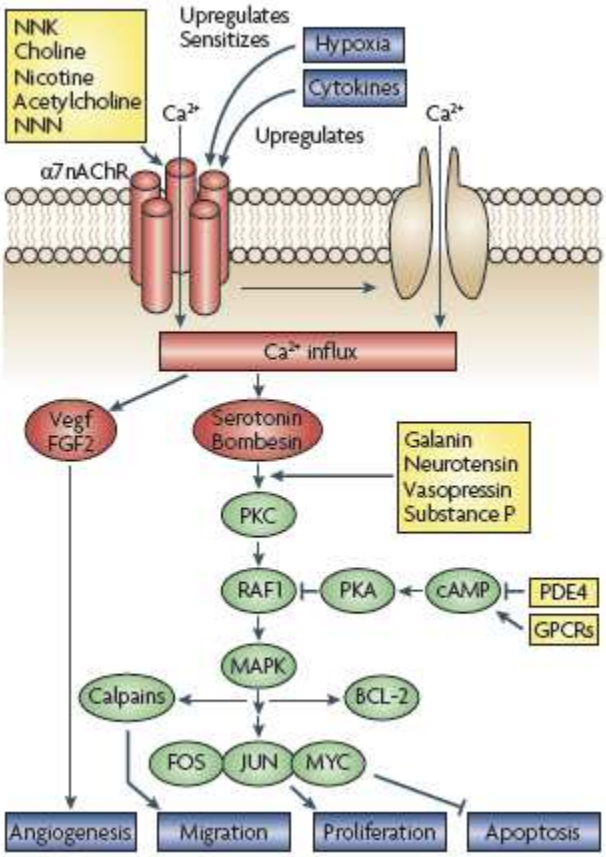

Fig. 6 Regulation of SCLC cells (Schuller, 2009)

SCLC cells *in vitro* are more sensitive to the growth-stimulating effects of nicotine in an environment with high CO_2 and low O_2, typical condition in tumor environments (Catassi et al., 2008; Schuller, 2009).

Serotonine is a potent mitogen for SCLC cells. Nicotine induces release of serotonine from these cells and stimulates SCLC growth. The proliferation effects caused by nicotine are blocked by mecamylamine and αBgtx (Codignola et al., 1994).

NSCLC, in contrast to SCLC cells, expresses multiple heteromeric nAChRs in addition to the α7 nAChR. In these cells, nicotine activates the p13K-Akt pathway and nuclear factor-κB (NF-κB), resulting in stimulation of proliferation and inhibition of the chemotherapy-induced apoptosis (Fig. 7; Schuller, 2009).

Human tissues express α7 nAChR. This expression is higher in smoking patients. Major expression of α7 nAChR can be related to major activation of the Rb-Raf1/phospho-ERK/phospho-p90RSK pathway. RB-Raf-1 interaction is an important early event in mediating cell proliferation. Antagonists of α7 nAChR such as α-cobratoxin inhibit the proliferation of NSCLC cells (Palerari et al., 2008; Paleari et al., 2009).

Nicotine can promote inhibitory phosphorylation of protein phosphatase 1 (PP1) In NSCLC. Loss of PP1 function decreases the levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, which in turn facilitates cell cycle progression (Egleton et al., 2008).

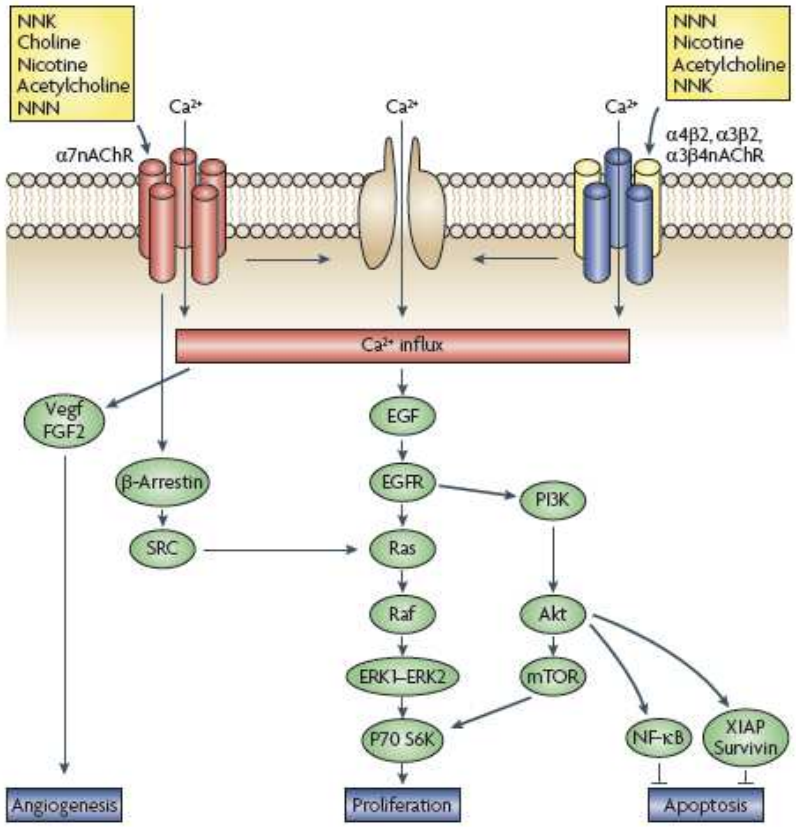

Fig. 7 Regulation in NSCLC cells (Schuller, 2009)

nAChR and survival pathways: In tumors, nicotine has been shown to protect cells from the apoptosis induced by anticancer drugs. Acquisition of drug resistance is a considerable challenge in cancer therapy. nAChR antagonists could be useful to enhance the therapeutic response to chemotherapy.

Activation of Akt causes apoptosis inhibition in many tissues in response to growth factor deprivation or oncogene stimulation

(Minna, 2003).

Nicotine causes activation of the Akt signaling pathway in lung epithelial cells. Akt is a multifunctional serine-threonine protein kinase. Usually, this kinase resides in the cytosol in a low-activity conformation. Upon cellular stimulation, Akt is activated through recruitment to cellular membranes by PI3K lipid products, resulting in the activation of a variety of downstream targets including NF-κB pathways. Activation of PKC and PKA and downregulation of the tumor suppressor p53 (Catassi et al., 2008; Egleton et al., 2008).

Nicotine also regulates the Bcl-2 family of apoptotic proteins. The different proteins that belong to this family can have an anti-apoptotic or pro-apoptotic role. The pro-apoptotic Bax belongs to this family. Bax may be an essential component in the nicotinic survival signaling pathway, through a mechanism involving activation of PI3K/Akt that directly phosphorylates and inactivates its function.

Bcl-2 is instead an anti-apoptotic protein that is phosphorylated and thus activated in SCLC, by treatment with nicotine (Egleton et al., 2008).

Furthermore, nicotine causes inactivation of the pro-apoptotic Bad protein by its phosphorylation in different sites (Egleton et al., 2008).

The α3 and α4 nAChR subunits mediate the anti-apoptotic effect of nicotine, as shown by the use of specific antagonists.

Not only does nicotine have an anti-apoptotic effect, it can also cause a loss of contact inhibition at high cell densities in culture (Minna, 2003).

The effects on proliferation and apoptosis are responsible for the mitogenic effects of nicotine.

nAChRs and angiogenesis: An important process for tumor growth and metastatic dissemination is angiogenesis, the development of new blood vessels. This process is regulated by different pro- and anti-angiogenic molecules. Angiogenic growth factors, such as VEGF and FGF, are released by induction of angiogenic stimuli (hypoxia or inflammatory cytokines). These growth factors stimulate endothelial cells to proliferate and to migrate to form new endothelialized channels (Heeschen et al., 2002).

Stimulation of nicotinic acetylcholine receptors results in a significant release of VEGF. Antagonists of nAChR significantly attenuate VEGF-induced angiogenesis.

In the endothelial cells, nAChR induce stimulation of angiogenesis through activation of PI3K/Akt pathways that results in NF-κB activation. Moreover, via Akt pathways, nicotine induces phosphorylation of endothelial nitric-oxide synthase (eNOS), thus nAChR activation increase production of nitric oxide (Heeschen et al., 2002).

Hypoxia *in vitro* and ischemia *in vivo* produce upregulation of α7 nAChR. The selective α7 nAChR antagonist αBgtx inhibits capillary network formation *in vitro*.

nAChR and cell migration: Lateral migration in eukaryotic cells is important to several biological processes. Different chemical stimuli can modulate random cell migration (chemokinesis), or directional migration (chemotaxis). Cell movement is generally controlled by intracellular Ca^{2+}, which may be altered by activation of nAChRs.

Both chemokinesis and chemotaxis have been observed to be modulated by nAChRs in human Keratinocytes (Chernyavsky et al., 2004).

For example the stratified epithelial cells in the uppermost division of the skin produce Ach and use it as an autocrine and paracrine hormone regulating their motility, particularly during wound healing. Both heteromeric and homomeric nAChRs are present in the plasma membranes of KCs. The α7 nAChR subtype stimulates directional migration, whereas the nAChRs containing the α3 subunit stimulate random migration (Chernyavsky et al, 2005).

The direction of migration is determined when a KC extends a cytoplasmic protrusion (lamellipodium) from the free basolateral side into the wound. In presence of an ACh gradient, nAChRs accumulate in the membrane at the front of the cell, leading to a membrane depolarization that causes the lamellipodium formation.

The Ca^{2+} ions entering through α7 nAChR can regulate, in various types of epithelial cells the CAMKII, PKC, PI3K, Jak2 and the Ras/Raf/MEK/ERK cascades. These signaling pathways are implicated in the physiologic regulation of cell motility. Activation of this pathway induces upregulation of α2 and α3 integrins, which are required for stabilization of the lamellipodium (Chernyavsky A. et al, 2005; Chernyavsky A. et al, 2009).

Nitrosamines

Nitrosamines are carcinogens formed in the mammalian organism from amine precursors contained in food, cosmetics, beverages and drugs. Nitrosamines are formed by nitrosation of simple amine precursors. Oxidative enzymes convert nitrosamines into a number of metabolites. Some of these bind to the DNA molecule thus form adducts associated with the activation of point mutations in genes implicated in numerous types of human cancer (Schuller, 2007).

The tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) are formed from nicotine (Fig. 8).

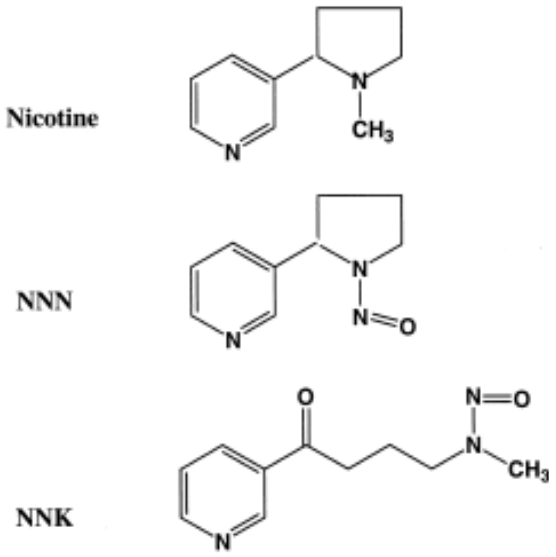

Fig. 8 Structure of nicotine, NNK and NNN (Schuller and Orloff, 1998)

Nicotine is an alkaloid consisting of a pyridine and a pyrrolidine ring. NNN has a similar structure, whereas NNK does not have the pyrrolidine ring, which is opening during the metabolic process.

The lung absorbs nicotine and both nitrosamines rapidly. Both nicotine and nitrosamines enter the bloodstream and are distributed in body tissues. In an individual who smokes one pack of cigarettes a day on average, the concentration of nicotine in the blood is ~ 1μM. The concentration of nicotine in the smoke of cigarettes is on average 5,000-10,000 greater than that of NNK, and about 2,000-3,000 times greater than that NNN. In the blood of a smoker, the proportions of unmetabolized nicotine and nitrosamines are similar (Schuller and Orloff, 1998).

Data obtained by radioligand binding assays suggest that NNK has a high affinity to α7 receptors, whereas NNN to heteromeric receptors (Fig. 9; Schuller and Orloff, 1998).

The binding of nitrosamines can activate the intracellular pathways involved in nAChR activation. In the organism, NNK and NNN undergo α-hydroxylation, a process that leads to the generation of electrophilic species that react with DNA. DNA can be attacked at several positions; the major sites of reaction are the 7- and O^6-positions of guanine.

The lung is one of the major target organs of tobacco specific nitrosamines and was found to accumulate the promutagenic lesion O^6mGua during exposure to NNK. In the lung, the accumulation of promutagenic methylated DNA adducts may cause respiratory tumors (Belinsky et al., 1986).

Therefores nitrosamines can produce carcinogenic effects in two

ways; *a)* directly on the genome by causing the formation of DNA adducts, and *b)* binding to nAChRs on the surface membrane, thus activating the intracellular signaling pathways that promote tumorigenisis (Nishioka et al., 2010).

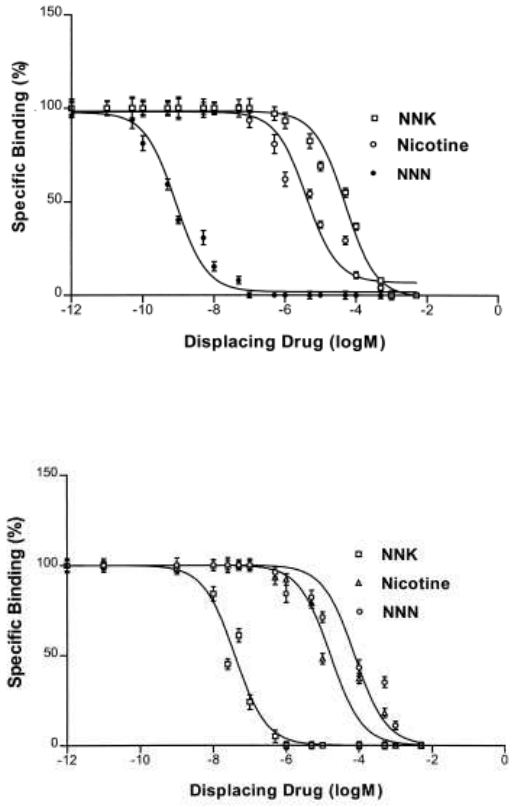

Fig. 9 Results of receptor binding assays in which nicotine, NNN and NNK competed with [^{3}H]epibatidine, for the epibatidine–sensitive nicotine binding sites (top panel), and with αBgtx, for the α7 sensitive nicotine binding sites (lower panel) (Schuller and Orloff, 1998)

Scope of the thesis

As I discussed above, neuronal nicotinic receptors are pivotal elements in the regulation of different physiological mechanis in several tumours, including lung cancers. This is suggestive because smoke is an established risk factor for cancer, and particularly lung cancer. Activation of nAChRs stimulates (directly and indirectly) Ca^{2+} influx, which triggers the release of growth factors and other transmitter molecules. These produce autocrine and paracrine effects that promote proliferation, inhibit apoptosis and stimulate angiogenesis. Such effects occur in both small and non-small cell lung cancer cell lines, although the intracellular signalling cascades and the nAChRs involved are different. Radioligand competition data suggest that several carcinogens produced by tobacco inhalation, particulary the N-nitrosamines such as NNK bind with high affinity to nAChRs. It has thus been proposed that some of the oncogenous effects of these compounds depend on specific activation of nAChRs. Moreover these drugs are throught to easily cross the blood-brain barrier, therefore they could contribute to the addictive effects of tobacco mediated by nAChR engagement.

However rigorous studies exist on i) the functional membrane expression and the properties of different nAChR subtypes in lung cancer cell lines; ii) the effects produced by nitrosamines on specific nAChR subtypes. In particular, long-term exposure to ligands has complex effects on nAChRs and may lead, in different receptor types, to increased expression or prolonged desensitization, with radically different physiological implications.

The scope of my thesis was the study of these different aspects by two complementary approaches. To define the full complement of functional nAChRs in small and non-small cell lung cancer cell lines, I measured the nicotinic currents in the presence of agonists and several extracellular inhibitors specific for the different subtypes. This approach is a necessary first step to determine the physiological role of different nAChR types in processes such as cell proliferation and migration. On the other hand, to understand detailed mechanism of action of NNK on nAChRs, I applied patch-clamp methods to HEK cells stably expressing $\alpha 4\beta 2$ nAChRs, the most common heteromeric receptor in different tissues.

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CHAPTER 2

"Effect of carbamazepine an oxcarbazepine on wild-type and mutant neuronal nicotinic acetylcholine receptors linked to nocturnal frontal lobe epilepsy"

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Abstract

Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide) and oxcarbazepine (10,11-dihydro-10-oxo-5H-dibenz[b,f]azepine-5-carboxamide) are widely used for the treatment of partial epilepsy. Recent work indicates that these drugs, in addition to targeting voltage-gated Na^+ channels, can modulate ligand-gated channels. These compounds appear to be particularly effective for treatment of nocturnal frontal lobe epilepsy, which can be caused by mutant neuronal nicotinic receptors. We compared the effects of carbamazepine and oxcarbazepine on heteromeric nicotinic receptors to better understand the underlying mechanism of the effect of these drugs in epileptic patients.

Receptors were expressed in cell lines and studied by patch-clamp methods at -60 mV. For α2β4 receptors activated with 100 μM nicotine, IC_{50} for carbamazepine was 49 μM. Receptors in which α2 was substituted with α2-I279N, linked to autosomal dominant nocturnal frontal lobe epilepsy, had an IC_{50} of 21 μM. For oxcarbazepine, the estimated IC_{50} was larger than 500 μM for wild-type receptors and approximately 100 μM for mutant receptors. A similar inhibition was observed in the presence of 10 μM nicotine,

indicating a non-competitive mechanism. The monohydroxy derivative (MHD) of oxcarbazepine, clinically the most relevant compound, was tested on both α2β4 and α4β2 receptors, to obtain a broader view of its possible physiological effects. At the typical concentration present in blood (100 μM), MHD produced an approximate 40% channel block on α4β2 and 10% block on α2β4 receptors. Oxcarbazepine and MHD retarded the channel deactivation, suggesting that these compounds produce open channel block. These results may explain the particular efficacy of these drugs in nocturnal frontal lobe epilepsy.

Introduction

Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide) is commonly used for treatment of partial and generalized seizures. Oxcarbazepine (10,11-dihydro-10-oxo-5H-dibenz[b,f]azepine-5-carboxamide) is a newer compound that avoids some serious side effects caused by carbamazepine's metabolites and is considered a first choice drug for the treatment of partial-onset seizures (Beydoun et al., 2008). After absorption, oxcarbazepine is rapidly converted to 10,11-dihydro-10-hydroxy-carbamazepine (MHD; Fig. 1). The two compounds coexist in plasma at different concentrations. In humans, the peak concentration of oxcarbazepine is approximately 5 μM, whereas its steady state concentration is negligible (Lloyd et al., 1994). Hence, MHD is considered the clinically more relevant compound, with plasma therapeutic levels between 30 and 150 μM (Dam et al., 1989; Johannessen et al., 2003; Tecoma, 1999).

The molecular targets of these compounds are only partially known (Schmidt and Elger, 2004). Carbamazepine reduces action potential frequency by retarding recovery from the inactivation of voltage-gated Na^+ channels (McLean and MacDonald, 1986; Schwarz and Grigat, 1989). Oxcarbazepine and MHD probably produce similar effects

(Benes et al., 1999; MacDonald and Rogawski, 2008). Inhibition of voltage-gated Ca^{2+} currents has also been described, although the efficacy at therapeutic concentrations is debated (Ambrosio et al., 2002; Stefani et al., 1995). These actions alter neuronal excitability and may impair glutamate release (Sitges et al., 2007a, 2007b), although direct inhibition of glutamate receptors cannot be excluded (Giustizieri et al., 2008). Recent results show that carbamazepine and oxcarbazepine (but MHD only slightly) potentiate $GABA_A$ receptors (Zheng et al., 2009).

Another possible target was suggested by the efficacy of carbamazepine in treating nocturnal frontal lobe epilepsy (NFLE), characterized by frontal seizures usually arising during phase II of sleep (Provini et al., 2000). The Mendelian form of the disease (ADNFLE; Oldani et al., 1998) is often associated to mutant subunits of the heteromeric neuronal nicotinic receptors (Picard and Brodtkorb, 2008). These are heteropentamers of differing α and β subunits, widely expressed at both the pre- and postsynaptic level throughout the brain (Albuquerque et al., 2009). Indeed, carbamazepine was found to block α4β2 and α2β2 nicotinic receptors, and some mutant subunits linked to ADNFLE display altered sensitivity to this drug

(Bertrand et al., 2002; Hoda et al., 2009; Leniger et al., 2003; Picard et al., 1999).

Although oxcarbazepine is structurally related to carbamazepine and may be even more efficacious for treating NFLE (Raju et al., 2007; Romigi et al., 2008), its effects on nicotinic receptors are unknown. Thus, in this study we compare the effects of these carbamazepine-related compounds on α2β4 receptors with and without the α2-I279N mutant subunit, the only α2-related mutant presently known to be linked to ADNFLE (Aridon et al., 2006). Moreover, we test the effect of MHD on α4β2 receptors, the most widespread heteromeric form in the mammalian brain. We find that these drugs inhibit different types of human neuronal nicotinic receptors, which, because of the general excitatory role of acetylcholine in the brain, could contribute to their antiepileptic effects. Their therapeutic action may be particularly effective in NFLE, considering the involvement of the cholinergic system in this disease.

Materials and methods

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich Italia Srl (Milan, Italy).

Cell culture and transfection procedure

The α4, α2, α2–I279N, β2 and β4 subunits of the human neuronal nicotinic receptor, all subcloned into the pcDNA3 expression vector (InvivoGen, San Diego, CA), were transiently expressed in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories, South Logan, UT) supplemented with 10% fetal calf serum (HyClone) and 4.5 g/l glutamine, at 37°C and 5% CO_2. For patch-clamp experiments, cells were harvested by trypsinization and plated onto 35 mm Petri dishes (Corning Incorporated, NY). To obtain wild-type or mutant receptors, cells were transfected with either α2 or α2-I279N, respectively, plus an equimolar amount of β4. The receptor subunits were co-expressed with the Enhanced Green Fluorescent Protein (EGFP) pcDNA3 (Clontech Laboratories, Palo Alto, CA). The transfection mixture contained: 50 μl of $CaCl_2$ 0.5 M, 100 μl of Bes medium, 4 μg of

nicotinic subunit pDNA, 0.2-0.4 μg of EGFP pDNA. Bes medium contained (mM): Bes 50, NaCl 280, Na_2HPO_4 1.5, pH 6.96 (adjusted with NaOH). The mix was left for 20 min at room temperature to allow the formation of calcium phosphate precipitates. Subsequently it was dripped onto the cells until reaching 10% of the dish volume. After 6-8 h, cells were washed with fresh culture medium.

MHD (Toronto Research Chemicals Inc., North York, Ontario, Canada) was tested on HEK cell clones stably expressing the α4β2 receptors (kindly gifted by Dr. L. Rampoldi and Prof. G. Casari, Istituto Scientifico San Raffaele, Milano, Italy). These clones were extensively characterized by patch-clamp. Whole-cell currents showed the typical kinetic and pharmacological features of α4β2 receptors. In particular, for agonists, the EC_{50} values were 12.7 ± 3.6 μM for acetylcholine and 19 ± 2.74 μM for nicotine. For inhibitors, the IC_{50} values were 49.7 ± 1.25 nM for dihydro-β-erythroidine and 1.43 ± 1.12 μM for methyllycaconitine. These values were estimated with a single-term Hill equation and are in line with literature on α4β2 receptors stably expressed in HEK cells (e.g. Buisson and Bertrand, 2001).

Solutions and drugs

The extracellular solution contained (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, D-glucose 5 (pH 7.3). Pipettes contained (mM): K-aspartate 120, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 1.3, EGTA-KOH 10, HEPES-KOH 10, MgATP 1, (pH 7.3). Stock solutions for nicotine were prepared weekly in our extracellular solution and kept refrigerated. Stock solutions of 40 mM carbamazepine or oxcarbazepine were prepared in 100% ethanol or dimethylsulfoxide, respectively, and diluted daily into our extracellular solution. No effect of solvent was observed at the highest concentrations applied. MHD powder was dissolved at the final concentration in our extracellular solution.

Patch-clamp experiments

We have applied the whole-cell configuration of the patch-clamp technique. Currents were registered 36-48 h after transfection, with an Axopatch 200B amplifier (Molecular Devices, Union City, CA), at room temperature. Micropipettes (2-3 MΩ) were pulled from borosilicate capillaries with a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Company, Novato, CA). The cell capacitance and series resistance were always compensated.

Fluorescent cells were detected with an Eclipse TE200 microscope (Nikon Instruments, Sesto Fiorentino, Italy), equipped with TE-FM epifluorescence attachment. Currents were low-pass filtered at 2 kHz and acquired on-line at 5-10 kHz with pClamp 9 hardware and software (Molecular Devices). Drugs were applied with an RSC-160 Rapid Solution Changer (BioLogic Science Instruments, Claix, France).

Analysis of data

Data were analyzed with Clampfit 9.2 (Molecular Devices) and OriginPro 8 (OriginLab, Northampton, MA). The concentration-response data were fitted to a single-term Hill-Langmuir equation:

$$I_L/I_{max} = \{1 + ([L]/IC_{50})^{nH} \}^{-1} \qquad (1)$$

where I_{max} is the maximal current (i.e. the peak current in the absence of inhibitor), I_L is the current at a given concentration of inhibitor L, IC_{50} is the concentration of inhibitor causing a 50% block, and n_H is the Hill coefficient (expressing the degree of apparent cooperativity). Data are generally given as mean values ± standard error of the mean. Statistical significance was determined with two-tailed Student's t test

for paired or unpaired samples, as indicated. The level of significance was set to $P < 0.05$.

Results

Expression of the α2 and α2-I279N subunits with β2 and β4

To understand the functional properties of α2-containing receptors, it is necessary to study at least the α2β2 and α2β4 forms, which present distinct pharmacological features when expressed in *Xenopus* oocytes (Chavez-Noriega et al., 1997; Luetje and Patrick, 1991). Moreover, recent results show that α2β2 and α2β4 receptors can be expressed in different cerebral nuclei and thus exert distinct physiological effects (Whiteaker et al., 2009). From an epileptologic standpoint, current evidence suggests that both β2 and β4 subunits are implicated in seizure susceptibility (De Fusco et al., 2000; Kedmi et al., 2004; Phillips et al., 2001; Salas et al., 2004).

We thus tested the effect of coexpressing either β2 or β4 with α2 and α2-I279N subunits. Expression of these receptor forms in HEK cells was tested at -60 mV with 300 μM acetylcholine. Overall, α2β2 receptors showed the lowest functional expression (Fig. 2). With saturating acetylcholine doses, these receptors showed an average current density of 0.79 ± 0.30 pA/pF, less than one twentieth the value obtained with α4β2 receptors. The expression of α2-I279Nβ2 was even lower than that of α2β2.

The data shown in Fig. 2 summarize registrations from about 100 cells, carried out after 12 runs of transfection, in different cell batches. Similar results were obtained with other HEK cell clones (such as Phoenix, or TsA) and by using nicotine instead of acetylcholine (data not shown). Higher ligand concentrations (up to 3 mM) did not produce greater currents, suggesting that the above results were not due to a shift to the right of the concentration-response to agonists. Considering that our α2 subunits produced robust currents when coexpressed with β4 subunits (see also Aridon et al., 2006) and that β2 gave normal expression with α4 (Fig. 2), it seems unlikely that the low expression of α2β2 receptors in our cells is due to altered transcription or trivial artifacts such as degradation of our clones. The molecular reasons for these differences are not further investigated here. In the rest of the paper, the α2 subunit was expressed with β4, because the generally low current amplitudes observed with the α2β2 form were insufficient for extensive pharmacological characterization with the patch-clamp method.

The maximal current densities of α2β4 and α2-I279Nβ4 receptors were similar whether channels were activated with acetylcholine or nicotine, which suggests that α2-I279N is unlikely to cause important alterations of α2β4 channel expression. Moreover, no major difference

was observed in the monoexponential time constant of channel desensitization, in the presence of the same agonist, for the different receptor's forms. These results are summarized in Table 1.

Because the properties of our receptors tested with acetylcholine or nicotine were overall comparable, the effect of antiepileptics was studied in the presence of nicotine. This avoids possible alteration of the nicotinic current kinetics because of superimposition of other currents activated by the muscarinic receptors that may be endogenously expressed by HEK cells (e.g. Oldfield et al., 2009).

Effect of carbamazepine and oxcarbazepine on α2β4 and α2-I279Nβ4 receptors

Cells expressing α2β4 and α2-I279Nβ4 receptors were voltage clamped at -60 mV, a membrane potential close to the typical resting potentials of neocortical neurons. Carbamazepine and oxcarbazepine were applied for 20 s, to reach equilibration of drug concentration, before nicotine was applied to elicit whole-cell currents. The antagonists' effect was tested in the presence of 100 μM nicotine, which was applied for 2 s. Stimulations were spaced 2-3 min apart to allow full channel recovery from desensitization. The response to nicotine alone was tested at regular intervals to exclude artifacts

caused by the spontaneous nicotinic current decay that is sometimes observed in cell lines. Our experimental procedure is illustrated in Fig. 3A for mutant receptors treated with oxcarbazepine.

Picard et al. (1999) have shown that carbamazepine produces a non-competitive channel inhibition in heteromeric neuronal nicotinic receptors, probably caused by an open-channel block. Our results suggest that the same applies to oxcarbazepine, as indicated by the transient current 'rebound' observed upon drug removal (Fig. 3A). This suggests that oxcarbazepine is flushed from the channel pore during the wash, thus temporarily increasing the current amplitude before receptor deactivation. Quantification of this effect can be obtained by measuring the time course of the nicotinic current decay during wash. Table 2 gives the half-time values of current deactivation in the presence of the indicated inhibitor, for different receptor types. This parameter was chosen because its calculation does not depend on any specific assumption about the mechanism of channel blocking/unblocking by our inhibitors. Channel deactivation turned out to be generally slower in the presence of oxcarbazepine. For instance, 100 μM oxcarbazepine produced a 30-40% delay of channel closure, in both wild-type and mutant receptors.

Finally, the blocking effect produced by 100 μM oxcarbazepine was similar when this antiepileptic was applied in the presence of either 100 μM or 10 μM nicotine, in line with a non-competitive mechanism (Fig. 4).

The general use of a quasi-saturating nicotine concentration (which maximizes the probability of a single channel being open) allowed us to obtain a quick time course of channel blocking. At a given antagonist concentration, the fractional current (the ratio between currents in the presence and in the absence of antagonist) at the peak was scarcely different from that measured 1.0 s and 1.8 s after agonist application. Fig. 3B and 3C plot the fractional currents for wild-type and mutant receptors respectively at i) the peak, ii) after 1 s and iii) after 1.8 s at the indicated antagonist concentration. Thus, blockade fully developed within the typical apparent time of nicotinic current activation, which was usually around 200 ms (in the experiments shown in Fig. 3, the 10-90% current rise-time was 120 ms). Therefore, the concentration-response relations for carbamazepine and oxcarbazepine (Fig. 4) plot peak fractional currents at the indicated antagonist concentrations for wild-type and mutant receptors. The dose-response experiments with carbamazepine and oxcarbazepine were capped at 200 μM and 100 μM, respectively, to avoid the

interference of high concentrations of solvent. These concentration-response relations were fitted with equation 1. Full statistics are given in the figure legend. Briefly, IC_{50} for carbamazepine was approximately 50 μM for wild-type and 20 μM for mutant receptors (Fig. 4, upper panel). For oxcarbazepine, the estimated IC_{50} was lager than 500 μM for wild-type and approximately 100 μM for mutant receptors (Fig. 4, lower panel). Overall, the α2β4 receptors were inhibited by both drugs, with carbamazepine being considerably more effective. Moreover, both compounds were capable of discriminating wild-type and mutant receptors. The IC_{50} we measured with carbamazepine in wild-type receptors was intermediate between the values previously reported for α2β2 (15 μM; Hoda et al., 2009) and α4β2 (about 150 μM; Picard et al., 1999; Leniger et al., 2003). The IC_{50} for α2-I279Nβ4 was somewhat lower than the value reported for α2-I279Nβ2 receptors (about 37 μM; Hoda et al., 2009). These results confirm that different heteromeric nicotinic receptors, containing or not ADNFLE-linked subunits, exhibit distinct pharmacological properties.

Effect of MHD on α2β4 and α4β2 neuronal nicotinic receptors

As discussed earlier, to determine the possible therapeutic relevance of the inhibition produced by oxcarbazepine on neuronal nicotinic receptors, it is also necessary to study the effects of its main metabolite, MHD. Nothing is known about the action of this compound on any nicotinic receptor, to the best of our knowledge. We thus tested MHD on both α2β4 and α4β2 receptors, which should represent a significant fraction of the cerebral heteromeric receptors. This drug was applied at 1 and 100 μM, to cover the typical therapeutic range, in the presence of 100 μM nicotine at -60 mV. The experimental protocol was analogous to the one used for carbamazepine and oxcarbazepine. Typical current traces for α4β2 receptors in the presence and in the absence of MHD are illustrated in Fig. 5 (upper panel). MHD was less effective than carbamazepine and oxcarbazepine on α2β4 receptors, producing about 10% channel block at 100 μM. On the other hand, MHD was considerably more effective onto α4β2 receptors, with nicotinic currents inhibited by 40% in the presence of 100 μM MHD (Fig. 5; lower panel). Full statistics are given in the figure legend. As was also the case of carbamazepine and oxcarbazepine, the inhibitory effect of MHD was completely reversible on washout.

Once again, the current deactivation phase was prolonged by the antiepileptic, as estimated by the half-time of current decay of α4β2 receptors upon nicotine (or nicotine plus drug) removal. These results are summarized Table 2 and support the idea that carbamazepine, oxcarbazepine and MHD exert at least part of their action through an open channel blocking mechanism. Irrespective of the detailed mechanism, we conclude that the main metabolite of oxcarbazepine inhibits heteromeric neuronal nicotinic receptors at therapeutically significant doses, with particularly strong effects on α4β2 receptors.

Conclusions

By analyzing our data alongside those of the available literature (Bertrand et al., 2002; Hoda et al., 2009; Leniger et al., 2003; Picard et al., 1999), we propose the following conclusions. First, carbamazepine inhibits α4β2, α2β2 and α2β4 receptors and thus appears to be capable of producing very widespread effects on neuronal nicotinic receptors. Although the IC_{50} values vary between receptor subtypes, they are generally compatible with the notion that partial inhibition of these ion channels occurs at the therapeutic doses. Second, the oxcarbazepine/MHD combination also blocks heteromeric nicotinic receptors, with a markedly higher efficacy on α4β2 compared to α2β4. Considering the relatively high levels reached by MHD in therapeutic use, administration of oxcarbazepine is also likely to modulate nicotinic receptors in the brain. Finally, the pattern of inhibition observed in receptors expressing mutant subunits linked to ADNFLE is complex and depends both on the specific mutation and on the combination of subunits.

Discussion

Many antiepileptic drugs modulate ion channels. Because the drug-channel interaction is rarely specific, the control of seizures may depend on a combination of effects on different channel types (MacDonald and Rogawski, 2008). Comparative studies of the full spectrum of molecular targets are thus necessary for full understanding of therapeutic action as well as side effects. Since growing evidence points to the involvement of heteromeric neuronal nicotinic receptors in human epilepsy (Steinlein and Bertrand, 2009), we have studied how carbamazepine, oxcarbazepine and MHD affect several types of these ion channels.

Expression of α2-containing receptors

Among the mammalian subunits, α2 is somewhat neglected, partly because of its restricted expression in rodent brains (Ishii et al., 2005; Son and Winzer-Serhan, 2006; Wada et al., 1989). However, recent results in both rodents and primates, including humans, indicate the potential interest of further studies on this subunit. The amino acid substitution α2-I279N has been found to be linked to a form of ADNFLE (Aridon et al., 2006). Moreover, polymorphisms of the α2-

coding gene has been associated with overweight in human populations (Kim, 2008). Therefore, the role of α2 in humans seems more important than previously thought, in agreement with the wider distribution of α2 in primates' brains compared to those of rodents (Aridon et al. 2006; Gotti et al., 2006; Han et al. 2000; Quik et al. 2000). Nonetheless, distinct important physiological roles of α2 are also emerging in rodents (Nakauchi et al., 2007).

The functional study of the different forms of cerebral nicotinic receptors is still in its infancy (Gotti et al., 2009). At first approximation, when studying α2, it seems reasonable to coexpress it with either β2 or β4, considering that α2β2 and α2β4 receptors present distinct pharmacological, kinetic and expression features (Chavez-Noriega et al., 1997; Luetje and Patrick, 1991; Whiteaker et al., 2009). Both forms, including the human α2β2 (Hoda et al. 2009), yield good functional expression in *Xenopus laevis* oocytes. We have focused on the α2β4 form, because the generally smaller whole-cell currents we have observed with α2β2 channels in HEK cells were incompatible with extensive pharmacological characterization by patch-clamp. Whether the lower expression in HEK cells has physiological relevance remains to be determined. Irrespective of expression issues, the available evidence about β4 distribution suggests that the α2β4

receptor may be of considerable interest *per se*. In squirrel monkeys, β4 largely prevails over β2 throughout the brain (Quik et al. 2000). Widespread expression of β4 has been also observed in the adult mouse (Gahring et al., 2004) and in several *Macaca* species (Kulak et al., 2007). Importantly, from our standpoint, in human fetuses and aged post-mortem samples the transcript for β4 is widely distributed throughout the brain. The expression of β2 is more restricted, and the two subunits overlap in the neocortex (Hellstrom-Lindahl et al., 1998). The common expression of β4 in the thalamocortical system and hippocampus suggests that this subunit may be implicated in the susceptibility to seizures. Work carried out in mice supports this notion, as knocking out β4 confers resistance to nicotine-induced seizures (Kedmi et al. 2004; Salas et al. 2004).

The effects of carbamazepine and oxcarbazepine/MHD on neuronal nicotinic receptors

Carbamazepine and oxcarbazepine appear to have multiple molecular targets, but few studies have directly addressed their effects on specific ion channel types (Ambrosio et al., 2002; Schmidt and Elger, 2004). Carbamazepine has been shown to inhibit α4β2 (Picard et al., 1999) and α2β2 (Hoda et al., 2009) neuronal nicotinic receptors,

probably through an open channel blocking mechanism. Here, we show that carbamazepine and oxcarbazepine/MHD also inhibit α2β4 receptors, with the former being considerably more effective. In addition, we found that MHD significantly blocked α4β2 receptors at the therapeutically relevant concentrations.

The therapeutic concentration of carbamazepine is 20-50 μM in plasma and 5-15 μM in the cerebrospinal fluid (Dam et al., 1989; Picard et al., 1999; Shorvon, 2000). These values indicate that this drug can exert widespread effects on different types of nicotinic receptors in both neuronal and non-neuronal tissue. On the other hand, peak plasma levels of oxcarbazepine in primates are in the micromolar range, whereas the steady state levels are negligible because of rapid conversion to MHD. The latter compound exhibits a slow decay and its therapeutic levels range between 30 and 150 μM (Dam et al., 1989; Johannessen et al., 2003; Lloyd et al., 1994). Similarly to carbamazepine, these drugs easily cross the blood brain barrier (Tecoma, 1999). Their concentrations in the cerebrospinal fluid are thus comparable to those in plasma, particularly for MHD, which binds weakly to plasma proteins (Shorvon, 2000). Patients taking daily doses of oxcarbazepine are exposed to the above steady levels of MHD plus pulsatile micromolar levels of oxcarbazepine. Under these

conditions, our results suggest that significant inhibition of heteromeric nicotinic receptors (particularly α4β2) may occur. In general, the range of effective concentrations of the carbamazepine-related drugs on voltage-gated Na^+ channels overlaps with that observed for nicotinic receptors (McLean and MacDonald, 1986; McLean and Rogawski, 2008). For example, carbamazepine interacts with voltage-dependent Na^+ channels with approximate IC_{50} of 30 μM (Willow et al, 1985). Therefore, the contribution to the antiepileptic effect of nicotinic receptors' inhibition should be taken into account. It should also be noted that, in rats and dogs, oxcarbazepine persists in blood at concentrations much higher than those measured in humans (Tecoma, 1999). Hence, caution should be exerted when interpreting the response to this drug in animal models of human epilepsy.

How partial blockade of neuronal nicotinic receptors could contribute to the antiepileptic effect is matter of speculation, as the complex roles of these ion channels in the brain are still debated. Nicotinic receptors control both glutamate and GABA release in the hippocampus and the thalamocortical system and can also play excitatory postsynaptic roles (Albuquerque et al., 2009; Aracri et al., 2009; Couey et al., 2007; Lambe et al., 2003; Lendvai and Vizi, 2008; Zolles et al., 2009). Considering that carbamazepine and oxcarbazepine have been

recently shown to produce potentiation of $GABA_A$ receptors (Zheng et al., 2009), we hypothesize that inhibition of glutamate release and stimulation of postsynaptic $GABA_A$ channels may cooperate to dampen network excitability.

Finally, many subunits of the 'neuronal' nicotinic receptors are in fact expressed in non-neuronal tissue, where they exert physiological roles as diverse as hormone release, cell migration and cell proliferation (Wessler and Kirkpatrick, 2008). As a consequence, another point that deserves further study is the possible contribution of the modulation of neuronal nicotinic receptors to the development of peripheral side effects.

Implications for treatment of nocturnal frontal lobe epilepsy

Carbamazepine is one of the most effective drugs for NFLE (Provini et al., 2000). Because alteration of the cholinergic system can be involved in the pathogenesis of this disease, the efficacy of carbamazepine has been partly attributed to its capability of blocking neuronal nicotinic receptors. However, approximately 30% of the patients remain unresponsive to therapy. Recent reports indicate that better results may be obtained with oxcarbazepine (Raju et al., 2007; Romigi et al., 2008). This drug allowed to control seizures in several

patients unresponsive to other treatments, included (for one of them) carbamazepine (Raju et al., 2007). Our results suggest a straightforward interpretation of these findings. The typical doses of oxcarbazepine administered to patients allow MHD to reach cerebrospinal concentrations high enough to produce effective inhibition of heteromeric nicotinic receptors (particularly α4β2). Equally effective concentrations of carbamazepine cannot be reached without running the risk of serious side effects. More extensive studies aimed at comparing the efficacy and tolerability of carbamazepine and oxcarbazepine on NFLE patients will be necessary to substantiate this hypothesis.

The link between ADNFLE and mutant nicotinic receptors suggests that specific targeting of these receptors may be a preferred treatment, particularly for the genetic forms of the disease caused by mutant receptors hypersensitive to these drugs. Hence, it could be helpful to seek drugs more effective in blocking nicotinic receptors compared to voltage-gated channels. Molecular dynamics models indicate that the structure of the aromatic ring is fundamental for carbamazepine to target the nicotinic receptor pore (Ortells and Barrantes, 2002), whereas the additional oxygen of oxcarbazepine and its metabolites does not seem to impair Na^+ channel targeting (Ambrosio et al.,

2002). Our results show that the aromatic ring substituents decrease the blocking efficacy, at least on α2β4 channels. These observations suggest the possibility of calibrating the drug's efficacy on this and other receptor types by inserting different substituents on the dibenzazepine ring.

Besides therapeutic issues, the general efficacy of antiepileptics targeting nicotinic receptors in patients suffering from NFLE supports the idea that the cholinergic system is a very sensitive modulator of excitability during sleep, irrespective of the presence of specific mutations on nicotinic subunits. These observations will thus be useful in guiding further research on the pathogenic mechanism of sleep-related frontal epilepsy and the role of the cholinergic system in slow-wave sleep.

Acknowledgments

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Table 1

Receptor	Agonist	I density[a] (pA / pF)	Desensitization τ (s)[b]
α2β4	Nicotine	69 ± 13 (12)	4.0 ± 0.8 (6)
	Acetylcholine	83 ± 16 (25)	5.7 ± 1.2 (8)
α2-I279Nβ4	Nicotine	50 ± 7 (26)	5.2 ± 1.0 (8)
	Acetylcholine	71 ± 10 (20)	6.9 ± 1.9 (7)

Functional properties of α2β4 and α2-I279Nβ4 receptors

[a] Peak whole-cell current density values at -60mV, in the presence of either 100 μM nicotine or 300 μM acetylcholine. The differences between wild-type and mutant receptors are not significantly different (with t-test for unpaired samples).

[b] Time constant of channel desensitization, calculated from monoexponential decay functions best fittine the experimental traces. Average values refer to experiments carried out in the same cell batch. Number of experiments is given in brackets.

Table 2

Receptor	Ctrl	OXC20	Ctrl	OXC100	Ctrl	MHD100
α2β4	3.6±0.5	4.4 ± 1	3.9±1.2	6.2 ± 2[a]	7.5±0.6	10.9±1.4[a]
α2I279Nβ4	5.1±0.6	7.0 ± 1	5.3±0.7	7.6 ±0.9[a]		
α4β2					3.3±1.06	6.1±1.6[b]

Half-times of channel deactivation, for the indicated receptor type, in the presence of oxcarbazepine or MHD

Ctrl: control; OXC 20: oxcarbazepine 20 μM; OXC 100: oxcarbazepine 100 μM; MHD 100: MHD 100 μM.

[a] Significant differences from the corresponding controls ($0.01 < P < 0.05$; t-test for paired samples), N = 4-6

[b] Significant difference from the corresponding controls ($P < 0.01$; t-test for paired samples), N = 8.

Figure 1

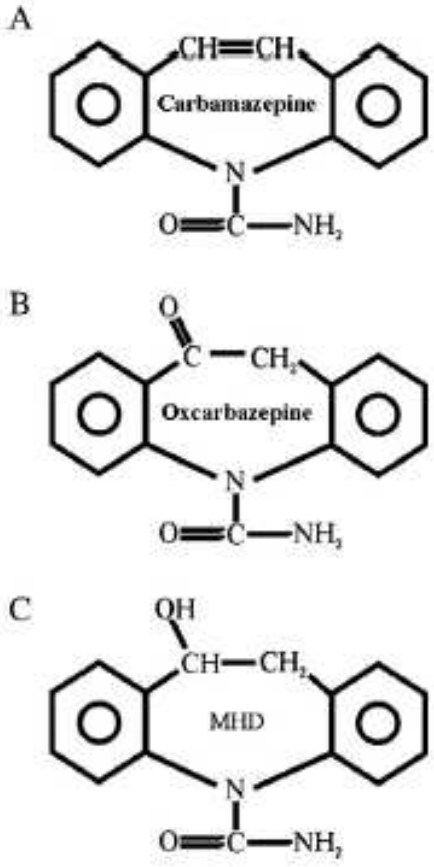

Fig. 1 Molecular structures of carbamazepine (A), oxcarbazepine (B) and 10-hydroxycarbamazepine (MHD;C). MHD is the main active metabolite of oxcarbazepine and is also know as GP 47779 or licarbazepine

Figure 2

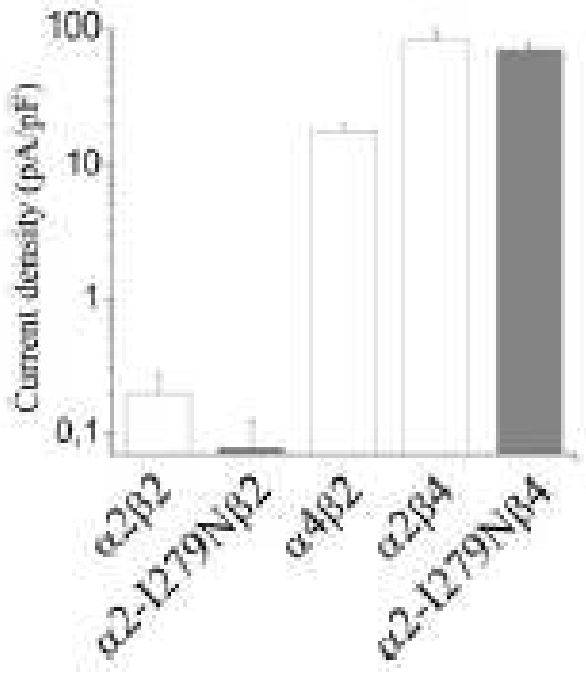

Fig. 2 Functional expression of different heteromeric nicotinic receptors in HEK cells

Bars indicate average peak whole-cell current densities evoked at -60mV by 300 μM acetylcholine. Cells were transieltly transfected with the indicated subunit combinations. Both α2 and α2-I279N produced particularly strong current expression when cotransfected with β4. In particular, the average current density values were (pA/pF): 0.197±0.076 (n=91), for α2β2; 0.08±0.043 (n=38); for α2-

I279Nβ2; 18.2±2.1, for α4β2 (n=57); 83±16 (n=25), for α2β4; 71±10 for α2-I279Nβ4 (n=20). Experiments summarized the results obtained after 12 runs of transfection

Figure 3

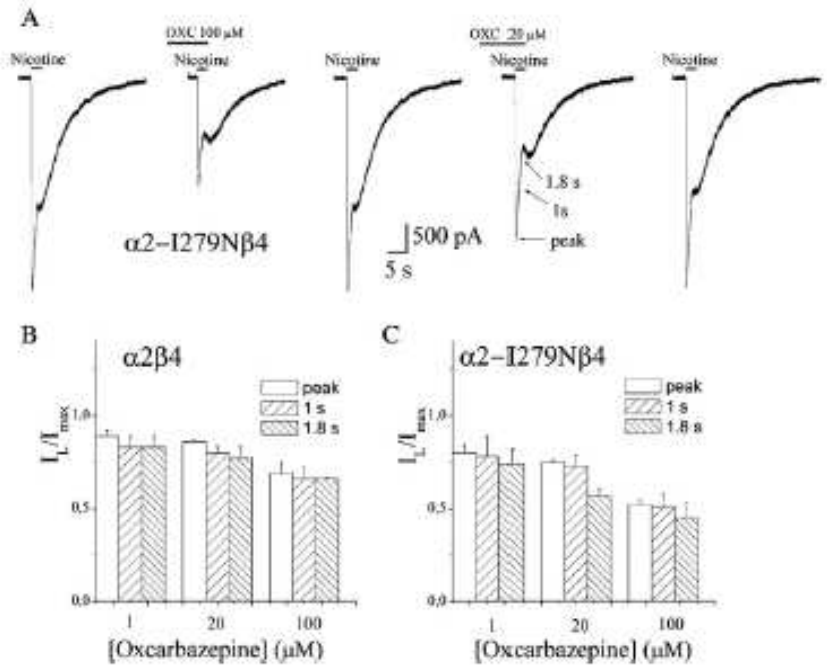

Fig. 3 Effect of oxcarbazepine on nicotinic currents

A. Typical whole-cell currents for α2-I279Nβ4 receptors activated by 100 μM nicotine at -60 mV, in presence or absence of the indicated antagonist concentration. Stimulations were spaced three min apart. Continuous lines mark the time of oxcarbazepine (OXC) and nicotine application, as indicated. Arrows mark the times at which inhibition was calculated to generate panels B and C. **B.** Wild-type receptors. Bars show the fractional currents (i.e. the ratio of currents in the

presence and in the absence of the indicated concentration of oxcarbazepine) calculated at the current peak (left bars), 1 s (middle bars) and 1.8 s (right bars) after nicotine application. Data are averages of at least five determinations in the same cell batch. **C.** Same as panel B, for mutant receptors.

Figure 4

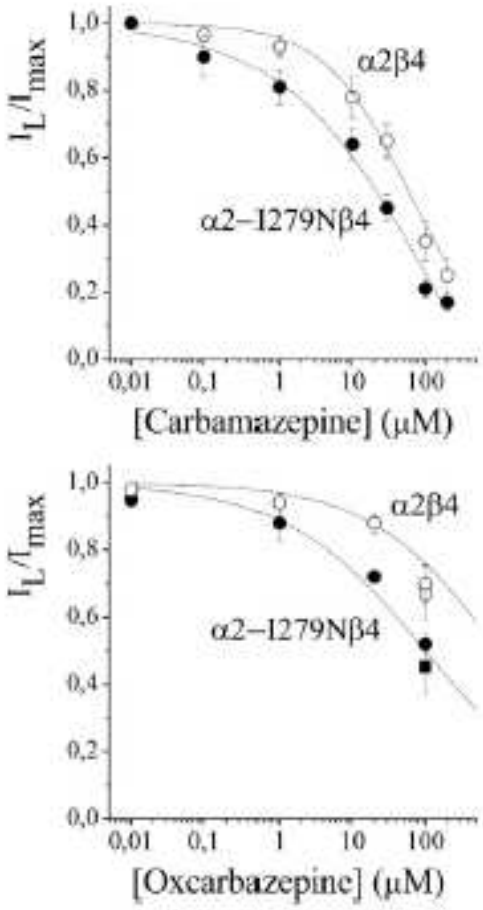

Fig. 4 Concentration-response curves for carbamazepine and oxcarbazepine

The experimental procedure for both drugs was as illustrated in Fig. 3. Data points are average peak currents, at the indicated inhibitor concentration (I_L), normalized to the current obtained at 100 μM

nicotine (I_{max}). Data were fitted with equation (1). **A.** Carbamazepine. Values obtained from best fits were IC_{50} = 49 μM (n_H = 0.75) for wild-type (white circles) and IC_{50} = 21 μM (n_H = 0.6) for mutant receptors (black circles). At 100 μM, the fractional current was 0.35 ± 0.06 for wild-type and 0.21 ± 0.03 for mutant receptors (averages of five determinations; $p \leq 0.05$ with unpaired t-test). **B.** Oxcarbazepine. Plot was generated as in panel A. Best fit estimates gave IC_{50} > 500 μM (n_H = 0.65) for wild-type (white circles) and 100 μM (n_H = 0.5) for mutant receptors (black circles). At 100 μM, the fractional current was 0.70 ± 0.05 for wild-type and 0.52 ± 0.03 for mutant receptors (averages of five determinations; $p \leq 0.05$ with unpaired t-test). Squares give fractional current values obtained in the presence of 100 μM oxcarbazepine, when nicotinic currents were activated with 10 μM nicotine. Values obtained for wild-type (white squares) and mutant (black squares) receptors were not significantly different from the corresponding values obtained in presence of 100 μM nicotine.

Figure 5

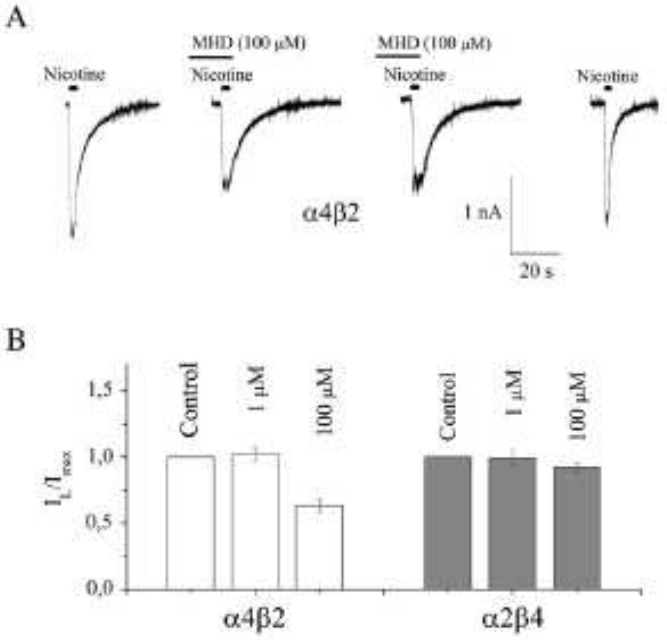

Fig. 5 MHD inhibits heteromeric nicotinic receptors

A. Typical whole-cell currents activated on α4β2 receptors by 100 μM nicotine at -60 mV, in presence or absence of the indicated antagonist concentration. Stimulations were spaced three min apart. Continuous lines mark the time of MHD and nicotine application, as indicated. **B.** Bars show the fractional peak currents (i.e. the ratio of currents in the absence and in the presence of MHD), at the indicated inhibitor

concentration, for α4β2 and α2β4 receptors, as indicated. Data are averages of at least five determinations in the same run of transfection. For α4β2 receptors treated with 100 μM MHD, the peak current densities were (pA/pF) 5.9 ± 1.67 before treatment and 3.8 ± 0.99 in the presence of MHD ($p < 0.05$, with paired t-test; $N = 8$).

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CHAPTER 3

“What knowledge about neuronal nicotinic receptors in the nervous system can tell us about the nicotinic role in cancer”

Abstract

The neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels activated by ACh or exogenous agonists such as nicotine. They are permeable to cations and can generate fast excitatory postsynaptic potentials. nAChRs are also expressed at presynaptic and extrasynaptic sites, where they regulate neurotransmitter release and neuronal excitability in response to diffuse ACh release. These receptors are also widely expressed outside the nervous system, in normal as well as neoplastic tissue, which is intriguing as smoking is an established risk factor for cancer. Besides nicotine, several tobacco-derived compounds, such as nitrosamines and their metabolites, are tumor-promoting agents. Although nitrosamines have mutagenic effects, they are also potent nAChR ligands. Therefore, many of the cellular effects of nicotine and nitrosamines, such as stimulation of proliferation and inhibition of apoptosis, seem to be mediated by nAChRs. In analogy with what happens in the brain, nAChR activation regulates the secretion of autocrine and paracrine factors that stimulate cell proliferation, migration and inhibit apoptosis. Other mediators, such as γ-amino-butyric acid (GABA), may function as inhibitors of proliferation and

neoplasia. Activation of nAChR can also produce direct control of the Ca^{2+}-dependent intracellular signals related to these processes. However, interpreting the precise nAChR roles in neoplastic cells is made considerably difficult by the overlap of many different receptor's subunits and the general lack of detailed studies in single cells. Different nAChR types present different pharmacological and kinetic features and may regulate different cellular processes. Moreover, the mechanistic effects of nitrosamines on these receptors, e.g. the balance of excitatory and inhibitory effects, are poorly known.

Introduction

The nicotinic acetylcholine receptors are pentameric ionotropic receptors permeable to cations, belonging to the superfamily of ligand-gated ion channels, which includes the $GABA_A$, glycine and serotonin receptors (Hogg et al., 2003). They were originally discovered in the postsynaptic membrane of the neuromuscular junction and isolated from the electroplaxes of electric fishes such as *Torpedo*. The composition of the muscular receptor is typically $(\alpha_1)_2\beta_1\gamma\delta$ during development. In the adult, ε substitutes γ. Two binding sites for ACh are located at the interface between each α and the γ (or ε) subunit. Two molecules of agonist are normally necessary to produce a significant probability that the channel is open (Albuquerque et al., 2009).

Based on knowledge about the muscle receptors, subsequent work identified and cloned several neuronal subunits, which were found to be widely expressed in the central and peripheral nervous system. To date nine α (α2-α10) and three β (β2-β4) neuronal subunits are known. The pattern of possible subunit combinations is very complex (Dani and Bertrand, 2007). The α2-α6 and β2-β4 can associate to form heteromeric αβ nAChRs with various stoichiometries. The α7-α9

subunits can form homopentameric receptors, such as the commonly found $(\alpha7)_5$. However, these subunits can also participate in heteromeric receptors. For example, α9 forms heteromers with α10 and some evidence suggests that α7 can associate with β2, at least in expression systems (Khiroug et al., 2002). The physiological meaning of this diversity is still unclear. The α8 subunit is restricted to avians. A9 and α10 are mainly expressed in the cochlea. In the mammalian brain, the heteropentamers α4β2, with prevalent stoichiometry $(\alpha4)_2(\beta2)_3$ and the homopentamers $(\alpha7)_5$ seem to be the most common forms, at least in rodents, but the contribution of the other subunits is under intense investigation. α5 is thought to modulate the properties of heteromeric receptors. In the peripheral nervous system, there is a prominent contribution of α3 and β4 (Gotti et al., 2009).

The structure of the neuronal and muscle receptors is essentially similar. Pseudo-crystalline forms of the receptors isolated from *Torpedo californica* gave a structural resolution at 0.36 nm (Unwin, 2005). Receptors have an extracellular water-filled vestibule with a diameter around 2 nm. This vestibule is formed by the long N-terminal extracellular domains of the five subunits. The N-terminal segment of each subunit is followed by 4 trasmembrane domains (M1-M4). The M2 segments line the channel pore. The short C-terminal

domains also face the extracellular side. The ligand-binding site is formed by the α subunits and the adjacent subunits in heteromeric receptors, and by the interaction of adjacent α subunits in homomeric receptors. Therefore, the number of agonists that can bind the receptor depends on the number of α subunits. For example, five ACh can bind to $(\alpha7)_5$ receptors and two can bind to $(\alpha4)_2(\beta2)_3$ receptors (Dani and Bertrand, 2007).

Postsynaptic nicotinic receptors mediate fast excitatory neurotransmission, as in the neuromuscular junction and autonomic ganglia, where nAChR activation quickly depolarizes the postsynaptic membrane. In the brain, nAChRs can also exert slower paracrine actions (Lendvai and Vizi, 2008), for example by modulating neurotransmitter release (Lambe et al., 2003; Aracri et al., 2010). In fact, cholinergic innervation in many cerebral regions, including the neocortex, presents a diffuse, non-synaptic nature. Moreover, the distribution of acetylcholinesterase (AChE) does not precisely matches that of nAChRs and low steady ACh levels are measured in the cerebrospinal fluid even when cholinesterase inhibitors are not applied (Dani and Bertrand, 2007; Lendvai and Vizi, 2008; Pepeu and Giovannini, 2008).

Expression of nAChRs in non-neuronal tissue and cancer cells

Growing evidence shows that the 'neuronal' nAChR subunits are frequently expressed in non-neuronal differentiated tissue, such as skin keratinocytes, bronchial, oral and gastrointestinal epithelia, vascular endothelium, muscle tissue, lymphocytes and macrophages (Wessler and Kirkpatrick, 2008). These results derive mainly from expression studies, as functional studies are still very scarce (Dasgupta et al., 2006; Fu et al., 2009). The functional meaning of nAChR expression in non-neuronal tissue is still debated and may be different in different cells. One thoroughly studied cell type is the human keratinocyte, in which α7 nAChRs regulate directional cell migration along a gradient of cholinergic agonists. The nicotinic receptors cluster at the cell's leading edge along with M1 muscarinic and β1-integrin receptors. These interact to produce a combination of calcium-dependent and independent signals that converge on the Ras/Raf/MEK1/ERK pathway. In keratinocytes, heteromeric nAChRs seem instead to be mainly involved in controlling random chemokinesis (Chernyavsky et al., 2004; Chernyavsky et al., 2005).

The nAChRs are also frequently observed in cancer cells, including lung cancer, in which they regulate cell proliferation, apoptosis and angiogenesis (Maneckjee and Minna, 1990; Dasgupta et al., 2006;

Lam et al., 2007; Paleari et al., 2008; Guo et al., 2008; Egleton et al., 2008; Calleja-Macias et al., 2009; Schuller, 2009). Such findings are suggestive because tobacco inhalation is an established risk factor for cancer, and particularly lung cancer, in which these effects have been studied in depth. Widely used experimental models are the small and non small cell lung cancer cells, respectively SCLC and NSCLC (Schuller, 2009). SCLC has epithelial origin, but displays many neuroendocrine features and may derive from neuroendocrine-oriented cell populations. It is very aggressive and highly correlated with smoking (about 98%). NSCLC is a heterogeneous family, with three main histological types (adeno-, squamous cell and large cell carcinoma). The correlation with smoking is approximately 60%. In smokers, prolonged or even chronic nAChR stimulation is possible because nicotine is not degraded by AChE. Moreover, several carcinogens produced by tobacco inhalation, namely NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone], NNN (N-nitrosonornicotine) and DEN (diethylnitrosamine), bind with high affinity to nAChRs, as indicated by radioligand competition studies (Schuller and Orloff, 1998; Schuller, 2007). These compound are in fact structurally analogous to either ACh or nicotine. It has thus been proposed that some of the oncogenic effects of these compounds

depend on specific activation of nAChRs, which may supplement the well-known effects caused by DNA targeting of several nitrosamine metabolites.

Because of the heterogeneity of lung cancer cells, it is not surprising that the full spectrum of nAChR types and signaling cascades involved in regulating different aspects of the physiology of these cells are still matter of debate. Most data concern NSCLC cells, in which nAChRs not only exhibit an altered expression (Egleton et al., 2008), but have been shown to stimulate cell proliferation through different mechanisms that include up-regulation of signaling pathways downstream to integrin engagement (Zheng et al, 2007). Nicotine has been also found to confer resistance to the chemotherapeutic-induced apoptosis. Hence, the molecular network centered on nAChRs is currently considered a promising target for the tobacco-related cancer therapy (Grozio et al., 2008).

The functional properties of nAChRs

How nAChRs respond to tonic stimulation seems the more relevant aspect for understanding the regulation of the above processes in normal and neoplastic non-neuronal tissue. This notion is reinforced if one remembers the slow, multistep nature of the neoplastic

progression (DeVita et al., 2008). As mentioned earlier, few direct studies in real time are yet available. Nevertheless, the general working hypothesis is that prolonged nAChR stimulation can lead to sustained cell depolarization and calcium entry (through nAChR themselves or other pathways) with ensuing effects on exocytosis of several autocrine/paracrine factors that promote proliferation, migration, angiogenesis and inhibit apoptosis. Moreover, calcium entry can modulate directly some of the cytoplasmic signaling pathways implicated in these processes. To deepen our understanding of the mechanism underlying these physiological effects, it is thus very important to thoroughly consider the functional features of nAChRs during steady stimulation.

Calcium permeability (P_{Ca}) and modulation by external Ca^{2+}

The neuronal nAChRs differ from the muscular in that their permeability to Ca^{2+} (P_{Ca}) is generally higher. P_{Ca} depends on subunit composition. Rigorous determinations for the different forms are still scarce, but the ratio between P_{Ca} and P_{Na} is generally about 2-3 in heteromeric receptors, while in homomeric receptors it can be 10 or higher (Fucile, 2004).

Besides Ca^{2+} permeability, nAChRs are modulated by extracellular Ca^{2+} ($[Ca^{2+}]_o$) in a complex way. They are progressively potentiated by $[Ca^{2+}]_o$ up to the physiological concentration (Mulle et al., 1992; Vernino et al., 1992), whereas higher $[Ca^{2+}]_o$ produces channel inhibition (Buisson et al., 1996). Thus, nAChRs present a bell-shaped response to $[Ca^{2+}]_o$ which is caused by Ca^{2+} binding to several metal binding sites, located within the N-terminal domain. These sites contain typical EF-like helix-loop-helix motifs, with conserved terminal glutamate residues, whose neutralization decreases or completely inhibits the regulation by $[Ca^{2+}]_o$ (Galzi et al., 1996).

From an oncologic standpoint, P_{Ca} is the most relevant parameter. In principle, prolonged nAChR activation could lead to an increase in the steady state calcium influx. This applies to most nAChR types, although the effect is probably stronger in α7 receptors. On the other hand, at difference with the situation in the synaptic cleft, the modulatory effect of $[Ca^{2+}]_o$ seems less important, because the calcium levels in blood and interstices are unlikely to undergo appreciable long-term variation.

Kinetics of nAChRs and long-term effect of ligands

In the presence of tonic agonist levels, the extent of steady calcium entry through a given nAChR type depends not only on P_{Ca}, but also on the steady state open probability of that receptor. This depends on the intrinsic kinetic properties of each nAChR. Typical agonists such as ACh and nicotine activate nAChRs at concentrations between 100 nM and 1 mM. On agonist application, receptors activate (open) within microseconds (Sine and Engel, 2006) and quickly deactivate (close) when the agonist is removed. If the agonist is not degraded or otherwise removed, the receptor desensitizes with a kinetics that depends on subunit composition and is generally quicker in homomeric receptors, such as $(\alpha 7)_5$.

For an ensemble of ion channels i, the current amplitude I across the plasma membrane is generally given by the following relation:

$$I_i = \gamma_i N_i P_o (V_m - E_{rev}),$$

Where γ_i is the single-channel conductance for I; N_i is the number of active channels expressed onto the plasma membrane; P_o is the probability that the channel is open in the conditions being considered; V_m is membrane potential and E_{rev} is the channel reversal potential (generally between -20 and 0 mV, for nAChRs). P_o is generally not a constant, but depends on concentration of ligands and

other regulators, time, V_m, etc. In ligand-gated channels, the steady state P_o essentially depends on the product of the probability that the channel is open times the probability that it is not desensitized. These parameters depend on the agonist concentration and must be determined experimentally by using patch-clamp methods. In general, lower agonist concentrations are less effective in activating the channel, but also determine slower (or negligible) desensitization, and *viceversa* for higher doses. The range of concentrations for which the steady state current is not null defines a so-called 'window current', in analogy with the terminology used for voltage-gated ion channels. For heteromeric receptors such as the α4β2, steady state currents can be measured even at 10-100 nM of ACh or nicotine, consistent with tonic levels of ACh in the cerebrospinal fluid between 0.1 and 1 μM (Pepeu and Giovannini, 2008) and comparable levels of nicotine in smokers (Russell et al., 1981). The maximal steady state current is obtained at intermediate levels of agonists, approximately 5-10 μM, for many heteromeric and homomeric nAChRs (e.g. Fenster et al., 1997). At high agonist doses, the channel desensitization is essentially complete. Besides intrinsic kinetic response to agonists, other nAChR properties are modulated by chronic agonist concentrations. Steady exposure to agonists regulates the surface expression of nAChRs, mainly by post-

transcriptional mechanisms, in a manner that depends on subunit composition (Govind et al., 2009). Moreover, when exposed to chronic agonists, several heteromeric receptors undergo deep desensitization (also named inactivation), differently from the α7-containing receptors (Kawai and Berg, 2001). Therefore, prolonged exposure to nicotine and the other tobacco metabolites can either stimulate or depress the nicotinic signal, depending on the nAChR subunits expressed by the cell. It is thus clear that the long-term physiological effects of nicotinic ligands in the blood or cerebrospinal fluid depend in a very complex way on the balance of the nAChR types expressed by a certain cell and on the extracellular levels of agonists.

Implication of nAChRs in the neoplastic progression: critical points and perspectives

Current evidence indicates two main aspects of nAChR signaling as related to neoplasia. First, prolonged α7-mediated stimulation is thought to lead to sustained cell depolarization and Ca^{2+} influx. These receptor's features could modulate in complex ways the release of autocrine messengers with effects on proliferation, migration and angiogenesis. For example, in pulmonary neuroendocrine cells and

their neoplastic derivative SCLC, activation of α7-containing receptors regulates cell proliferation by stimulating the release of local mediators such as serotonin and bombesin. These stimulate proliferation through the PKC/RAF1/MAPK pathway (Cattaneo et al., 1993; Jull et al., 2001; Schuller, 2009). As discussed above, calcium influx through nAChRs can also regulate directly these pathways. However, considering the complexity of calcium homeostatic control and the variety of pathways for calcium exchange with the different intra- and extracellular compartments (Roderick and Cook, 2008), it remains to be seen whether the effect of α7 receptor activation really exerts its effects mainly through calcium signaling. Demonstration of this would require detailed measurements of nicotinic currents and calcium influx at the steady state. These experiments are needed also in the light of increasing evidence suggesting the existence of non-conducting ion channel roles. These are mediated for example by direct interaction of the channel protein with cytosolic enzymes, membrane adhesion receptors and membrane growth factor receptors (Arcangeli and Bechetti, 2006).

A second interesting hypothesis regards the possible role of GABA as a tumor suppressor and the possible implication of nAChRs in this process. This hypothesis is founded on evidence about the

GABAergic effects in both cancer cells and embryos. First, GABA has been observed to exert a tumor suppressing action in colon carcinoma and lung adenocarcinoma cells. In the latter cell type, stimulation with GABA blocks the cAMP-dependent DNA synthesis and cell migration (Joseph et al., 2002; Schuller et al., 2008). Moreover, recent observations show that the proliferation of embryonic stem cells and peripheral neural crest stem cells is inhibited by local release of GABA, which activates $GABA_A$ receptors (Andäng et al., 2008). In this case, E_{rev} for the $GABA_A$ receptor is approximately -80 mV and stimulation with GABA determines cell hyperpolarization. Therefore, GABA seems to exert a variety of effects during development. The above stem cells respond to GABA as neurons do in the adult brain, where GABA is generally (although not necessarily) an inhibitory transmitter. However, the role of GABA during early neuronal development is known to be excitatory, because of a switch in the expression of different Cl^- transporters that alters E_{rev} for $GABA_A$ receptors (Ben-Ari et al., 2007). Evidence in rodents indicates that the transporter NKCC1, which absorbs Cl^- and is mainly expressed in early stages, is progressively substituted with the Cl^- extruder KCC2 (Rivera et al., 1999). Interestingly, from our standpoint, several lines of evidence show that nAChR activation

controls the GABAergic function in both adult and developing brain. For example, spontaneous nAChR activity regulates the switch between the excitatory and inhibitory roles of GABA during development (Liu et al., 2006). Moreover, in adult neocortical circuits, steady-state nAChR activation has been found to regulate GABA release (e.g. Aracri et al., 2010).

Overall, the above evidence has suggested the conclusion that prolonged inactivation of heteromeric nAChRs in peripheral tissue may promote neoplasia by decreasing GABA release (Schuller et al., 2008). Once again however, caution should be exerted in drawing such conclusions, because very few functional studies exist about the functional interaction between nAChRs and GABAergic transmission in cancer cells. A general puzzling issue is that, on first sight, the subtle kinetic differences between different nAChR types would seem to be of minor importance for controlling the neoplastic cell behavior. Therefore the steady state effect of stimulating α7-containing receptors on exocytosis should be similar to that produced by activating heteromeric nAChRs, especially considering that most of the cell lines studied so far express many nicotinic subunits. To hypothesize that stimulation of heteromeric receptors would mainly control GABA release, whereas stimulation of homomeric nAChRs

would produce other cellular effects comes down to the following hypotheses. Either homo- and heteromeric receptors are differentially expressed in cellular subpopulations that are specialized to release different paracrine mediators, or in every cell there is a close molecular interaction between specific nAChR types and specific release complexes or intracellular signaling pathways. Virtually no evidence about these issues is available, to the best of our knowledge, which certainly constitutes ample matter for future studies.

Pharmacological implications

The above discussion presents relevant pharmacological implications. It has been repeatedly suggested that targeting ion channels could be an effective way of treating some forms of neoplasia, because of the general accessibility of channel proteins from the extracellular side and the potential calibration of the side effects. In fact, recent results indicate that antagonizing nAChRs produces protective effects from tumorigenesis *in vivo* (Paleari et al., 2009). However, choosing the best strategy is not straightforward. Determining the relative importance of the changes in V_m, calcium influx or non-conducting pathways on the downstream processes will be important to attempt the development of drugs with more specific molecular targets (for

example the channel pore or some regulatory domain, etc.) and hopefully less side effects.

Another example of the complications involved in understanding the pharmacology of nAChR stimulation in cancer concerns the roles of nitrosamine compounds. Nitrosamines bind to nAChRs with high affinity and tend to activate the receptors. However, when considering high affinity ligands, it is also very important to consider how potent they are in activating the ion channel. If their potency is lower that than of ACh or nicotine, by displacing these agonists they may produce inhibitory instead of excitatory effects onto the cellular processes. More precisely, if nitrosamines behave as partial competitive agonists with high affinity (Hogg and Bertrand, 2007), they may turn out to produce either excitatory or inhibitory effects depending on the level of the main agonist. In fact, some evidence exists along this line (unpublished results).

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CHAPTER 4

“Neuronal nicotinic acetylcholine receptors and lung cancer cell lines”

Abstract

The neuronal nicotinic acetylcholine receptors are ligand-gated ion channels permeable to cations. Several α and β subunits can associate to form homo- or heteropentamers, which exhibit distinct kinetics, ion permeability and pharmacological properties. The nAChRs are also expressed in non-neuronal tissues and recent evidence indicates that they can regulate cell proliferation, apoptosis and angiogenesis in a variety of neoplastic cells, including small (SCLC) and non-small (NSCLC) cell lung cancer. These observations are suggestive because smoking is an established risk factor for cancer, especially in the lung. The mechanisms of these effects are debated. One possibility is that nAChRs modulate the release of growth factors and other molecules that exert complex autocrine and paracrine effects on lung cancer cells.

We studied nAChRs in SCLC (U2020) and NSCLC (A549) cell lines. These were voltage-clamped in the whole-cell configuration of the patch-clamp methods, at room temperature. During the experiments, cells were maintained in physiological saline solutions. In both cell lines, V_{rest} was usually between -17 and -19 mV. At -60 mV, application of 100 μM nicotine consistently elicited inward desensitizing currents in both cell types, although the average current density was generally higher in NSCLC. In SCLC and NSCLC the nicotinic currents was significantly blocked by 10 nM methyllycaconitine (MLA, specific for homomeric receptors) and 1 μM dihydro-β-erythroidine (DHβE, specific for heteromeric nAChRs). In both cell lines, the inhibition profile suggests that the

majority of the cells expressed both homo- and heteromeric nAChRs. Thus, lung cancer cells express nAChRs with distinct functional properties that may be implicated in physiological processes occurring at different time scales, such as transmitter release and cell migration.

Introduction

As I discussed in the first chapter, growing evidence shows that the 'neuronal' nAChR subunits are frequently expressed in non-neuronal differentiated tissue, such as skin keratinocytes, bronchial, oral and gastrointestinal epithelia, vascular endothelium, muscle tissue, lymphocytes and macrophages (Wessler and Kirkpatrick, 2008). These results derive mainly from expression studies, as functional studies are still very scarce (Dasgupta et al., 2006; Fu et al., 2009). The functional meaning of nAChR expression in non-neuronal tissue is still debated and may be different in different cells.

nAChRs and lung cancer cell lines

Recent evidence points to neuronal nicotinic receptors as pivotal elements for the regulation of cell proliferation, apoptosis and angiogenesis in several tumours, including lung cancers. Smoking is a well-established risk factor for cancer, particularly lung cancer. Several lines of evidence suggest that nAChRs are implicated in tumourigenesis. These receptors are found in bronchial epithelial cells, small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC; Egleton *et al.*, 2008). Binding of labeled ligands/antibodies and messenger expression show that the pattern of nAChR subunit expression in tumours is complex. For example, both NSCLC primary samples and cell lines express a variety of α and β subunits, whose pattern of association to express homo- and heteromeric receptors onto the plasma membrane is unclear (Egleton *et al.*, 2008; Schuller 2009). Subunit expression is commonly altered in lung cancer samples

and cell lines. Tumors often upregulate β4 and downregulate α4 (Lam *et al.*, 2007). What is more, frequent single nucleotide polimorphisms in the chromosomal region containing the genes coding for α3, α5 and β4 have been found to be risk factors both for nicotine dependence and for lung cancer (Spitz et al., 2008).

In lung cancer cells, nAChRs regulate proliferation and apoptosis (Schuller 1989; Maneckjee and Minna, 1990). Moreover, a combination of nicotine and hyperoxia can induce lung tumours in hamsters (Schuller *et al.*, 1995). Activation of nAChRs increases Ca^{2+} influx, which stimulates the release of paracrine factors that promote cell-cycle and inhibit apoptosis. In SCLC cells, α7 activation stimulates serotonin and bombesin release, with consequent activation of the RAF1 and MAPK pathways. In NSCLC cells, activation of both homo- and heteromeric nAChRs leads to release of EGF, which stimulates proliferation through ERK1-ERK2 and inhibits apoptosis through PI3K/Akt/NF-kB.

None of the above studies have rigorously characterized either the nAChRs expressed by lung cancer cells. In fact antibodies raised against nAChRs tipically crossreact with different subunits and, probably, other membrane proteins (Moser *et al*., 2007). This inconvenience applies to other ligands as well. Moreover, subunit mRNA quantification is scarcely informative about functional subunit association onto the membrane.

Moreover, it none of the above studies provide unequivocal information about the contribution of specific nAChR types to the control of cell physiology. The patch-clamp technique allows us to

characterize the nAChRs physiological and pharmacological properties and the physiological role of different nAChRs.

Materials and Methods

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich Italia Srl (Milan, Italy).

Cell cultures

The NSCLC and the SCLC cell lines were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum and 2.25g/l glutamine, at 37°C and 5% CO_2. For patch-clamp experiments, cells were harvested by trypsinization and plated onto 35 mm Petri dishes (Corning Incorporated, NY).

Solutions for patch-clamp experiments

The extracellular solution contained (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES-NaOH 10, glucose 5, pH 7.4. Pipette contained (mM): K-aspartate 130, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 1.3, EGTA-KOH 10, HEPES-KOH 10, MgATP 1, pH 7.3. Stock solutions of the pharmacological compounds were prepared afresh each week and the appropriate concentrations dissolved in the extracellular solution daily.

Patch-clamp experiments

We have applied the whole-cell configuration of the patch-clamp technique. Cells were voltage-clamped with an Axopatch 200B amplifier (Molecular Devices, CA), at room temperature. Patch pipettes (2-4 MΩ) were pulled from borosilicate capillary tubes, with

a P-97 Flaming/Brown puller (Sutter Instruments). Cell capacitance and series resistance are always compensated before each voltage-clamp experiment. Currents are low-pass filtered at 2 kHz and acquired on-line at 5-10 kHz with pClamp hardware and software (Molecular Devices).

Extracellular solutions are applied through an RSC-160 Rapid Solution Changer (BioLogic Science Instruments, France). A syringe-fed automated 9-way solenoid valve system is connected to 9 borosilicate pipettes fixed onto a cylinder, whose rotation is controlled by a fast-step motor. The current flowing through nAChRs is isolated from the background leak, by subtracting the current obtained in the absence of the agonist to the current obtained in the presence of the agonist. Between consecutive agonist applications, at least 120 s are left to allow full channel recovery from desensitization.

Analysis of data

Data were analyzed with Clampfit 9.2 (Molecular Devices) and OriginPro 8 (OriginLab, Northampton, MA). Data are generally given as mean values ± standard error of the mean. Statistical significance was determined with two-tailed Student's t test. The level of significance was set to *$P < 0.05$ and **$P < 0.01$.

Boyden chamber assays

The Boyden chamber assay (BioMap, Milan) is used to study cell migration and cell invasion. This assay is based on a chamber formed by two medium-filled compartments separated by a microporous membranes (∅8 µm). These membranes were incubated overnight in RPMI-1640 supplemented with matrigel 250 µg/ml (a solution containing different proteins of the extracellular matrix) and 2% L-glutamine (L-gln). NSCLC cells were harvested by trypsinization and were diluted in a serum free media. Subsequently, cells were placed in the upper compartment and were allowed to migrate through the pores of the membrane into the lower compartment filled with RPMI-1640 medium supplemented with 2% L-gln and bovin serum albumin (BSA) 25 µg/ml, which contains chemotactic agents. After 8 hours of chamber incubation at 37° C, the membrane between the two compartments was fixed and stained with hematoxylin-eosin coloration. The number of migratory cells to the lower side of the membrane was determined by optical microscope.

Results

Functional nAChR expression in NSCLC and SCLC cell lines

We studied nAChRs in SCLC (U2020) and NSCLC (A549) cell lines (Fig. 1)

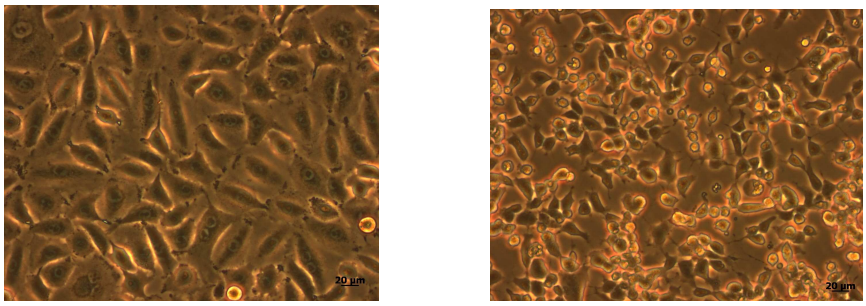

Fig. 1 Lung cancer cell lines

Left panel: NSCLC (A549)

Right panel: SCLC (U2020)

The presence of functional nAChRs was studied in presence of nicotine, instead of the physiological agonist Ach, to avoid possible interference with other currents activated by the muscarinic receptors that may be endogenously expressed in lung cell lines.

In both cell lines, V_{rest} was -17, 62 ± 2,60 (n=29) for NSCLC and -18,5 ± 1,62 (n=26) for SCLC (table 1).

Nicotinic currents were activated by using 100 μM nicotine, which is an almost saturating dose, which thus maximizes the probability of

channel opening. Higher doses of agonist can produce undesirable effects, such as open channel block (Sine and Steinbach, 1984).

At -80 mV, application of the agonist consistently elicited inward desensitizing currents in both cell types (Fig.2).

The multiple possible combinations of nAChR subunits produce a multitude of nicotinic receptors with distinct pharmacological and physiological profiles. On agonist application, receptors activate (open) within microseconds (Sine and Engel, 2006) and quickly deactivate (close) when the agonist is removed. If the agonist is not degraded or otherwise removed, the receptor desensitizes with a kinetics that depends on subunit composition.

In both cell lines the same agonist concentration evoked currents with different kinetics, suggesting the presence of different nicotinic receptor subtypes in these cancer cells. Principally we saw two different profiles with different desensitization time-course. The left panel of figure 1 shows a current trace with fast desensitization. This kind of kinetics is typical of homomeric receptors such as $(\alpha 7)_5$. The right panel of the same figure shows a current trace with slow desensitization, typical of the heteromeric receptors.

In order to obtain comparable data between the different single cells and the two cell lines, we determined the current density values for each cell. This was calculated by dividing the current amplitude (pA) by the cell capacitance (pF). The current density values were 8.47 pA/pF ± 1.99 for NSCLC and 1.71 pA/pF ± 0.43 for SCLC (Fig. 3). Because the cell capacitance is proportional to the cellular surface area, the current density allows to exclude the differences between cells caused by different dimensions. Therefore, the above results

indicate that the functional expression of nAChRs in NSCLC cells was higher as it was in SCLC cells (table1).

Effect of MLA and DHβE on nicotinic currents in SCLC and NSCLC

The different current kinetics we have observed suggest that different cells express different combinations of nicotinic receptors. We next determined the contribution of hetero- and homomeric nAChRs, the two main subtypes, by using specific inhibitors. Homomeric receptors were distinguished by using 10 nM methyllycaconitine (MLA; Fig. 4) whereas heteromeric nAChRs were distinguished by applying 1 μM dihydro-β-erythroidine (DHβE).

MLA and DHβE were applied at -80 mV for 20 s, to reach equilibration of antagonist concentration, before 100 μM nicotine was added for 2 s to elicit whole-cell currents. Stimulations were spaced 2-3 min apart to allow full channel recovery from desensitization.

The results obtained indicate that both MLA and DHβE produced significant block of the nicotinc currents in SCLC and NSCLC cell lines.

The current density values in the presence of these antagonists were: for NSCLC, 2.34 pA/pF ± 0.99 for MLA and 2 pA/pF ± 0.68 for DHβE; for SCLC, 1.2 pA/pF ± 0.5 for MLA and 0.69 ± 0.23 for DHβE (Fig. 5). The current density values of the control (100 μM nicotine) were 4.67 pA/pF ± 1.3 for MLA and 5 pA/pF ± 1.3 for DHβE, in NSCLC; 2.35 pA/pF ± 0.95 for MLA and 1.74 pA/pF ± 0.4 for DHβE, in SCLC.

Sartelet et al. (2008) have establish that SCLC cell express high levels of α7 mRNA whereas the expression of mRNA for heteromeric

receptors containing the β2 subunit are low. Our study confirms α7 nAChR expression in SCLC, however our results suggest that heteromeric receptors are also express in the membrane of these cell lines. Moreover our data indicate the presence of both homo- and heteromeric receptors also in NSCLC, these results are agreement with those obtained by the same group (Sartelet et al., 2008).

Lung cell migration and nAChRs

As I discussed in chapter 1, activation of nAChRs stimulates (directly and indirectly) Ca^{2+} influx, which stimulates the release of paracrine factors that promote cell-cycle, cell migration and inhibit apoptosis.
Preliminary experiments of cell migration in NSCLC cells, by using a Boyden chamber assay, indicate that incubation with 5 μM nicotine produces an approximate 4-fold increase in cell migration. The effect was largely abolished by cotreatment with 10 nM MLA, whereas the inhibition produced by 1 μM DHβE was less potent. These results indicate that both homo- and heteromeric nAChRs regulate cell migration in NSCLC (Fig.6), but the former are more effective. Overall, they are in agreement with the results obtained in human keratinocytes. In these, α7 receptors are more involved in directional migration (chemotaxis), whereas heteromeric receptors seem to be preferentially implicated in random cell migration (chemokinesis, Chernyavsky et al., 2004).

Table 1

Cell line	V_{rest}	pF	pA	N
NSCLC	- 17,6 ± 2,60	36,38	308,21	~ hundreds
SCLC	- 18,5 ± 1,62	10,79	18,45	~ tens

Table 1 Physiological properties of NSCLC and SCLC cell lines

V_{rest} : resting membrane voltage, pF: average cell capacitance, pA: average nicotinic current, N: approximated number of active channels expressed onto the plasma membrane, estimate based on the single channel current of nAChR(between 1,5 and 3 pA at -80 mV) and the average of total current.

Figure 2

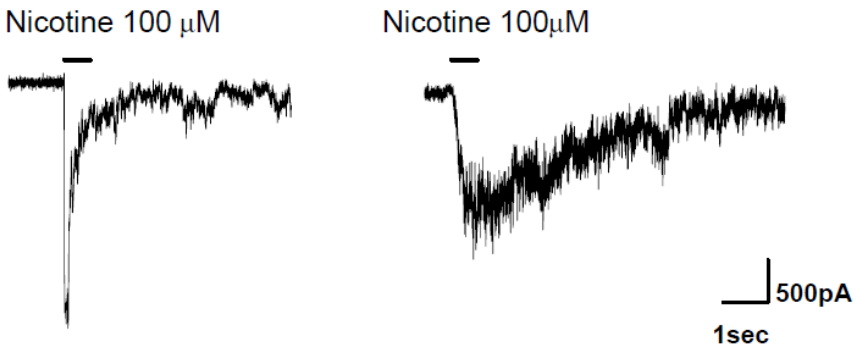

Fig.2 Nicotinic currents in SCLC and NSCLC

Whole-cell current traces evocated by nicotine 100 μM in NSCLC (left) and in SCLC (right), at -80 mV

Figure 3

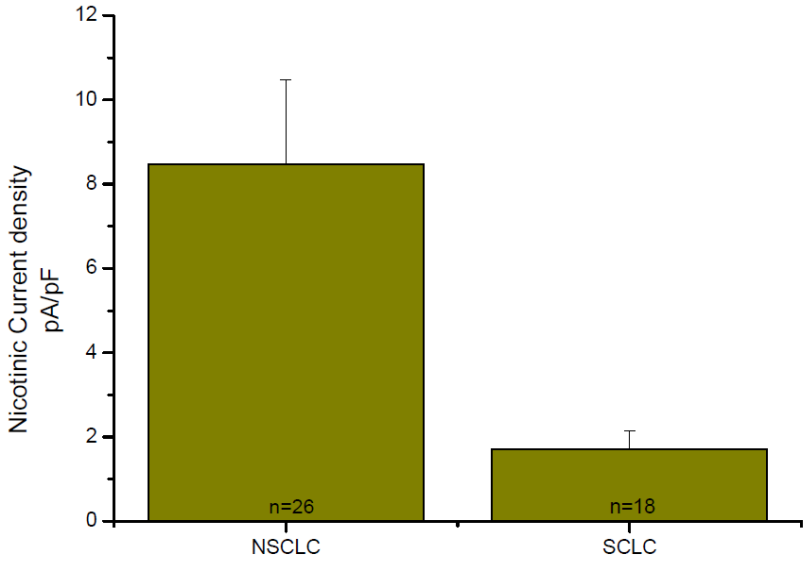

Fig. 3 Functional expression of nAChRs in SCLC and NSCLC

Bars indicate average peack whole-cell current densities evoked at -80 mV by nicotine 100 μM. In particular, the average current density values were (pA/pF): 1.71 ± 0.43 (n=18), for SCLC; 8.47 ± 1.99 (n=26), for NSCLC

Figure 4

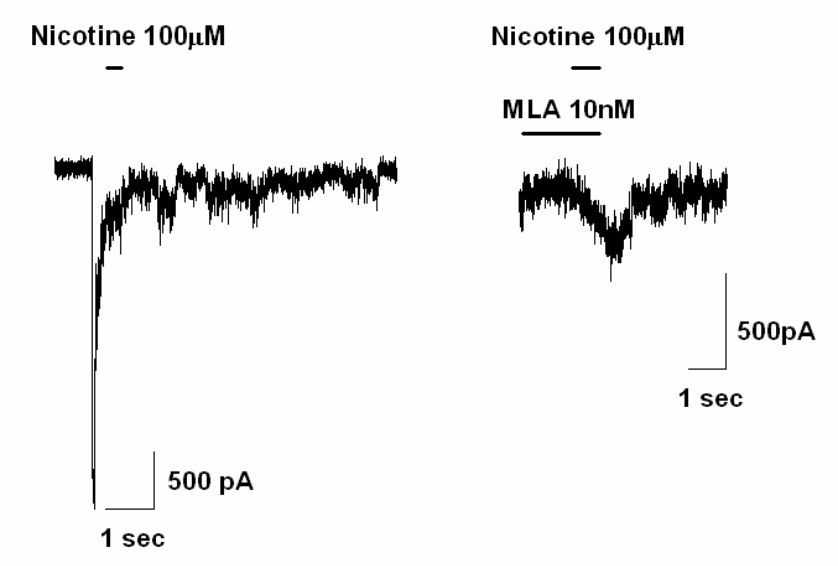

Fig. 4 Effect of MLA on nicotinic currents in NSCLC

Whole-cell nicotinic currents activated by 100 μM nicotine at -80 mV, in presence and absence of 10 nM MLA. Stimulations were spaced three min apart. Continuous lines mark the time of MLA and nicotine application.

Figure 5

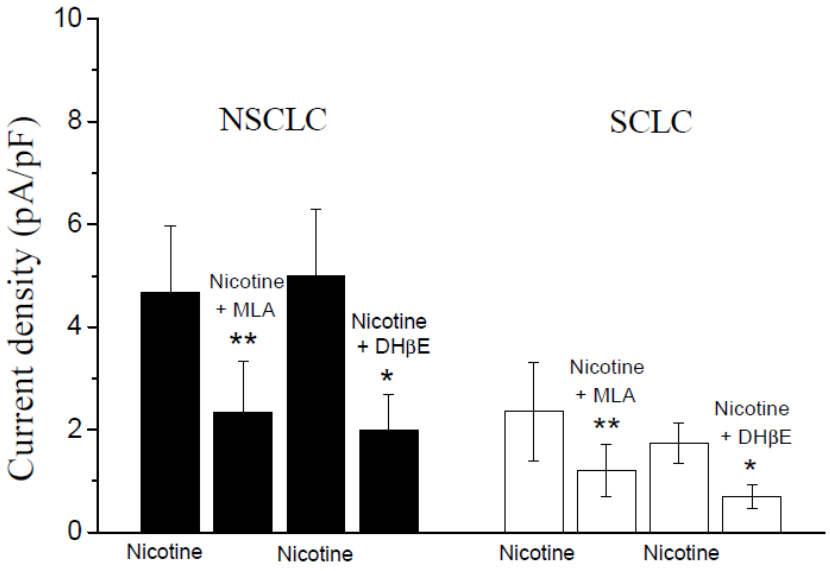

Fig. 5 Effect of MLA and DHβE on nicotinic currents in NSCLC and SCLC cell lines (pA/pF)

Bars indicate average peak whole-cell current densities evoked at -80 mV by 100 μM nicotine. The average current density values of the control (100 μM nicotine) were 4.67 pA/pF ± 1.3 for MLA and 5 pA/pF ± 1.3 for DHβE, in NSCLC; 2.35 pA/pF ± 0.95 for MLA and 1.74 pA/pF ± 0.4 for DHβE, in SCLC. The average current density values of the antagonists were: for NSCLC: 2.34 pA/pF ± 0.99 for MLA and 2 pA/pF ± 0.68 for DHβE; for SCLC: 1.2 pA/pF ± 0.5 for MLA and 0.69 ± 0.23 for DHβE. Each antagonist blocks significantly the nicotinic current (** $p < 0.01$; * $p < 0.05$ with paired t-test)

Figure 6

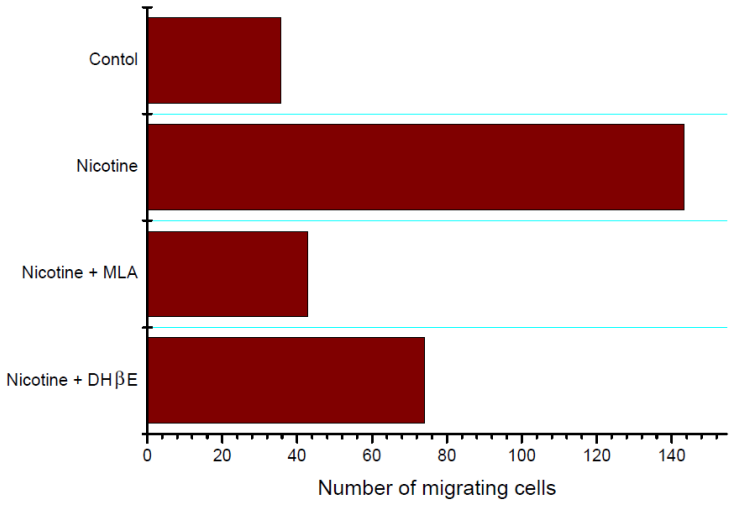

Fig.6 Effects of MLA and DHβE on cell migration

NSCLC number of migrating cells in different conditions: control (n=35), nicotine 5µM (n=143), nicotine 5µM + MLA 10nM (n=42), nicotine 5µM + DHβE 1µM (n= 74)

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CHAPTER 5

“The tobacco-related nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is a partial agonists with high affinity of human α4β2 neuronal nicotinic acetylcholine receptors”

Abstract

The neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels widely distributed in both neuronal and non-neuronal tissue, including neoplastic cells. Several tobacco-derived carcinogens structurally similar to nicotine, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) bind to nAChRs with high affinity. This is suggestive, as smoking is an established risk factor for cancer. Moreover, modulation of nAChRs is thought to be implicated in cerebral tobacco addiction. However, very little is known about the mechanism of action of these drugs on nAChRs.

We studied the effect of NNK on human α4β2 nAChRs stably expressed in human embryonic kidney cells, with patch-clamp methods. At negative membrane potentials, NNK activated inward desensitizing whole-cell currents. These were inhibited by 1 μM dihydro-β-erythroidine, which blocks heteromeric nAChRs. The effect of NNK had a threshold of approximately 0.01 nM and saturated at concentrations higher than 10 nM. The peak of the steady state current was approximately 0.05 nM, close to the typical concentrations observed in smokers' plasma. Saturating doses of NNK activated

about 10% of the maximal current activated by nicotine. These results indicate that NNK is a partial agonist with high affinity for α4β2 nAChRs. Accordingly, it produced nAChR potentiation in the presence of low concentrations of the full agonists (with a threshold around 0.1 μM), and nAChR competitive inhibition at higher concentrations of the full agonist. In keeping with a competitive action on the ligand binding site, the effect of NNK showed negligible voltage-dependence. We conclude that NNK can stimulate or inhibit heteromeric nAChRs, depending on the concomitant concentration of the full agonists.

Introduction

The nAChRs are pentameric ligand-gated ion channels formed by different combinations of α and β subunits. Subunit composition determines the receptor's pharmacology, kinetics and permeability. These channels are generally permeable to cations, including Ca^{2+}. In the central nervous system (CNS), nAChRs mediate or regulate synaptic transmission and excitability. The prevalent cerebral forms in rodents are the heteropentamer α4β2 and the homopentamer $(\alpha7)_5$. The contribution of other subunits in different species is still debated (Gotti et al., 2009). Many nAChR subunits are also expressed outside the nervous system and can be found in human cancers (Song et al., 2008) and tumor cell lines (Wessler and Kirkpatrick, 2008).

Besides the physiological agonist ACh, nAChRs are activated by tobacco-related compounds such as nicotine, which partly explains the multiple effects of smoking. In the brain, uncontrolled stimulation of nAChRs mediate the addictive effects of tobacco, with mechanisms that are only partially understood (Changeux, 2010). In other tissues, smoking has toxic as well as carcinogenic effects. These latter are produced by several tobacco metabolites, such as the N-nitrosamines (Hecht and Hoffmann, 1988). When metabolically activated, these

compounds can cause DNA mutations, particularly G to T transversions that may lead to mutation of k-ras and p53 (Pfeifer et al., 2002). What is more, the most potent carcinogenic nitrosamines are structural analogs of either ACh (such as diethylnitrosamine) or nicotine (such as NNK and the related N'-nitrosonornicotine, NNN; Fig. 1). In fact, radioligand binding assays showed that these drugs potently bind to nAChRs *in vitro* (Schuller and Orloff, 1998; Schuller, 2007) and growing evidence indicates that some of the toxic effects of these compounds derive from their action on nAChRs. Prolonged stimulation of nAChRs with nicotine or nitrosamines alters the normal nAChR functions during cell proliferation and differentiation (Schuller, 1989; Minna, 2003; Egleton et al., 2008; Grando, 2008; Schuller, 2009). For example, treatment with nicotine or NNK produces similar Akt-mediated stimulus of proliferation and protection from apoptosis in both normal human airway epithelial cells (West et al., 2003) and in lung cancer cells (Tsurutani et al., 2005). In general, exposure to nAChR agonists stimulates cell proliferation, angiogenesis, migration and inhibits apoptosis, thus promoting tumorigenicity or tumor-like features in a variety of experimental models (Arredondo et al., 2006; Guo et al., 2008; Paleari et al., 2008; Al-Wadei and Schuller, 2009; Calleja-Macias et al., 2009;

Dasgupta et al., 2009; Paleari et al., 2009). These effects are thought to depend on nAChR-mediated depolarization and Ca^{2+} influx, which can lead to the exocytosis of autocrine factors (Cattaneo et al., 1993; Jull et al., 2001), or direct stimulation of the calcium-dependent signaling pathways that regulate the above processes. Other possible mechanisms are still debated (Grando, 2008; Schuller, 2009).

Although current work is leading to clarification of the signaling pathways downstream to nAChR engagement, the mechanism of action of the tobacco-related nitrosamines on these receptors is poorly understood. Radioligand assays show that NNK and NNN potently compete with the main agonists' binding (Schuller et al., 1998; Schuller, 2007). Considering the indirect functional evidence, such as the increase in $[Ca^{2+}]_i$ or stimulation of the signaling pathways which depend on nAChR activation, these compounds are assumed to cause nAChR opening. However, no direct functional studies exist about the action of nitrosamines on any nAChR subtypes. In particular, the possible interference with the other agonists has not been addressed until now, which is unfortunate as smokers are exposed to a mixture of tobacco-related nAChR ligands. These are present at different concentrations and sum their effects to those of the physiological agonist ACh. Even less is known about the possible cerebral effects of

nitrosamines and their possible roles in tobacco addiction, although NNK is thought to easily traverse the blood brain barrier (Jorquera et al., 1992; Gerde et al., 1998) and has been recently found to activate microglia *in vivo* (Ghosh et al., 2009).

To study the mechanism of action of these compounds, we carried out patch-clamp experiments to determine the effect of NNK, the most potent carcinogenic nitrosamine, on α4β2 nAChRs stably expressed in human embryonic kidney (HEK) cells. This receptor type is probably the most widespread heteromeric nAChR. We found that NNK is a partial competitive agonist with high-affinity for α4β2 receptors. From a patophysiological standpoint, these results suggest that this and analogous compounds can have stimulatory or inhibitory effects on nAChRs, depending on the concentration of the full agonist.

Materials and Methods

Unless otherwise indicated, all chemicals and culture media were purchased by Sigma-Aldrich Italia (Milano, Italy).

Cell culturing

Human embryonic kidney (HEK) 293 cells stably expressing α4β2 human neuronal nicotinic receptors (kindly gifted by Prof. G. Casari, San Raffaele Scientific Institute, Milano) were cultured in Minimum Essential Medium Eagle's alpha modified, supplemented with 10% fetal calf serum, 4.5 g/l glutamine, 0.05 ng/ml hygromycin B and 0.25 μg/ml amphotericin B, at 37°C and 5% CO_2. For patch-clamp experiments, cells were harvested by treatment with trypsin and plated onto 35 mm Petri dishes (Corning Incorporated, NY). These HEK clones were previously extensively characterized with patch-clamp methods (Di Resta et al., 2010).

Solutions and drugs

For patch-clamp experiments, the extracellular solution contained (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, D-glucose 5 (pH 7.3). Pipettes contained (mM): K-aspartate 120, NaCl 10, $MgCl_2$

2, $CaCl_2$ 1.3, EGTA-KOH 10, HEPES-KOH 10, MgATP 1, (pH 7.3). Stock solutions (1 mM) of nicotine were prepared in our extracellular solution, the pH was calibrated again after nicotine addition. Stock solutions of NNK (0.05 mM) were prepared in distilled water. All of the above solutions were kept refrigerated for not longer than 1 week. Nicotine and NNK were added daily to the extracellular solution at the final concentration.

Electrophysiological recordings

Cells were voltage-clamped in the whole-cell configuration of the patch-clamp method. Currents were registered with an Axopatch 200B amplifier (Molecular Devices, Union City, CA), at room temperature. Micropipettes (3-4 MΩ) were pulled from borosilicate capillaries with a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Company, Novato, CA). The cell capacitance and series resistance (up to about 75%) were always compensated. Currents were low-pass filtered at 2 kHz and acquired on-line at 5-10 kHz with pClamp hardware and software (Molecular Devices). Drugs were applied with an RSC-160 Rapid Solution Changer (BioLogic Science Instruments, Claix, France). This allows an acceptable compromise between rapid application of drugs (with a 10-90% risetime between 10 and 100 ms)

and the availability of multiple tubes for delivering solutions with different ligand concentrations. After patch rupture we allowed 1-2 min for pipette solution exchange and signal stabilization, before applying our stimuli. Consecutive stimuli were generally spaced 2-3 min apart, to permit full recovery from receptors' desensitization. To check for possible channel rundown, the maximal agonist concentration was repeatedly applied at regular intervals. The nicotinic currents were isolated by subtracting the non specific currents measured in the absence of the agonist to the total currents obtained in the presence of the agonist.

Analysis of data

Data were analyzed with Clampfit 9.2 (Molecular Devices) and OriginPro 8 (OriginLab, Northampton, MA). For the activation and desensitization curves (Fig. 3 and Fig. 4), functions best fitting to the data were determined by a nonlinear least-squares method (Levenberg-Marquardt algorithm).

The theoretical curve for activation was a single-term Hill-Langmuir equation of the form:

$$I_L/I_{max} = \{1 + (EC_{50}/[L])^{nH} \}^{-1} \quad (1)$$

where I_{max} is the maximal current, I_L is the current at a given concentration of agonist L, EC_{50} is the concentration of L that yields 50% of the maximal current, and n_H is the Hill coefficient.

The theoretical curve for desensitization was:

$$I_L/I_{max} = \{1 + ([L]/IC_{50})^{nH} \}^{-1} \quad (2)$$

where IC_{50} is the concentration of agonist in which the steady state current is half the value of the maximal current and the other symbols are as described above.

The nicotinic current decay (Fig. 3) was fitted with a single exponential function of the form:

$$y = - A\exp(-t/\tau) - B \quad (3)$$

where A is the amplitude of the decaying current, *t* is the time, τ is the exponential time constant in seconds, and *B* is the amplitude of the current at the plateau phase. Time 0 for the exponential was set at the peak of the evoked current.

Statistics

Data are generally given as mean values ± S.E.M. Statistical significance was determined with two-tailed *t* test for paired or unpaired samples, as indicated. Asterisks mark the level of significance (* = $p < 0.05$; ** = $p < 0.01$).

Results

The effect of NNK on α4β2 nAChRs expressed in HEK cells

The α4β2 receptors are one of the most widespread heteromeric receptor type in both brain (Changeux, 2010) and non neural tissue, including tumor cell lines (Egleton et al., 2008). We studied the effect of NNK on these receptors stably expressed in HEK cells (Di Resta et al., 2010). Application of NNK at -60/-80 mV consistently elicited rapidly activating inward currents, with a threshold around 10 pM. The maximal effect was generally obtained between 1 and 10 nM. The current stimulated by NNK at -80 mV was potently blocked by 1 μM dihydro-β-erythroidine (DHβE), which is a selective inhibitor for α4β2 receptors, at this concentration. To allow equilibration of the DHβE concentration, this compound was applied for 30 s before adding nicotine. Subsequently, the peak current in the presence of the antagonist was compared to the current measured with nicotine alone. In particular, this inhibitor brought the average peak current density from 5.75 ± 1.22, in the presence of NNK, to 0.53 ± 0.199, in the presence of 10 nM NNK plus 1 μM DHβE ($n = 9$; $p < 0.05$). Fig. 2B (bottom panel) compares the residual currents measured in the presence of DHβE, when the nAChRs were activated by either 10 nM

NNK, or 100 μM nicotine, or 100 μM ACh. The average blocking efficacy of DHβE was very similar for the three agonists. Overall, the above results support our conclusion that NNK activates the heteromeric nAChRs expressed in our cells, as is also indicated by the virtual identity of the current reversal potentials observed when nicotine was applied in the presence and in the absence of NNK, as will be discussed below (Fig. 5).

To compare the effects of NNK to those of the main agonists, we carried out a series of experiments in which the current activated by different doses of NNK was compared to the current activated by 10 μM nicotine. This concentration was chosen to avoid extensive channel desensitization and facilitate the drug washout, as high concentrations of ACh and nicotine are known to produce open channel block which can considerably delay channel deactivation (Sine and Steinbach, 1994; Ogden and Colquhoun, 1985). Nicotine was preferred to ACh, to avoid possible interference with the endogenous muscarinic receptors sometimes expressed in cell lines. The effect of muscarinic receptor blockers on nAChRs is not fully defined (Zwart and Vijverberg, 1997). The activation curve for NNK was generated from the normalized peak current values. To avoid underestimating the peak current because of slow drug application, the

desensitizing current in the presence of NNK was fitted with a monoexponential function that was extrapolated to the time of agonist application (Buisson and Bertrand, 2001; Fig. 3). For each NNK concentration, the value of the function at this initial time was normalized to the current obtained with 10 μM nicotine. These values were plotted in Fig. 2C and fitted with equation 1. Fig. 2C also reports the concentration-response relations for nicotine and ACh, obtained by using an analogous procedure. Once again, the data points were normalized to the current elicited by 10 μM nicotine, for direct comparison. The EC_{50} for NNK estimated from the theoretical curve turned out to be approximately 0.07 nM. Full statistics are given in the figure legend. The maximal current obtained with NNK was 0.12 of the value obtained with maximal doses of ACh or nicotine, which indicates that NNK is a partial agonist with high affinity for heteromeric nAChRs. The maximal open probability (P_o) of α4β2 nAChRs obtained with the full agonists in HEK cells was recently estimated to be between 0.8 and 1 (Li and Steinbach, 2010). Hence, we conclude that NNK produces a maximal P_o between 0.1 and 0.15, at V_ms close to the resting potential. This estimate assumes that the single channel conductance in the presence of NNK is similar to the one observed in the presence of the other agonists, which is generally

a reasonable assumption for nAChRs (Li and Steinbach, 2010). From a pathological point of view, the range of effective NNK concentrations we observed overlaps with the typical range of plasma concentrations of this compound observed in smokers, in the range 10-100 pM (Schuller, 2007).

Steady-state activation and desensitization curves in the presence of NNK

To minimize distortion of channel kinetics due to solution exchange, we have considered only those experiments where the apparent channel activation required less than 200 ms. In these cases, the desensitizing phase of the currents recorded in the presence of NNK was fitted with a monoexponential decay function, as for the study of the activation process (Fig. 3A). To determine the desensitization curve, we plotted the fractional steady state current at each NNK concentration, obtained by dividing the steady state current (obtained by the fitting procedure) by the corresponding peak current. The average values calculated from a series of such experiments are given in Fig 3B. The data points were fitted with equation (2), giving an NNK concentration for half-desensitization of approximately 0.06 nM. Full statistics are given in the figure legend. For comparison, Fig.

3B also gives the corresponding activation curve, which was generated as illustrated in Fig. 2, except that the current values at the different NNK concentrations were normalized to the maximal current obtained with NNK.

The α4β2 nAChR 'window current' as a function of NNK

The long-term physiological effects of treating cell cultures with NNK or those produced by long-term smoking depend on the level of tonic activation of nAChRs. At the steady state, a macroscopic current through an ensamble of ligand-gated channels of the same kind is proportional to the product of the activation and desensitization parameters, at a given agonist concentration. The crossover of the activation and desensitization curves illustrated in Fig 3B defines a 'window current', a bell-shaped curve representing the steady state fractional channel open probability, as a function of NNK concentration. It is clear that the nAChR P_o is significant within a relatively broad range of NNK concentrations, which indicates that this compound could maintain tonic nicotinic currents in different physiological conditions.

The effect of NNK in the presence of the full agonist

As a partial agonist with high affinity, NNK is expected to compete with the full agonists in a complex way. This could lead to either increase or decrease of the current elicited by the main agonist, depending on the concentration of both compounds (Stephenson, 1956; Ariëns et al., 1964; Hogg and Bertrand, 2007). Clarifying this point presents considerable importance for interpreting the patophysiological effects of nitrosamines in the organism.

We thus tested the effect of different NNK concentrations in the presence of different concentrations of nicotine. A typical experiment is shown in Fig. 4A. Current was activated at -60 mV by 1 μM nicotine. After 0.5 s, 10 μM NNK was added for 2.5 s and then removed, in the presence of nicotine. Finally, nicotine was also removed. The experiment indicates that NNK, contrary to its effect in the absence of nicotine, partially inhibits α4β2 nAChRs, when applied in the presence of nicotine (Fig. 4B). The extent of the inhibitory effect was quantified by dividing the residual current in the presence of NNK just before NNK removal by the peak current measured after NNK was washed out. By comparing these two measures very close in time, we minimized the possible distortion of the result because of the progressive channel desensitization.

The effect of V_m

We next studied the voltage dependency of the NNK effect (Fig. 5). Currents were elicited by applying 100 nM nicotine for the indicated time. During nicotine application, 1 nM NNK was applied and then removed soon after the full blocking effect was obtained. The same procedure was carried out at different V_ms. Typical experimental traces are shown in Fig. 5A, for experiments carried out at the indicated V_m. In these conditions, NNK produced partial inhibition of the nicotine effect. Fig. 5B plots the current voltage relations in the presence of nicotine alone or nicotine plus NNK. Data points are average current density values calculated from 9 experiments. At each V_m, currents were measured at the steady state (for NNK) and immediately after NNK washout (for nicotine). The current reversal potentials were estimated by eye after fitting each I/V curve with the polynomial function best fitting the experimental data (Haghighi and Cooper, 1998). These values were not significantly different in the presence and in the absence of NNK. Moreover, the fractional block produced by NNK was similar independently of the applied V_m. Analogous results were obtained with NNK concentrations up to 10 μM. These data support the notion that NNK competes with the full agonist for binding to the nAChRs, without exerting significant open

channel block, at least at the concentrations we used, which are well above the maximal concentrations reached by NNK *in vivo*.

Discussion

In this paper, we have shown by using patch-clamp methods that the tobacco-specific nitrosamine NNK is a partial agonist with high affinity of one of the most common neuronal nAChR subtypes, namely α4β2. These results have clear implications for interpreting the patophysiological effects of this and other structurally related N-nitrosamines, as they indicate: i) that NNK can activate heteromeric nAChRs at concentrations consistent with those typically observed in smokers; ii) that the drug's effect on the nicotinic currents also depends on the concentration of the full agonist.

Comparison with previous studies

The available studies about the effects of NNK and other nitrosamines on nAChRs concern radioligand competition assays carried out with labeled epibatidine (to target heteromeric nAChRs) or α-bungarotoxin (specific for the α7 subunit), in lung cancer cell lines (Schuller and Orloff, 1998; Schuller, 2007). Most of the results obtained with heteromeric receptors are not directly comparable with ours, because many cell lines express multiple nAChR subunits (Egleton et al., 2008) and the apparent affinity of most nicotinic ligands is different in

specific nAChR subtypes (e.g. Xiao and Kellar, 2004). Moreover, patch-clamp experiments specifically reveal the population of active channels, thus excluding the possible contribution of silent receptors (e.g. McNerney et al., 2000). Nonetheless, the competition experiments carried out with labeled α-bungarotoxin on cells that prevalently express homomeric α7 receptors indicate that NNK has a high affinity for this receptor type. Because our results suggest that NNK is also very effective on α4β2 nAChRs, we conclude that the tobacco-related nitrosamines are likely to produce generalized binding to nAChRs *in vivo*, at the typical concentrations observed in smokers.

Physiological implications for non-neuronal and cancer tissues

As mentioned earlier, previous work in cultured cell lines showed that nicotine and several N-nitrosamines, including NNK, produce similar effects on cell physiology, particularly a stimulation of proliferation and inhibition of apoptosis (e.g. West et al., 2003; Tsurutani et al., 2005). Our results are in agreement with the general interpretation that these effects are caused by activation of nAChRs produced by either nicotine or NNK. Fig. 3 indicates that steady NNK concentrations between 0.1 and 10 nM induce a tonic nAChR activation which can determine steady Ca^{2+} influx accompanied by cell depolarization.

However, our results also suggest that future work should devote considerably more attention to the interaction between nitrosamines and the full agonists in specific nAChRs. N-nitrosamines in smokers display steady concentrations of 10-50 pM (Hecht and Hoffmann, 1988; Schuller, 2007). As shown in Fig. 2, these concentrations can produce significant activation of α4β2 nAChRs. Therefore, a mixture of tobacco-related nitrosamines in plasma at these concentrations should potentiate the effects of low levels of nicotine, although a full picture of the effects on different nAChR types is lacking. However, the outcome of interacting nitrosamines and nicotine is not straighforward, given that the typical peak levels of plasma nicotine in smokers can vary between 20 and 500 nM (e.g. Russell et al., 1980). According to our results, this range of concentrations is close to the threshold for the transition between the stimulatory and the inhibitory effect of NNK, at least in α4β2 nAChRs. Therefore, to predict the possible long-term effects of the tobacco-related nitrosamines in humans, it will be probably necessary to consider the fluctuating levels of nAChR agonists in specific individuals, which depend on frequency of smoking, individual metabolic capacity, etc.

Implications for neurophysiology

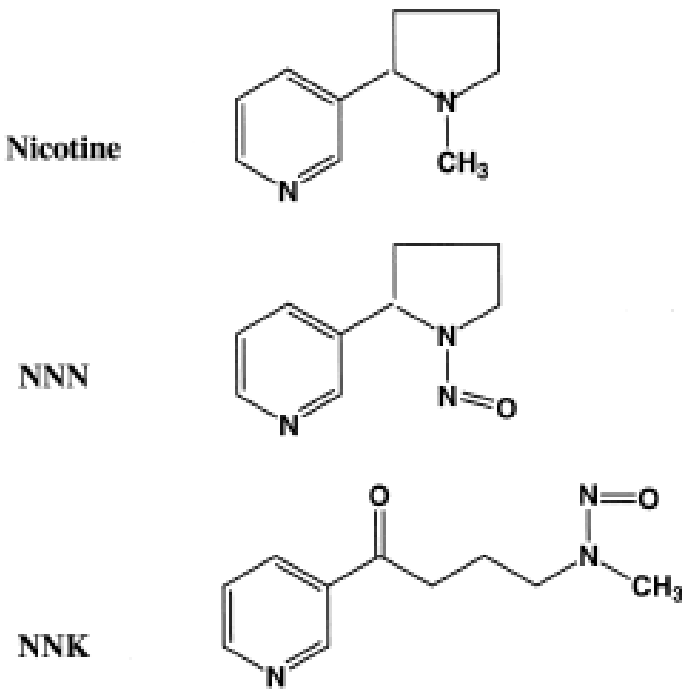
The above considerations also apply to the CNS and particularly to the brain. In the CNS, interpreting the effects of tobacco-related nitrosamines is even more complex than in non-neuronal tissue, for two reasons.

First, the steady concentration of nicotine in the cerebrospinal fluid of smokers, which is not far from the plasma levels (Berridge et al., 2010), is superimposed to the tonic concentration of ACh. These steady levels of cerebral ACh depend on the fact that the distribution of acetylcholinesterase does not appear to precisely match the distribution of the other cholinergic elements. Therefore, the tonic effects of nitrosamines in the brain should be considered in the context of the total level of the full agonists. Once again, these levels are generally close to the threshold for the NNK-dependent inhibition that we have observed.

Second, in the CNS it is also important to consider the kinetics of ACh release. In *bona fide* cholinergic synapses, on vesicle release, [ACh] in the synaptic cleft quickly reaches millimolar levels. Subsequently, ACh is degraded by acetylcholinesterase with a time constant usually lesser than 10 ms. In this case, the presence of NNK could alter the kinetics of current decay, being poorly effective at the

peak, when [ACh] is very high, but becoming more and more effective as ACh is degraded. Once again, the precise patophysiological outcome depends on the nAChR subtype expressed by a give synaptic population in a certain cerebral region.

Figure 1



Molecular structures of nicotine, N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNN and NNK are two of the most common tobacco-related nitrosaminic metabolites and are structural analogs of nicotine.

Figure 2

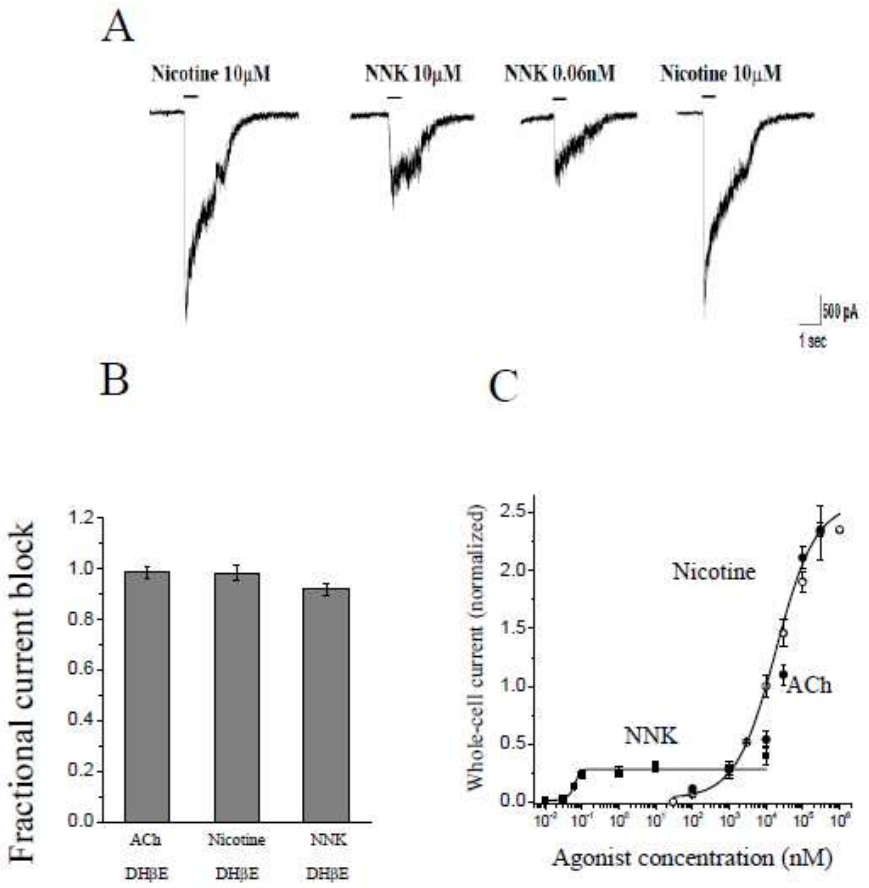


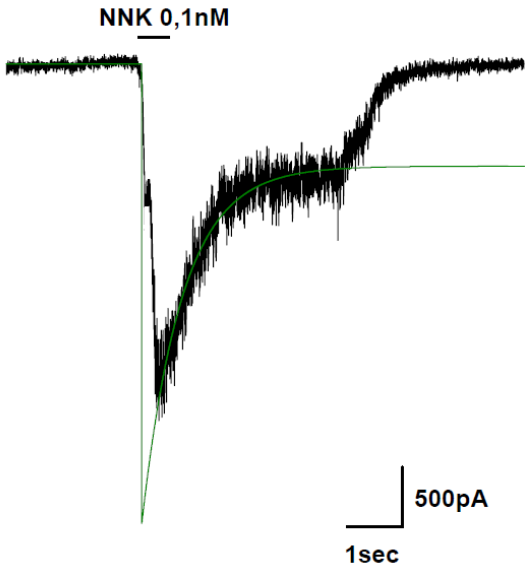
Fig. 2 The effect of NNK on α4β2 nAChRs expressed in HEK cells

Concentration-response curves for α4β2 nAChRs treated with ACh, nicotine or NNK. A, typical whole-cell current traces, recorded at -80 mV. The panel illustrates nicotinic currents elicited by application of the indicated agonist concentration. Notice that NNK stimulated

currents whose maximal amplitude was about one third of the peak current activated by 10 μM nicotine or NNK at different concentrations. Nicotine was repeatedly applied during the experiment, to check for possible channel rundown. Continuous bars above the current traces indicate the time of agonist application. B, concentration response curves generated from experiments analogous to those illustrated in panel A. Data points are average peak currents plotted as a function of the agonist concentration and normalized to the current activated by 10 μM nicotine. Data summarize the results of 5-6 determinations for each concentration, carried out in the same run of experiments. White circles: nicotine; black circles ACh; black squares: NNK. The concentration-response relations for NNK and nicotine were fitted with equation one (continuous lines). For clarity, the corresponding fitting curve for ACh was omitted.

Figure 3

A


NNK 0,1nM
500pA
1sec

B

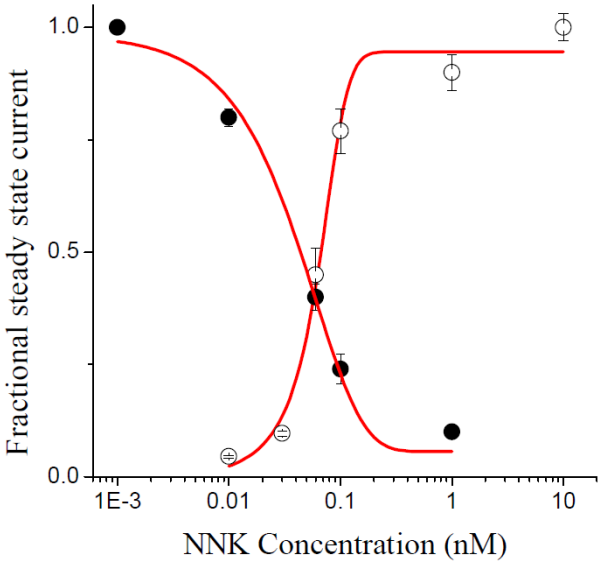

Fractional steady state current
1.0
0.5
0.0
1E-3
0.01
0.1
1
10
NNK Concentration (nM)

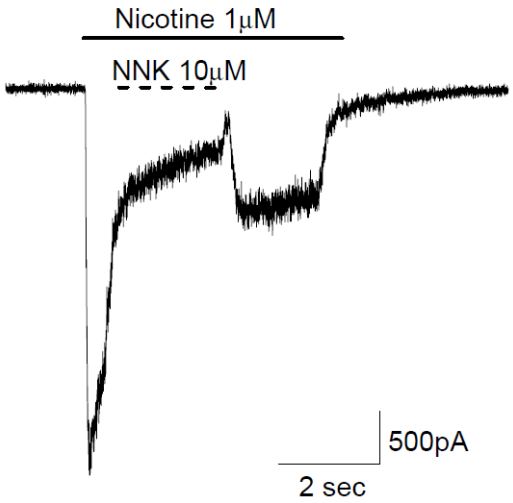
Fig. 3 Steady-state activation and desensitization curves in the presence of NNK.

Steady-state activation and desensitization of α4β2 nAChRs in the presence of NNK. A, typical whole-cell current trace activated by the indicated concentration of NNK, at -80 mV. From analogous current traces obtained at different NNK concentrations, we determined the maximal and the steady state current, by fitting the decaying phase as indicated. The fitting curve was extrapolated back to the time of agonist application, to estimate the maximal current activated by NNK. B, from the procedure illustrated in panel A, we generated the activation and desensitization curves for NNK. The activation curve was generated by plotting the average normalized peak currents at the different agonist concentrations. The desensitization curve was obtained by plotting the average fractional currents at the steady state as a function of NNK concentration. Continuos lines were obtained by fitting the data with equation 1 (activation) and equation 2 (desensitization). Notice that the maximal steady state current, as shown by the crossover of the two curves, is produced by NNK

concentrations around 0.06 nM. Panel B summarizes the results of 6-7 dterminations carried out in the same run of experiments.

Figure 4

A


Nicotine 1μM
NNK 10μM
500pA
2 sec

B

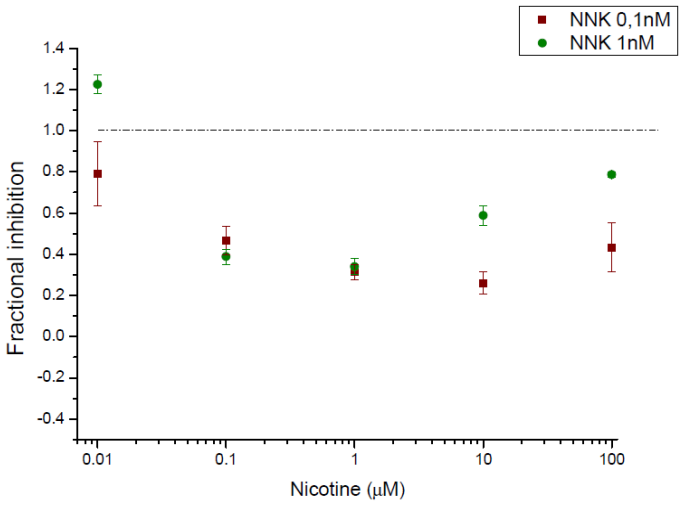

NNK 0,1nM
NNK 1nM
Fractional inhibition
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
0.01
0.1
1
10
100
Nicotine (μM)

Fig. 4 The effect of NNK in the presence of the full agonist

A. Representative protocol of patch-clamp experiments to tested the effect of different NNK concentrations in the presence of different concentrations of nicotine. Continuous lines marks time of nicotine application, dot lines mark time of nicotine + NNK application.

B. Fractional inhibition of the nicotinic currents by two different concentrations of NNK (0,1 and 1 nM). NNK behaves as a partial agonist. It has the property of being able to act both as agonists or antagonist depending on the concentration of the main agonist.

Figure 5

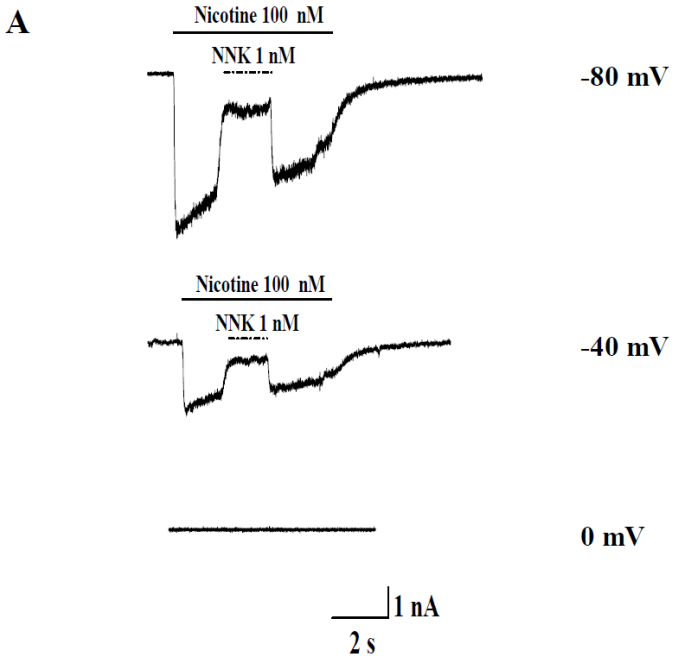

A
Nicotine 100 nM
NNK 1 nM
-80 mV
Nicotine 100 nM
NNK 1 nM
-40 mV
0 mV
1 nA
2 s
B
Vm (mV)
-80
-60
-40
-20
0
20
40
-10
-20
-30
-40
-50
pA/pF

Fig. 5 Voltage dependence of nicotinic currents

A. Whole-cell current traces evoked by nicotine 100 nM and nicotine 100 nM + NNK 1 nM. The effect of voltage on nicotinic current amplitude was tested at the indicated potentials. Continuous lines marks time of nicotine application, dot lines mark time of nicotine + NNK application.

B. Current density-voltage relation between -80 to +40 mV for nicotine 100nM (squares) and nicotine 100nM + NNK 1nM (circles). For both curves the reversal potential is approximately 0 mV, as estimated by fitting the data points with a third order polynomial curves.

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CHAPTER 6

“Summary, conclusione and future perspectives”

Summary and conclusions

The nAChRs are distributed widely throughout the nervous system and are also expressed by non-neuronal tissue.

In the brain, the activation of nicotinic receptors induces the release of a number of neurotransmitters including Ach, GABA, NA, DA, 5-HT and glutamate, many of which are known to play a role in mediating/modulating a number of behavioural tasks (Paterson and Nordberg, 2000; Alkondon et al, 2000; Mesulam, 2004; Dani and Bertrand, 2007). Moreover, nAChRs play an important role in cognition and memory.

Recent evidence points to nAChRs as pivotal elements for the regulation of cell proliferation, apoptosis and angiogenesis in several tumours, including lung cancers (Egleton et al., 2008; Wessler and Kirkpatrick, 2008). This is suggestive because smoke is an established risk factor for cancer, and particularly lung cancer. Activation of nAChRs stimulates (directly and indirectly) Ca^{2+} influx, which triggers the release of growth factors and other transmitter molecules. These produce autocrine and paracrine effects that promote proliferation, inhibit apoptosis, stimulate angiogenesis and migration (Schuller 1989; Maneckjee and Minna; 1990; Chernyavsky et al., 2004; Egleton et al., 2008). Moreover, nicotine confers resistance to the chemotherapeutic-induced apoptosis (Minna, 2003; Egleton et al., 2008). Such effects occur in both small and non-small cell lung cancer cell lines, although the intracellular signalling cascades and the nAChRs involved are different, as nAChRs stimulate intracellular signaling pathways in a cell-type specific manner (Schuller, 2009).

nAChRs in SCLC and NSCLC cell lines

Expression on nicotinic receptors in lung cancer derives from the expression of nAChRs in normal lung cells. Normal bronchial epithelial cells express nAChRs as part of a cholinergic autocrine loop in which all proteins needed for cholinergic signaling are present (Song et al., 2008). The nicotinic subunit expression changes between normal lung cells and tumor cells (Lam et al., 2007; Sartelet et al., 2008).

Binding of labelled ligands/antibodies and messenger expression show a complex pattern of nAChR subunit expression in SCLC and NSCLC cell lines. The expression of different subunits does not imply that functional receptors are expressed onto the plasma membrane. Moreover, it provides no information about the contribution of specific nAChR types to the control of cell physiology.

We studied the nAChR expression in SCLC (U2020) and NSCLC (A549) cell lines. The data obtained by the patch-clamp experiments confirm the presence of functional nicotinic receptors in both tumor cell lines. The current density values were higher in NSCLC indicating that more nAChRs are expressed in the cell membrane of these cells.

In both cell lines, saturating doses of nicotine evoked nicotinic currents with variable kinetics. These could be broadly classified as quickly and slowly desensitizing currents, tipically representative of homomeric $(\alpha 7)_5$ receptors and heteromeric receptors. To confirm the expression of the different subtypes we used selective inhibitors to distinguish $\alpha 7$ –containing receptors (MLA 10 nM) from those that do not contain it (DHβE 1 μM). Overall, in SCLC cells and NSCLC both

MLA and DHβE produced significant block of the nicotinic currents. This inhibition profile suggests that the majority of the cells of both cell lines expressed both homo- and heteromeric nAChRs.

Preliminary results obtained by cell migration of NSCLC cell lines confirm the presence of both homo- and heteromeric receptors in NSCLC, moreover indicates that these nicotinic receptors, especially the homomeric receptors, are involved in the regulation of cell motility.

NNK and human α4β2 nAChRs

Nitrosamines are carcinogens formed in the mammalian organism from amine precursors. One of the major target organs of tobacco specific nitrosamines is the lung, in which these can activate the intracellular pathways involved in nAChR activation.

The tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed from nicotine.

Very little is known about the mechanism of action of these drugs on nAChRs. The complex expression of nAChRs subtypes in SCLC and NSCLC makes difficult to determine in detail the mechanism of action of NNK on nicotinic receptors. Therefore, we used HEK cell lines stably expressing the α4β2 nAChR. This nicotinic receptor subtype is one of the most common in both nervous system and tumor cell lines (Egleton et al., 2008, Changeux, 2010).

Data obtained by patch-clamp experiments in presence of NNK and in presence of NNK and the specific inhibitor DHβE, show that this compound activates the α4β2 nicotinic receptor subtype. Moreover, the results obtained by experiments carried out to compare the effects

of NNK to those of the main agonist, indicates that NNK is a partial agonist with high affinity for α4β2 nAChRs. A partial agonist has the unique property of being able to act both as agonists or antagonist depending on the concentration of the main agonist (Stephenson, 1956; Ariëns et al., 1964; Hogg and Bertrand, 2007). The presence of agonist causes desensitization of nicotinic receptors. Since in smokers NNK remains in the plasma and in the cerebrospinal fluid for indefinite time, we estimated the fraction of active receptors at the steady-state. The data indicate that the range of concentrations of NNK at which channels have a significant probability of being open overlaps with the NNK concentrations typically found in smokers blood, i.e. 10-50 pM (Hecht and Hoffmann, 1988; Schuller, 2007). Our results indicate that these concentrations can produce significant activation of α4β2 nAChRs, but that the contemporary presence of nicotine or Ach can considerably influence the effect of NNK. In fact, the agonist concentration threshold for NNK behaving as activator or inhibitor is around 100 nM, which is close to the average plasma concentrations of nicotine observed in smokers. Therefore, to interpret the pathophysiological effects of nitrosamines, it is necessary to consider the steady level of the full agonist in individual patients and the fluctuations of both nicotine and ACh on specific tissues.

These conclusions also apply to the CNS, where the cerebrospinal steady concentrations of nicotine are similar to the plasma levels (Berridge et al., 2010) and are superimposed to the tonic and fluctuate levels of ACh, which vary in different cerebral regions.

Future Perspectives

A first issue for future studies is understanding the physiological role of different nAChR types in cancer cells. Our preliminary results on the control of cell migration of lung tumor cell lines by nAChRs indicates that migrating cells are more, although not exclusively, sensitive to the blockade of α7 receptors. One possibility, suggested by work in keratynocytes, is that different receptor types give a different contribution to chemotaxis and chemokinesis (Chernyavsky et al., 2004). The asymmetric distribution of homomeric nAChRs is thought to determine front-rear calcium gradients that control directional cell migration, whereas the more uniform distribution of heteromeric receptors tends to control the cytoplasmic calcium levels in a more global way, thus regulating the cell's propensity to move (and thus random cell movement), but not so much the direction of migration. No data are however available on these issues in tumor cell lines, which may have interesting implications for the metastatic process. As to the role of different nAChRs in cell proliferation, it has been hypothesised that α7-containing receptors control the secretion of stimulatory autocrine factors, whereas heteromeric receptors (particularly α4β2) principally regulate the release of GABA, which

has been shown to have inhibitory effects on the proliferation of certain cancer cells (Joseph et al. 2002; Schuller et al., 2008). However, these hypotheses mainly derive from knowledge about the physiology of nAChRs in the adult and developing nervous system. Once again, virtually nothing is known about the existence of different tumor cell populations expressing different nAChRs associated with different secretion machineries (e.g. serotonergic versus GABAergic) or about the possible compartimentalization of these mechanisms in individual cells.

As to the effects of nitrosamines on cell physiology, our results with NNK show that the usual interpretation, that assumes essentially analogous effects of nicotine and nitrosamines, may be overly simplistic. Considering the typical steady levels of nicotine in smokers' plasma and cerebrospinal fluid, it is uncertain whether nitrosamines exert steady stimulatory or inhibitory effects. To understand how nitrosamines affect specific cell types, it will be necessary to study: i) the effect of nitrosamines on other nAChR types; ii) the effects of other tobacco-related nitrosamines such as NNN and DEN; iii) the effect of these compounds on processes such as cell proliferation in the presence of the full agonists, an approach that has been rarely adopted until now, to the best of our knowledge.

Moreover, to reach a comprehensive picture, it will be also necessary to understand the time course of the nitrosamine action in those physiological contexts where the levels of the full agonists are known to fluctuate. In such cases, the drug's effect could oscillate between inhibition and potentiation, thus potentially altering the dynamics of the normal process. This is probably the case in cerebral synapses, which opens interesting perspectives for deeper studies about the addictive effects of tobacco, as is increasingly recognized that the kinetics of administration of an addictive drug considerably affects its addictive efficacy (Samaha and Robinson, 2005).

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Publications

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Abreviations

5-HT	serotonin
αBgtx	α-bungarotoxin
ACh	acetylcholine
AChE	ACh hydrolyzing enzymes acetylcholinesterase
ADNFLE	autosomal dominant nocturnal frontal lobe epilepsy
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
BChE	butyrylcholinesterase
CBZ	carbamazepine
ChAT	Choline acetyltransferase
CNS	Central nervous system
DA	dopamine
DHβE	Dihydro-β-Erythroidine
DMPP	1,1-dimethyl-4-phenylpiperazinium
EEG	electroencephalogram
EGFR	epidermal growth factor receptor
eNOS	nitric-oxide synthase
ERK	extracellular signal-regulated kinase
FGF	Fibroblast growth factors
GABA	γ-aminobutyric acid
HEK	Human embryonic kidney
KC	Keratinocytes
LCC	Large cell lung carcinoma
MHD	monohydroxy derivative
MLA	Methyllicaconitine
NA	noradrenaline
nAChR	nicotinic acetylcholine receptor

NE	neuroendocrine
NF-κB	nuclear factor-κB
NFLE	nocturnal frontal lobe epilepsy
NMDA	*N*-Methyl-D-aspartic acid
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
NSCLC	non-small cell lung carcinoma
OXC	oxcarbazepine
P13-K	phosphoinositide3-kinase
PAC	Polmonary Adenocarcinoma
PET	positron emission tomography
PKA	Protein kinase A
PKC	Protein kinase C
PP1	protein phosphatase 1
REM	rapid eye movement
SCC	Squamous cells carcinoma
SCLS	Small cell lung carcinoma
TMA	tetramethylammonium
TNFα	tumor necrosis factor
VAChT	vesicular ACh transporter
VEGF	Vascular endothelial growth factor
V_m	membrane potential
V_{rest}	resting membrane potential
V_{rev}	channel reversal potential