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REVIEW ARTICLE

Proteins and chemical chaperones involved in neuronal nicotinic receptor expression and function: an update

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Neuronal nicotinic ACh receptors (nAChRs) are a family of ACh-gated cation channels, and their homeostasis or proteostasis is essential for the correct physiology of the central and peripheral nervous systems. The proteostasis network regulates the folding, assembly, degradation and trafficking of nAChRs in order to ensure their efficient and functional expression at the cell surface. However, as nAChRs are multi-subunit, multi-span, integral membrane proteins, the folding and assembly is a very inefficient process, and only a small proportion of subunits can form functional pentamers. Moreover, the efficiency of assembly and trafficking varies widely depending on the nAChR subtypes and the cell type in which they are expressed. A detailed understanding of the mechanisms that regulate the functional expression of nAChRs in neurons and non-neuronal cells is therefore important. The purpose of this short review is to describe more recent findings concerning the chaperone proteins and target-specific and target-nonspecific pharmacological chaperones that modulate the expression of nAChR subtypes, and the possible mechanisms that underlie the dynamic changes of cell surface nAChRs.

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Abbreviations

ER, endoplasmic reticulum; ERAD, ER-associated degradation; GPI, glycosylphosphatidylinositol; LY6, lymphocyte antigen-6; Lynx1, Ly6/neurotoxin1; Lynx2, Ly6/neurotoxin2; nAChRs, neuronal nicotinic ACh receptors; PBA, 4-phenylbutyric acid; PD, Parkinson's disease; PSCA, prostate stem cell antigen; RIC3, resistance to inhibitors of cholinesterase 3; SNP, single nucleotide polymorphism; SLURP-1, secreted mammalian Ly6/urokinase plasminogen-type activator receptor-related protein-1; SLURP-2, secreted mammalian Ly6/urokinase plasminogen-type activator receptor-related protein-2; TM, transmembrane; VPA, valproic acid

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Introduction

Neuronal **nicotinic ACh receptors (nAChRs)** are a family of **ACh**-gated cation channels that are ubiquitously expressed in virtually all neurons, where they contribute to many physiological processes in the central and peripheral nervous systems (Albuquerque *et al.,* 2009; Hurst *et al.,* 2013). They are also expressed in some non-neuronal cell types where their physiological role is still being investigated (Grando, 2014; Mucchietto *et al.,* 2016). In the brain, nAChRs are expressed at presynaptic sites (where they modulate the release of many different neurotransmitters), at postsynaptic sites (where they influence excitability), and extra-synaptically, where they participate in non-synaptic communication (Albuquerque *et al.,* 2009). Deficits in nAChR-mediated or -modulated neurotransmission are involved in the pathogenesis of many neurological and neuropsychiatric disorders (Lewis and Picciotto, 2013; Picciotto *et al.,* 2015), and variants of the nAChR genes coding for the **α3**, **β4**, **α5** and **α9 subunits** have been implicated in **nicotine** dependence and lung cancer susceptibility (Improgo *et al.,* 2010a,b; Chikova *et al.,* 2012). A detailed understanding of the mechanisms that regulate the functional expression of nAChRs in neurons and non-neuronal cells is therefore important in order to clarify the causes of these pathologies. Very exhaustive reviews of this matter have been previously published (Millar and Harkness, 2008; Govind *et al.,* 2012; Colombo *et al.,* 2013; Sadigh-Eteghad *et al.,* 2015), and the purpose of this short review is to describe more recent findings concerning the chaperone proteins and target-specific and non-target-specific pharmacological chaperones that modulate the expression of nAChR subtypes, and the possible mechanisms that underlie the dynamic changes of cell surface nAChRs.

Structure of nAChRs

nAChRs are transmembrane receptors of approximately 300 kDa, consisting of five identical or homologous subunits (α2–α10 and β2–β4) (Albuquerque *et al.,* 2009; Hurst *et al.,* 2013; Cecchini and Changeux, 2015). Each of the 12 vertebrate nAChR subunits is coded by a distinct gene and has the same architecture: a large extracellular N-terminal domain followed by four transmembrane (TM) domains, a large cytoplasmic loop between TM3 and TM4, and an extracellular C terminal. The N-terminal and TM domains are well conserved among the different subunits, but the TM3-TM4 cytoplasmic loop is more diverse in length and amino acid composition (Stokes *et al.,* 2015). It contains many sequences that are important for receptor export from the endoplasmic reticulum (ER) and trafficking to the plasma membrane, sequences for post-synaptic scaffold protein interactions, and phosphorylation sites for various serine/threonine and tyrosine kinases (Millar and Harkness, 2008; Colombo *et al.,* 2013). Moreover, it has recently been shown that the intracellular loop of the **α7 subunit** contains a G protein binding cluster that promotes intracellular signalling (King *et al.,* 2015; King and Kabbani, 2016). This finding together with the fact that α7 receptors are also found in non-neuronal cells such as the immune cells, where no ACh-dependent currents can be recorded at the plasma membrane, indicate that α7 receptors can function not only as ionotropic receptors, highly permeable to Ca^{2+}, but can also activate metabotropic-like second messenger signalling see Treinin *et al.,* 2017).

Each receptor subtype consists of five distinct subunits that co-assemble to form the channel, and determine the pharmacological properties of the ligand binding sites of the receptor and the cation preference of the channel. Although there is the potential for a large number of subunit combinations in neurons, the assembly of nAChRs seems to be highly regulated, with certain subunit combinations being favoured (reviewed in Gotti *et al.,* 2009). The most expressed subtypes in the brain are the **α4β2*** (* means that additional subunits may be present) and the homomeric α7 receptors, whereas the α3β4* is the most expressed subtype in the peripheral nervous system. In addition to differences in subunit composition, nAChRs may have the same subunit composition but different subunit stoichiometries, as in the case of the α4β2 and α3β4 subtypes (Hurst *et al.,* 2013; Zoli *et al.,* 2015). The two $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ stoichiometries have different calcium permeability and agonist or antagonist sensitivity, with the latter having a higher affinity and sensitivity to ACh; in the case of the α3β4 subtype $(\alpha3\beta4)_2\alpha3$ and $(\alpha3\beta4)_2\beta4$ have markedly different single-channel conductance and kinetics, and different sensitivities to zinc enhancement (reviewed in Zoli *et al.,* 2015).

The mRNAs for nAChR subunits are frequently expressed in non-neuronal cells although functional studies characterizing these receptors are still very few (Fu *et al.,* 2009). A large repertoire of nAChR subunits is present in bronchial cells and in the airway epithelium, α7 nAChRs participate in the control of the airway ion transport processes (Maouche *et al.,* 2013). Various studies have also shown the involvement of nAChRs in the development and progression of lung tumours (see Schaal and Chellappan, 2014; Mucchietto *et al.,* 2016). Linkage analyses, and candidate gene and genome-wide association studies, have shown that variants in the human α3, α5, β4 and α9 nAChR subunit genes are associated with the risk of nicotine dependence, smoking, and lung cancer (see Improgo *et al.,* 2010b; Chikova *et al.,* 2012). Transcripts of the α3 and β4 genes are significantly over-expressed in small-cell lung carcinoma cells, while the level of the α5 transcript is high in both normal and cancer lung cell lines (Improgo *et al.,* 2010a). Other studies have shown that the non-synonymous rs16969968 single nucleotide polymorphism (SNP) in the α5 gene, which leads to an aspartic acid to asparagine substitution (D398N), is also associated with lung cancer (see Improgo *et al.,* 2010b). As non-smokers bearing this SNP are at increased risk of lung cancer, the cancer may not only be the consequence of smoking but directly influenced by this SNP (Amos *et al.,* 2008).

nAChR assembly and trafficking

nAChRs are membrane proteins and their homeostasis or proteostasis is essential for the correct physiology of the CNS and peripheral nervous system. The proteostasis network

regulates the folding, assembly, degradation and trafficking of nAChRs in order to ensure their efficient functional cell surface expression. However, as nAChRs are multi-subunit, multi-span, integral membrane proteins, the folding and assembly of even the wild-type nicotinic subunits is a very inefficient process, and only a small proportion of subunits can form functional pentamers. Synthesis, folding and assembly take place inside the ER, and require the cleavage of the signal peptide, the oxidation of disulfide bonds, and the N-glycosylation of some residues. One very important role in this context is played by chaperone proteins, which guarantee tight quality control by assisting the subunits to assume the correct folded conformation or eliminating misfolded or unassembled proteins by means of ER-associated degradation (ERAD) (Millar and Harkness, 2008). This quality control prevents dysfunctional subunits from reaching the membrane, and only the few correctly assembled pentamers can leave the ER through vesicles that first reach the Golgi apparatus and then the plasma membrane. A very important role in the trafficking of nAChRs is played by specific signal sequences in the subunits, such as ER retention/retrieval sequences and signals that promote ER export. An overview of the chaperones, adaptor proteins and signals involved in these mechanisms has been given by Colombo *et al.,* (2013). The efficiency of assembly and trafficking varies widely depending on the nAChR subtypes and the cell type in which they are expressed. This has led to the definitions of 'permissive' cell types (in which nAChRs are expressed, assembled and localized on the plasma membrane) and 'non-permissive cells', in which nAChR subunits are mainly retained in the ER membrane. In contrast to α4β2–expressing cells which produce robust levels of receptor binding and function, expression of **α6**β2-containing or α7 receptors in the HEK and SH-EP1 cell lines yielded very low cell surface expression, ligand binding and functional responses, under typical cell culture conditions (see Letchworth and Whiteaker, 2011; Valles and Barrantes, 2012).

Much effort has been dedicated to clarifying the molecular mechanisms responsible for this difference and finding a way to improve the expression, assembly and trafficking of nAChRs. Some of the ER-resident chaperone proteins needed for the proper folding and assembly of some nAChR subtypes (resistance to inhibitors of cholinesterase 3 (RIC3), NACHO, lymphocyte antigen-6 (LY6) protein family and UBXN2A) are discussed below and summarized in Table 1.

In addition to chaperone proteins, there are target specific and non-target-specific pharmacological compounds that are cell permeable and by facilitating biogenesis and/or preventing/correcting subunit misfolding enhance assembly of the nAChRs in the ER and trafficking through the Golgi to the plasma membrane (Table 1).

Chaperone proteins

RIC3

RIC3 is an ER membrane protein (Cheng *et al.,* 2007) that has chaperone activity on the assembly of nAChRs (particularly the α7 subtype) and **$5HT_3$ receptors** (Castillo *et al.,* 2005; Lansdell *et al.,* 2005; Williams *et al.,* 2005; Castillo *et al.,* 2006). Full length human RIC3 consists of an N-terminal domain, a membrane-spanning domain, and a cytosolic C-terminal domain with one coiled-coil domain. Both the N- and the C-terminal regions are needed to enhance nAChR expression; however, deletion analyses of RIC3 suggest that ablating the coiled-coil domain does not modify the capacity of RIC3 to modulate the expression of AChRs or $5\text{-}HT_3$ receptors and, depending on the subtypes, differently affects their functional responses (Castillo *et al.,* 2005; Castillo *et al.,* 2006; Lansdell *et al.,* 2008). The level of RIC3 expression seems to be crucial; its overexpression usually helps the assembly of nAChRs, but an excessive amount has the opposite effect of retaining nAChRs (mainly in the ER) probably because the excess RIC3 oligomerises itself through its coiled-coil domain (Alexander *et al.,* 2010; Dau *et al.,* 2013). The surface expression of α7 subunits is highly cell type-dependent, and the presence of RIC3 enhances the assembly of α7 nAChRs, their arrival and functional expression at the plasma membrane (Millar, 2008; Gong *et al.,* 2016). GH4C1 rat pituitary and human SH-EP1 human neuroblastoma cell lines, transfected with the α7 subunit alone express very different levels of surface receptors as measured by **α-bungarotoxin** binding. A high binding level is detected in GH4C1 cells whereas no binding is detected in 'non-permissive' SH-EP1 cells (Koperniak *et al.,* 2013). There are different ways of enhancing the expression of nAChRs in 'non-permissive cell lines', including treatment with cycloheximide, lowering the temperature (Schroeder *et al.,* 2003) or co-transfection with RIC3 (Valles *et al.,* 2009). However, many published data demonstrate that there is not a linear correlation between the cell concentration of RIC3 and the plasma membrane expression of α7 nAChRs. The mRNA coding for RIC3 is not abundant in both 'permissive' and 'non-permissive' cell lines, and the concentration of the protein itself is very low (not detectable by commercial antibodies). Moreover, the silencing of endogenous rat RIC3 does not impair the expression of functional α7 nAChRs in GH4C1 cells ('permissive' cell line), thus indicating that other chaperones play a critical role in α7 nAChR assembly and trafficking (Koperniak *et al.,* 2013). In agreement with these data, other investigators have found that RIC3 may be necessary but not sufficient for α7 nAChR expression because, even when co-expressed with RIC3, many α7 subunits are retained in the ER membrane in 'non-permissive' HEK cells (Kuryatov *et al.,* 2013).

Comparison of the interactome of cell lines stably expressing α7 subunits alone or in association with RIC3 has shown that 39 proteins specifically interact with α7 (isolated by means of α-bungarotoxin binding), only when RIC3 is expressed (Mulcahy *et al.,* 2015). These proteins include many of the ER resident proteins involved in protein insertion, folding and glycosylation, the Golgi proteins involved in trafficking and recycling, proteins that belong to protein degradation and turnover and proteins involved in intracellular signalling. RIC3 can act differently on nAChRs other than α7, such as α4β2 (Dau *et al.,* 2013), whereas RIC3 favours both the assembly and trafficking of α7 nAChRs, it had either positive or negative effects on the α4β2 and α3β4 subtypes depending on the experimental system (Halevi *et al.,* 2002, 2003; Williams *et al.,* 2005).

Table 1

Effects of protein chaperones and pharmacological chaperones on different nAChR subtypes

	nAChR subtype and effect	Reference
Protein		
RIC3	↑ arrival and functional expression at the PM of α7 nAChRs.	Millar, 2008 Gong *et al.*, 2016
	Necessary for PM α7nAChR expression in 'non-permissive' cells, dispensable in 'permissive' cells.	Kuryatov *et al.*, 2013
	↑ intracellular retention of α7 nAChRs when present in excess.	Dau *et al.*, 2013
	Allows the interaction of α7 with 39 different proteins involved in intracellular trafficking.	Mulcahy *et al.*, 2015
	↓acute nicotine-induced up-regulation of α4β2 nAChRs.	Dau *et al.*, 2013
	Expression of PD-related RIC3 mutants in PC12 cells ↓ the levels of endogenous α7 in the membrane fraction.	Sudhaman *et al.*, 2016
LY6 prototoxin family		
Lynx1	↓ responses of α4β2 and α7 nAChRs in heterologous systems.	Miwa *et al.*, 1999
	↓ α3β4*-nAChR function in *Xenopus* oocytes.	George *et al.*, 2017
	↑ the rate and extent of desensitization of α4β2 subtype.	Ibañez-Tallon *et al.*, 2002
	By stabilizing the assembly of α4-α4 dimers ↑ formation of low sensitivity stoichiometry $(\alpha4)_3(\beta2)_2$.	Nichols *et al.*, 2014
	Competes with β-amyloid$_{1-42}$ in binding to different nAChR subtypes.	Thomsen *et al.*, 2016
	Acting through nAChRs, it is critical for the loss of synaptic plasticity in the visual cortex that occurs in adulthood.	(Morishita *et al.*, 2010)
Lynx2	Forms a complex with α4β2 nAChRs in HEK-293 cells, and ↓ the amount of the receptor on the PM by 80%, by competing with nicotine-chaperone activity.	Wu *et al.*, 2015
	When co-expressed with α7 ↓ ACh-induced calcium fluxes.	Puddifoot *et al.*, 2015
	Is an allosteric modulator of α7 and α4β2 nAChRs.	Arvaniti *et al.*, 2016
Ly6h	↓ the trafficking of α7 to the PM and ↓ agonist receptor-induced currents.	Puddifoot *et al.*, 2015
	↑ the epibatidine sensitivity of the α4β2 receptor.	Puddifoot *et al.*, 2015
Ly6g6e	↓ α4β2 desensitization upon ACh stimulation by acting at the PM.	Wu *et al.*, 2015
Lypd6	↓ signalling downstream of α3β4 nAChR activation.	Arvaniti *et al.*, 2016
	↓ α7-induced currents in hippocampal neurons.	Arvaniti *et al.*, 2016
	Interacts with α7 at the orthosteric site.	Arvaniti *et al.*, 2016
Lypd6b	↑ the sensitivity to nicotine and ↓ ACh-induced whole-cell currents of the $(\alpha3)_3(\beta4)_2$ subtype.	Ochoa *et al.*, 2016
	Negatively regulates α3β4α5D but does not change the function of α3β4α5N.	Ochoa *et al.*, 2016
PSCA	↓ the activation of α7 by interfering with the α7-mediated increase in intracellular calcium concentration.	Hruska *et al.*, 2009
SLURP-1	Is an allosteric antagonist of α7 and ↓ its response to ACh by non-competitive inhibition.	Lyukmanova *et al.*, 2016a
SLURP-2	Binds to α3-containing nAChRs by competing with ACh at the binding site, thus delaying keratinocyte differentiation and preventing their apoptosis. ↓ ACh -evoked currents of oocyte expressed α4β2 and α3β2-nAChRs. ↑or ↓ (depending on the concentration) ACh -evoked currents of the α7 subtye.	Arredondo *et al.*, 2006 Lyukmanova *et al.*, 2016b Lyukmanova *et al.*, 2016b
NACHO	When co-transfected with α7, ↑ ACh-evoked currents and ↑ the amount of receptor at the PM.	Gu *et al.*, 2016
	Works synergistically with RIC3 increasing the ACh-evoked α7-mediated currents.	Gu *et al.*, 2016
	↑ α4β2 currents in heterologous systems.	Gu *et al.*, 2016
	Its knockdown ↓α4β2-mediated currents in hippocampal neurons.	Gu *et al.*, 2016

continues

Table 1 (Continued)

	nAChR subtype and effect	Reference
UBXN2A	Interacts with α3 and α4 nAChR subunits.	Rezvani *et al.*, 2009
	↑ the amount of α3β2 receptors at the PM (equal increase in the total amount of the receptor in the cells) of PC12 cells.	Rezvani *et al.*, 2009
	Interferes with the ubiquitination of α3 subunits, thus protecting them from proteasomal degradation.	Teng *et al.*, 2015
Pharmacological chaperones		
Target specific		
Nicotine	↑ *in vivo* α4 and β2 subunits at protein level and $(\alpha4)_2(\beta2)_3$ stoichiometry in the cortex but not in the thalamus.	Fasoli *et al.*, 2016
	↑ α3β4 receptors at the PM in heterologous system.	Mazzo *et al.*, 2013
	↑ α4-containing nAChRs in GABAergic neurons.	Henderson *et al.*, 2016
Cotinine	↑ the trafficking of α4β2 receptors to the PM (1 μM)	Fox *et al.*, 2015
	↑ receptors with the highly sensitive stoichiometry $(\alpha4)_2(\beta2)_3$.	Fox *et al.*, 2015
Menthol	↑ α4- and α6-containing nAChRs in murine midbrain dopaminergic neurons.	Henderson *et al.*, 2016
	↑ α4β2 and α6β2β3 receptors in N2A cells.	Henderson *et al.*, 2016
Non-target-specific		
PBA and VPA	↑ the assembly of α7 pentamers without altering the level of α7 subunits.	Kuryatov *et al.*, 2013

Abbreviations: ↑, increases; ↓, decreases; PM, plasma membrane.

Interestingly, acute nicotine treatment for 30 min up-regulated α4β2 nAChRs and favoured their assembly, but this up-regulation was prevented by RIC3 co-transfection (Dau *et al.*, 2013). A whole-exome sequencing analysis made in order to identify the putative causal variant in an Indian family with Parkinson's disease (PD), identified a heterozygous mutation (c.169C > A, p.P57T) in RIC3 in nine members that segregates with the disease, and another heterozygous mutation (c.502G > C, p.V168L) in the same protein in an unrelated PD patient. The expression of the two RIC3 mutants in PC12 cells reduces the levels of endogenous α7 in the membrane fraction. This is the first time that it has been suggested that mutations in RIC3 causing defects in nAChR density may contribute to the pathogenesis of PD (Sudhaman *et al.*, 2016).

LY6 prototoxin family

The LY6 prototoxin family consists of small modulatory proteins that can interact with, numerous targets, including nAChRs and muscarinic ACh receptors (see Tsetlin, 2015; Lyukmanova *et al.*, 2016b). We here only report the effect of these proteins on the modulation of nAChR signalling.

Proteins of this family are characterized by five disulfide bonds and three "three finger" motifs: a three dimensional structure that is similar to that found in snake venom neurotoxins and facilitates the binding with partner proteins. Some of these proteins are secreted as water-soluble proteins (such as secreted mammalian Ly6/urokinase plasminogen-type activator receptor-related protein-1 and -2 (SLURP-1 and SLURP-2)), but the majority (including Ly6/neurotoxin1 (Lynx1), Ly6/neurotoxin 2 (Lynx2), Ly6h, Ly6g6e, Lypd6, Lypd6b and prostate stem cell antigen (PSCA) that are discussed below) are bound to the membranes by a glycosylphosphatidylinositol (GPI) anchor (Adermann *et al.*, 1999; Miwa *et al.*, 2012; Lyukmanova *et al.*, 2013; Tsetlin, 2015; Loughner *et al.*, 2016).

The first member of the LY6 family to be identified was Lynx1, which can form a stable complex and negatively regulates the responses of α4β2 and α7 nAChRs in heterologous systems (Miwa *et al.*, 1999), enhances the rate and extent of desensitization of α4β2 nAChRs thus acting as a molecular brake on nAChR function (Ibanez-Tallon *et al.*, 2002). *In vivo,* Lynx1 is enriched on the plasma membrane and synapses of neurons (Thomsen *et al.*, 2014). During postnatal development and adulthood, this protein affects neuronal plasticity by limiting dentritic spine turnover in the visual cortex (Morishita *et al.*, 2010; Sajo *et al.*, 2016), and memory (Miwa *et al.*, 2006).

Although Lynx1 has always been considered a plasma membrane regulator of nAChRs, Nichols *et al.* (2014) have demonstrated that it also acts on the α4β2 subtype through an intracellular mechanism within the ER, where it favours the formation of low sensitivity stoichiometry $(\alpha4)_3(\beta2)_2$ receptor because it stabilizes the assembly of α4-α4 dimers (Nichols *et al.*, 2014). Recently, in *Xenopus* oocytes, Lynx1 reduced function of α3- and β4- containing nAChRs (α3β4*-nAChRs) (George *et al.*, 2017). Lynx1 has also been described as a competitor of oligomeric β-amyloid$_{1-42}$ in binding to different nAChR subtypes, thus suggesting its possible role in Alzheimer's disease (Thomsen *et al.*, 2016).

Lynx1 is expressed in normal and neoplastic lung tissue, where it limits the ability of chronic nicotine exposure to increase nAChR levels. Lynx1 levels are lower in lung cancers than in the adjacent normal lung. The knockdown of Lynx1 by siRNAs increases the growth of lung cancer cells, whereas the expression of Lynx1 in lung cancer cells decreased cell

proliferation, suggesting that it might regulate lung cancer growth (Fu *et al.,* 2015).

Lynx2, a homologue of Lynx1, also forms a complex with α4β2 nAChRs in HEK-293 cells, and a biotinylation assay has shown that it acts intracellularly on the trafficking of the receptor to the plasma membrane and, by competing with nicotine, blocks the chaperone activity of nicotine and, consequently, reduces the amount of the receptor on the surface by 80% (Wu *et al.,* 2015). Lynx2 also forms stable complexes with the α7-subunits and regulates the levels of nAChRs at the cell surface. Lynx2 coexpression in α7 nAChR transfected HEKtsa cells reduces the surface expression of α7 receptors to approximately the same extent (50%) as it reduces the functional activity of α7 receptors as measured by calcium fluxes (Puddifoot *et al.,* 2015). Like Lynx2, Ly6h decreases the trafficking of the α7 nicotinic receptors to the plasma membrane and reduces the receptor-induced currents by 75% (Puddifoot *et al.,* 2015). However, unlike Lynx2, it potentiates the α4β2 receptor by increasing its sensitivity to epibatidine. In this case, it does not interact directly with the receptor but it is found in the same plasma membrane complex. Moreover, Ly6g6e does not influence the trafficking of the receptor towards the plasma membrane, but acts on the receptor at the cell surface, where it slows down its desensitization upon ACh stimulation (Wu *et al.,* 2015).

Arvaniti *et al.* (2016) have described the interaction of native human nAChRs with Lypd6. In human cortex, it interacts with α3, α4, α5, α6, α7, β2 and β4 nicotinic subunits and, interestingly, blocks signalling downstream of α3β4 nAChR activation. In hippocampal neurons it diminishes α7-induced currents. Recently Lypd6b was shown to exert different effects on the α3β4 receptors in *Gallus* parasympathetic neurons, depending on their stoichiometry. It enhanced the sensitivity of the $(\alpha3)_3(\beta4)_2$ subtype to nicotine and diminished ACh-induced whole-cell currents, but did not have any effect on the other stoichiometry $(\alpha3)_2(\beta4)_3$. Interestingly, Lypd6b also acts differently on α3β4α5 receptors depending on the α5 variant present in the pentamer. It negatively regulates α3β4α5D containing the common variant, but does not change the function of the receptor containing the variant of α5 (N398D) associated with an increased risk of developing lung cancer and nicotine addiction (Ochoa *et al.,* 2016). In the same paper, the authors showed that Lypd6b does not affect the properties of α7 nicotinic receptors, thus suggesting that the modulation of nicotinic receptors by Ly6 prototoxins is highly subtype- and stoichiometry-selective.

The Ly6 prototoxin family also includes PSCA, a molecule originally identified as a prostate cancer marker. It is highly expressed in the nervous system, and its expression correlates with that of α7 nicotinic receptors. In ciliary ganglion neurons, PSCA suppresses the activation of α7-containing receptors because it interferes with the α7-mediated increase of intracellular calcium concentration (Hruska *et al.,* 2009).

Two components of the Ly6 family that are secreted and not linked to the membranes by GPI anchors are SLURP-1 and SLURP-2. SLURP-1 is an autocrine/paracrine hormone that regulates growth and differentiation of keratinocytes, and controls inflammation and malignant cell transformation. Point mutations in the *SLURP-1* gene cause the autosomal inflammation skin disease Mal de Meleda (Chimienti *et al.,* 2003). SLURP-1 acts as an allosteric antagonist of α7 receptors and significantly non-competitively inhibits the response of α7 receptors to ACh (Lyukmanova *et al.,* 2016a). Moreover, it is expressed in the spinal cord, where it seems to be involved in cholinergic pain regulation (Moriwaki *et al.,* 2009), and in murine ciliated bronchial epithelial cells where it can act, together with ACh, in an autocrine manner to maintain cell homeostasis (Horiguchi *et al.,* 2009).

SLURP-2 regulates the growth and differentiation of epithelial cells. Affinity purification of cortical extracts has revealed that SLURP-2 can interact with α3, 4, α5, α7, β2, and β4 nAChR subunits and has a broad pharmacological profile. In particular, it inhibits ACh -evoked currents of α4β2 and α3β2 -nAChRs in oocytes and can enhance or inhibit (at higher concentrations) ACh-evoked currents of the α7 subtype (Lyukmanova *et al.,* 2016b).

Arredondo *et al.,* 2006 showed that SLURP-2 binds to α3-containing nAChRs by competing with ACh at the binding site, and this interaction delays keratinocyte differentiation and prevents their apoptosis.

Lynx1 and Lynx2 are both considered allosteric modulators of nAChRs because they do not compete with agonist and antagonist orthosteric sites in α7 and α4β2 receptors whereas Lypd6 interacts with α7 at the orthosteric site, thus suggesting its possible role as a nAChR antagonist (Arvaniti *et al.,* 2016).

In conclusion the Ly6 proteins are a large family of proteins present in organisms from *Drosophila* to humans. Some members of this family have as target the nAChRs, but the effects and type of interactions depend on the Ly6 protein and nAChR subtype.

NACHO

A high-throughput screening, has very recently identified NACHO, a TM protein resident in the ER and playing an important role in folding and trafficking of α7 nAChRs (Gu *et al.,* 2016). When co-transfected with α7 receptors, it greatly increased ACh-evoked currents, (which were not recorded in HEK-293 cells expressing α7 alone), and enhanced the amount of receptor on the cell surface. Interestingly, NACHO is only expressed in specific areas of the brain such as hippocampus, cerebral cortex, and olfactory bulb where it is enriched in neurons and colocalises with the ER protein PDI but it is not expressed at the plasma membrane. The genetic deletion of NACHO abolishes surface functional α7 receptors and ^{125}I-α-bungarotoxin (a selective α7 receptor ligand) binding in hippocampal neurons. Gu *et al.* (2016) demonstrated that NACHO and α7 subunits do not form a stable complex, because they do not directly interact with each other, thus suggesting that NACHO is not a surface auxiliary subunit of the nAChRs, but rather an ER-chaperone for α7 subunits. NACHO can work synergistically with RIC3: the effect of RIC3 alone on α7 activity was much less evident than the effect of NACHO, but the co-transfection of RIC3 and NACHO led to the largest increase in ACh-evoked α7-mediated currents, suggesting that the two chaperones act through two different mechanisms (Gu *et al.,* 2016). NACHO also increased α4β2 currents in heterologous systems, and its knockdown significantly

decreased, but did not eliminate, α4β2-mediated currents in hippocampal neurons (Gu *et al.,* 2016).

UBXN2A

UBXN2A (also known as UBXD4) is a cytosolic protein that directly interacts with α3 and α4 nAChR subunits. It was found by means of a yeast-two hybrid screening in which the intracellular loop and the flanking M3 and M4 TM regions of the α3, α4, α7, β2 and β4 nicotinic subunits were used as bait (Rezvani *et al.,* 2009). UBXN2A colocalises with α3 in puncta around the nucleus. In PC12 cells, it specifically increases the amount of α3β2 receptors at the plasma membrane by 40%, which is due to an equal increase in the total amount of the receptor in the cells. During the maturation of α3 subunits, UBXN2A seems to interfere with their degradation *via* proteasome, thus reducing ubiquitination, probably as a result of a direct interaction (Rezvani *et al.,* 2009). More recently, the E3 ubiquitin ligase CHIP was found to participate in the ubiquitination process of α3 nAChR subunits. This mechanism seems to be specific, as CHIP does not change β2 subunit levels, but it remains to be clarified whether it acts on the other nAChR subunits. Interestingly, UBXN2A can directly interact with CHIP and, in this way, interferes with the CHIP-mediated ubiquitination of α3 subunits, thus protecting them from proteasomal degradation. Furthermore, this regulation of α3 degradation occurred at ERAD level, because UBXN2A is a cofactor of p97 (a complex responsible for the retro-translocation of misfolded proteins) and is present in a complex together with α3 subunits, CHIP and p97 (Teng *et al.,* 2015).

Pharmacological chaperones

Target specific

Nicotine. Radioligand binding studies have consistently shown that chronic nicotine exposure increases high affinity agonist binding sites in animal brain (Marks *et al.,* 2011) as well as in cells expressing nAChRs, including primary neuronal culture (Govind *et al.,* 2012). Up-regulation of nAChRs is also found in the *post mortem* brains of smokers (see Colombo *et al.,* 2013). The extent and dependence on nicotine concentration of this process, termed up-regulation, vary with the nAChR subtype and experimental systems and is the result of nicotine acting at several steps of nAChR biogenesis and trafficking: nicotine can stimulate subunit expression, enhance the assembly and folding of the pentamers, and/or favour exit from the ER membrane and arrival at the plasma membrane (see Colombo *et al.,* 2013).

Due to its lipophilicity, nicotine can easily penetrate the blood–brain barrier and concentrate in the brain where it can have many psychoactive effects. The half-life of nicotine in brain tissue is much longer than that of ACh because acetylcholinesterase does not hydrolyse nicotine, which is only metabolized by liver enzymes and nicotine that passes the plasma membrane can persist even for days inside cells, where it interacts with nAChRs. Nicotine is thus the best known pharmacological chaperone for nAChRs and acts on α4β2, α7-, α3- and α6-containing receptors, and exhaustive reviews have been recently published (Colombo *et al.,* 2013; Henderson *et al.,* 2014).

We have recently found *in vivo* that prolonged exposure to nicotine increases the number of α4β2 nAChR binding sites, as a result of an increase in α4 and β2 subunit protein levels, without any change in mRNA levels. This up-regulation in the brain is not uniform and exposure to chronic (14 days) nicotine differently affects the expression of α4β2 nAChRs in the cortex and thalamus. *In vivo* the α4 and β2 subunits under control conditions, are only present in assembled α4β2 receptors in the cortex and thalamus and in this latter region a significantly higher proportion of receptors have the $(\alpha4\beta2)_2\beta2$ stoichiometry. The chronic *in vivo* administration of nicotine at a concentration that is in the range of that found in the blood of heavy smokers, more markedly up-regulated α4β2 nAChRs in the cortex than those in the thalamus (Fasoli *et al.,* 2016). This change in stoichiometry is very important because it can greatly influence the physiological response of the cells to nicotine and ACh.

In vivo studies of animal models of chronic nicotine exposure have failed to reveal α3β4 up-regulation (Nguyen *et al.,* 2003), but we have found that chronic treatment with 100 μM–1 mM nicotine increases the surface expression of these receptors more than fivefold in heterologous systems (Mazzo *et al.,* 2013). This up-regulation is due to a nicotine-induced increase in the number of receptors with $(\alpha3)_2(\beta4)_3$ stoichiometry. The M3-M4 intracellular loop of the β4 subunit contains an export motif (LXM) and receptors with the $(\alpha3)_2(\beta4)_3$ stoichiometry, which have three LXM motifs, are more efficiently recruited to ER exit sites and delivered to the plasma membrane (Mazzo *et al.,* 2013). This is important because transcripts of the α3 and β4 are present in bronchiolar epithelial cells, as well as in lung cell lines and in lung tumours (Improgo *et al.,* 2010a). Although the nicotine concentration necessary to up-regulate α3β4 receptors is much higher than that present in the serum of smokers, it must be noted that the nicotine concentration in the sputum of human subjects who have smoked one cigarette (Clunes *et al.,* 2008) is in the same range. This result may suggest that nicotine may enhance the proliferative capacity of α3β4 expressing cancer cells not only by its pharmacological action on nAChRs, but also by increasing the concentration of these receptors on the cell surface.

Cotinine. Cotinine, a compound that has a longer half-life (24 h) than nicotine (2 h), is the primary metabolite of nicotine, (in humans, 80% of nicotine is metabolized to cotinine). It is a partial agonist of nAChRs and able to cross the blood–brain barrier. Fox *et al.* (2015) have used super-ecliptic pHluorin-based fluorescence imaging, to show that at low concentrations (up to 1 μM) favour the trafficking of α4β2 receptors to the plasma membrane, whereas higher concentrations (more than 5 μM) do not increase the presence of the receptor on the cell surface. No effects were observed if the pentamer includes the α5 subunit (both the common variant and the D398N polymorphic variant). Using single molecule analysis of receptor subunits, the specific stoichiometry of the receptors in cell membrane-derived vesicles was analysed after treatment with 500 nM nicotine or 1 μM cotinine. In both cases, the treatment

changed the stoichiometric distribution in favour of receptors with the high sensitivity stoichiometry $(\alpha4)_2(\beta2)_3$. On the contrary, cotinine does increase the expression and/or trafficking of the α6β2, α3β4 and α3β4α5 (D398 or N398) nAChRs (Fox *et al.,* 2015).

Menthol. **Menthol** is a flavouring present in almost all cigarettes. In 2013, Brody *et al.,* showed that smokers of menthol cigarettes were subject to greater up-regulation of β2-containing nAChRs, in comparison with non-menthol smokers. Henderson *et al.* (2016) demonstrated that a 10 day treatment with menthol alone up-regulated α4- and α6-containing nAChRs in murine midbrain dopaminergic, but not GABAergic neurons, thus complementing the effect of nicotine, which up-regulated α4-containing nAChRs in GABAergic but not dopaminergic neurons. Treatment with 500 nM menthol for 24 h, induced a twofold and threefold increase in N2A cells expressing α4β2 and α6β2β3 receptors respectively. Interestingly, menthol and nicotine have a similar mechanism of action: (i) the up-regulation seems to be post-translational as there are no differences in α4 and β2 subunit transcripts in menthol-treated cells; (ii) menthol also requires the cycling of nAChRs between the Golgi and ER in order to increase the number of receptors; and (iii) the increase in trafficking towards the plasma membrane is paralleled by an increase in ER exit sites (Henderson *et al.,* 2016).

Non-target-specific

Chronic treatment with chemical chaperones (4-phenylbutyric acid (PBA) or **valproic acid (VPA)** enhances the assembly of α7 pentamers, without altering the level of α7 subunits independently of the chaperone RIC3. They also favour the assembly of nAChR subunits still present in the ER of HEK-293 cells after the co-expression of α7 and RIC3 (Kuryatov *et al.,* 2013). PBA can act directly on α7 subunits to promote their renaturation (Perlmutter, 2002). As both PBA and VPA can alter transcription (Butler and Bates, 2006), they may induce the expression of chaperone proteins, which contribute to the expression of α7 proteins. VPA and PBA are potent up-regulators of the endogenous α7 subunits present in the SH-SY5Y neuroblastoma cell line and, interestingly, VPA can also act on primary cultures of hippocampal neurons (Kuryatov *et al.,* 2013).

Conclusions

Many neurological and psychiatric disorders are due to low or incorrect expression of nAChRs, which together with the fact that nAChRs play an important role in neurodegenerative diseases, such as Alzheimer's disease and PD, indicates that increasing localized nAChR expression may be therapeutically valuable (Hurst *et al.,* 2013; Lombardo and Maskos, 2015).

The number and/or function of nAChRs can be increased using nAChR-specific compounds but, as chaperones can also modulate the surface expression and function of nAChRs, targeting them with low MW compounds may be an alternative or complementary approach.

By increasing surface receptors the chaperone activity of nicotine (or other nicotinic ligands) may contribute to its addictive properties, its therapeutic effect on PD and its potential to treat epilepsies associated with nAChR mutants (Hurst *et al.,* 2013; Srinivasan *et al.,* 2014). However, in order to pursue further this possibility, it is important to clarify the pathways regulating chaperone protein expression and their effect on nAChRs, and the intracellular pathways activated by surface receptors. On the other hand, decreasing the surface number of expressed nAChRs in some pathological tissues such as cancer, may be extremely valuable as a complementary therapy to decrease cell proliferation without affecting cholinergic mechanisms of neuronal or cell communication.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.,* 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.,* 2015).

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Conflict of interest

The authors declare no conflicts of interest.

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