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Auxiliary protein and chaperone regulation of neuronal nicotinic receptor subtype expression and function

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of pentameric, ligand-gated ion channels that are located on the surface of neurons and non-neuronal cells and have multiple physiological and pathophysiological functions. In order to reach the cell surface, many nAChR subtypes require the help of chaperone and/or auxiliary/accessory proteins for their assembly, trafficking, pharmacological modulation, and normal functioning in vivo. The use of powerful genome-wide cDNA screening has led to the identification and characterisation of the molecules and mechanisms that participate in the assembly and trafficking of receptor subtypes, including chaperone and auxiliary or accessory proteins. The aim of this review is to describe the latest findings concerning nAChR chaperones and auxiliary proteins and pharmacological chaperones, and how some of them control receptor biogenesis or regulate channel activation and pharmacology. Some auxiliary proteins are subtype selective, some regulate various subtypes, and some not only modulate nAChRs but also target other receptors and signalling pathways. We also discuss how changes in auxiliary proteins may be involved in nAChR dysfunctions.

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of pentameric ion channels that mediate excitatory transmission in the central and peripheral nervous system and non-neural cells. By mediating cation flux across cell membranes, they modulate neuronal excitability and the release of a variety of neurotransmitters in order to influence multiple physiological and behavioural processes [1–3].

Mammalian nAChRs are assembled from a pool of 12 different subunits (α2-α7, α9, α10, β2-β4) with cell type-specific expression patterns. These homopentameric and heteropentameric combinations give rise to receptor subtypes that have different localisations and unique structural, functional, and pharmacological properties [4]. The stoichiometry and pentameric arrangement of the subunits are fundamental determinants of receptor signalling because the binding sites of agonists, antagonists and allosteric modulators are located on the interfaces of the subunits themselves. The different subunit arrangements within a receptor pentamer can therefore lead to receptors with different functional and pharmacological profiles.

2. nAChR structure and intracellular trafficking

The two main classes of nAChR subtypes are homomeric or heteromeric alpha-bungarotoxin(αBgtx)-sensitive receptors consisting of α7, α7β2, α9 and α9α10 subunits, and heteromeric αBgtx-insensitive receptors consisting of combinations of α (α2, α3, α4, α5, α6) and β (β2, β3, β4) subunits. Homomeric α7 nAChRs bind acetylcholine (ACh) or nicotine at their five subunit interfaces; heteromeric nAChRs have two conventional agonist binding sites at the interfaces between two pairs of "structural" α and β subunits, and a fifth "accessory" subunit that generally does not participate in conventional agonist binding, although a third unorthodox ACh-binding site has recently been described at

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specific interfaces of the accessory subunit of certain nAChR subtypes [5].

Recent studies have identified the 3D structures of α4β2 [6] and α7 [7] nAChRs (the two most widely expressed brain subtypes), and the α3β4 nAChRs [8] that are the most widely expressed subtype in the autonomic nervous system. They have also further confirmed that heteromeric and homomeric subtypes have a common structure of five subunits arranged pseudo-symmetrically around a central axis [9]. Each subunit has a common transmembrane topology consisting of a large N-terminal hydrophilic extracellular domain (ECD), four transmembrane domains (TMDs), a major intracellular domain (ICD) between TM3 and TM4, and a short extracellular C-terminal ECD [6]. The TM2 helices are located proximally to the central axis and line the channel pore, whereas the TM4 helices are the most distal and lipid-exposed, and may act as lipid sensors [10]. A structural transition zone between the N-terminal ECD and TMD (known as the coupling region) forms an essential functional link between agonist binding and channel opening. The ICD carries two α-helix sequences (a short MX connected by a short loop to TM3, and the MA that merges with TM4), and a disordered loop [11]. The N-terminal and TM domains of the different subunits are well conserved, but the TM3-TM4 cytoplasmic loop varies in length and amino acid composition [12]. The ICD loop contains many sequences that are crucial for ensuring that receptors are exported from the endoplasmic reticulum (ER) and trafficked to the plasma membrane, various phosphorylation sites [13], and sequences that are important for the neuronal localisation and/or targeting of the different subtypes and interactions with other intracellular proteins such as chaperones and G proteins [14,15].

In order to function, nAChRs have to reach the cell surface, which they do by undergoing a complex process involving the assembly of the newly synthesised subunits and post-translational processing in the ER. Transport-competent nAChRs are subsequently recruited at ER exit sites before passing through Coat Protein Complex II (COPII) vesicles to the Golgi apparatus and then to the plasma membrane, where they can be internalised in the endosomal compartment before being targeted to the lysosomal compartment. All of these passages involve the precise coordination of interactions between the nAChRs and numerous resident proteins, including molecular chaperones that control correct receptor folding, glycosylation, maturation and forward trafficking, as well as the degradation of misfolded receptors [16,17].

Multi-subunit, multi-span nAChRs are integral membrane proteins whose folding and assembly is very inefficient, and only a small proportion of the subunits form functional pentamers. An important role in this process is played by chaperones, which may be endogenous proteins or target-specific and non-target-specific pharmacological compounds: chaperone proteins assist the subunits in taking on the correct folded conformation and eliminate misfolded or unassembled proteins by means of ER-associated degradation (ERAD), whereas target-specific and non-target-specific pharmacological compounds are generally cell permeable compounds that facilitate biogenesis and/or prevent or correct subunit misfolding, and enhance the assembly of nAChRs in the ER and through trafficking through the Golgi to the plasma membrane.

Over the last ten years, significant progress has been made in identifying the proteins that interact with ionotropic nAChRs. In particular, the use of powerful genome-wide cDNA screening (reviewed in [18]) has allowed the characterisation of a number of molecules and mechanisms involved in the assembly and trafficking of receptor subtypes, including auxiliary or accessory ion channel proteins that do not directly participate in ion conduction, but which regulate receptor trafficking and/or modulate their functional and pharmacological properties, and are necessary for the normal functioning of receptor subtype in vivo.

Among the modulatory proteins, the lymphocyte antigen-6 (Ly6) prototoxin family consists of small proteins with a single domain characterised by a small, three-fingered motif and disulphide bonds, and are structurally similar to the snake venom α-neurotoxins [19–21] capable of interacting with numerous nAChR subtypes [22,23]. These proteins, which are either secreted or membrane anchored through the glycosylphosphatidyl-inositol (GPI) lipid moiety [21], are endogenous allosteric modulators that associate with nAChRs, alter the ability of ACh to induce their open conformational state [24], and regulate their properties during the different stages of biogenesis. However, as the characteristics of the Ly6 prototoxin family are thoroughly reviewed in this special issue of Pharmacological Research [22], we will here restrict ourselves to discussing their role in diseases (see Section 5).

3. Assembly and trafficking of nAChR subtypes

Our initial understanding of these processes largely came from studies of muscle-type ACh receptors and the neuronal α4β2 subtype, the most widely expressed in brain (reviewed in [16,17]). They involve subunit biogenesis on rough ER, which requires cleavage of the signal peptide, the oxidation of disulphide bonds, and the N-glycosylation of some of the residues with chaperone proteins, oligomerisation in the ER lumen, and transport through the Golgi to the cell surface. Chaperone proteins such as BiP, ERp57, and calnexin guarantee close quality control by assisting the subunits in taking on the correct folded conformation, eliminating misfolded or unassembled proteins by means of ERAD, and modulating the trafficking and maturation of nAChRs [25]. Overviews of the originally known chaperones, adaptor proteins, and signals involved in these mechanisms are available in [17,23].

The efficiency of nAChR assembly and trafficking varies widely depending on the subtype and the cell type in which they are expressed. This has led to the definition of 'permissive' cells' in which nAChRs are expressed, assembled and localised on the plasma membrane, and 'non-permissive' cells' in which the nAChR subunits are mainly retained in the ER membrane: for example, the expression of the α7 or α6β3β2 subtypes in HEK cells leads to very low levels of expression of non-functional subtypes on the plasma membrane.

The function, metabolism and intracellular trafficking of nAChRs are regulated by means of physically associated proteins that may be permanent or transient depending on the duration of their interactions. The wide range of transiently associated proteins reversibly interact with nAChRs and include enzymes that catalyse post-transcriptional and post-translational modifications, and chaperones that participate in the maturation of functional receptors and their cell surface expression [13].

What follows is a description of the main interactions between accessory proteins and receptor subunits, and their role in modulating receptor functions and trafficking.

3.1. α7 receptors

Mammalian α7 receptors bind the antagonist αBgtx with high affinity, are calcium permeable, and are mainly expressed as homopentamers in the brain, although heteromeric α7β2 subtypes have also been reported [26]. They are more broadly distributed than the other nAChR subtypes as they are expressed not only in neurons, but also in astrocytes, microglia, autonomic ganglia, pulmonary epithelial cells, lymphoid cells, and endothelial cells [1].

3.1.1. NACHO

Gu *et al.* [27] have recently used genomic cDNA screening to show that the product of the *TMEM35A* gene that they called NACHO (a small four-pass transmembrane protein that is enriched in the neuronal ER) can mediate the functional reconstitution of α7 receptors in non-neuronal cell lines and is essential for the assembly of α7 receptors in neurons. The genetic deletion of NACHO abolishes functional α7 receptors and the cell surface binding of ^{125}I-αBgtx throughout rodent brain [27–29].

Kweon *et al.* [30] used molecular and neurobiological approaches to identify the mechanisms underlying the effects of NACHO on α7 receptors. Their proteomic studies showed that NACHO and α7 subunits do not directly interact with each other, but that NACHO co-purifies

with ribophorin-1 and ribophorin-2 (components of the N-glycan oligosaccharyltransferase complex that links high levels of mannose sugars to asparagine (N) residues of nascent polypeptides in the ER) and with calnexin, an ER chaperone that retains unfolded N-linked glycoproteins. By constructing chimeras of α7 and 5-HT3A receptors (which belong to the same Cys-loop receptor super family as nAChRs), they found that defective α7 assembly and surface expression involves an impairment in the assembly of the first and second TM domains of α7 that is repaired by NACHO. In particular, they determined that the amino acids L264 and G265 in the second TM domain of α7 receptors are critical for NACHO-mediated assembly and trafficking, which other chimeras revealed involves the α7 extra-cellular N terminus region that needs to be correctly N glycosylated at N-46, N-90 and N-233, whereas mutations of the ACh-binding site on the α7 subunit does not affect NACHO chaperone activity.

These studies led to a proposed model in which NACHO engages co-translationally with nascent subunits by interacting with ribophorin-1 and ribophorin-2. Calnexin may assist the folding of the first two α7 TM domains by engaging the monoglycosylated N-linked glycans on α7 either by means of additional direct interactions or by recruiting downstream chaperones that have not yet been identified.

3.1.2. RIC-3

After their folding and disulphide bond formation, α7 subunits dissociate from calnexin and interact with Resistance to inhibitors of cholinesterase 3 (RIC-3) [31,32], which helps to complete their assembly in the form of pentameric receptors. Wang *et al.* [33] suggests that RIC-3 accompanies α7 nAChRs to the cell surface [5], but it has also been suggested that it remains in the ER or may only reach the Golgi [31,33,34]. In any case, these later steps are also facilitated by Bcl-2 family proteins [32].

RIC-3 is an ER membrane protein [35] that has chaperone activity on the assembly of nAChRs and can moderately enhance the expression of α7 and some other mammalian nAChRs [31,36]. However, it is neither necessary [29,37]) nor sufficient [38] for robust α7 receptor assembly or function. When NACHO and RIC-3 are both co-transfected, they synergistically increase ACh-evoked currents from α7 receptors, thus suggesting that they work through distinct mechanisms [27]. It was originally demonstrated that, on wild-type (WT) α7 receptors, RIC-3 alone had minimal effects on surface localisation but greatly increased NACHO-mediated cell surface α-Bgtx staining. Kweon *et al.* [30] studied the effects of RIC-3 and NACHO in an α7 chimera obtained by swapping the large intra-cellular loop from α3 to α7, and found that the chimera retained full NACHO activity but was almost devoid of the effects of RIC-3, thus indicating that the large intracellular loop is necessary for the effects of RIC-3 but not for those of NACHO.

3.1.3. Bcl2 proteins

By screening a genome-wide cDNA library in order to determine whether other proteins increase α7 nAChR expression in the presence of NACHO, Dawe *et al.* [32] found that a number of anti-apoptotic Bcl-2 family proteins [39] further upregulate α7 receptor assembly and cell surface functional expression, and determined that, albeit to a lesser extent, this upregulation is also present in the absence of NACHO. They also identified a BH3-like motif in the MA portion of the TM3-TM4 intracellular loop of α7 receptors that is necessary for α7 regulation by anti-apoptotic Bcl2 proteins. Mutation of this BH3 motif eliminates Bcl-2-mediated α7 upregulation, but has no effect on NACHO or RIC-3 upregulation, thus suggesting Bcl-2 proteins make use of unique structural pathways to enhance assembly.

As discussed in a number of reviews [29,40,41], αBgtx-binding α7 receptors are not only expressed in neurons, but also in non-neuronal cells such as glia or immune cells such as macrophages and lymphocytes. Deshpande *et al.* [29] have shown that the presence or absence of NACHO in various cell lines does not necessarily correlate with the cells' ability to support surface α7 nAChR expression, and that primary mouse peritoneal macrophages do not require NACHO to express surface α7 nAChR localised by ^{125}I-αBgtx binding. These findings suggest that there are additional factors regulating surface α7 nAChR expression on non-neuronal cells.

Fig. 1A and Table 1 briefly summarise the chaperone and accessory/auxiliary proteins involved in the assembly and trafficking of α7 receptors.

3.2. α6 receptors

Early in situ hybridisation studies revealed the presence of α6 and β3 subunit mRNA in dopaminergic cells of the substantia nigra (SN), ventral tegmental area (VTA), and retina [42]. Biochemical studies, ligand binding and functional assays, immunopurification procedures using subunit-specific antibodies, and studies of α6 knockout mice have shown that the striatum (a region that receives nerve terminals from mid-brain dopaminergic cells) and the superior colliculus and lateral geniculate nucleus (two retinal target regions) express α6β2* receptors (* indicates the possible presence of additional subunits): α6β2β3, α4α6β2β3, and a small number of α6β2 subtypes [43]. Although the presence of the β3 subunit is not essential for the expression of high-affinity α-conotoxin MII binding to α6* receptors [44,45], it is important for the correct assembly, stability and/or transport of α6* nAChRs in dopaminergic neurons. These studies indicate that there is a sub-family of closely related nAChR subtypes containing the α6 subunit, the members of which vary in terms of the other subunits in the pentameric complex. As all of these subtypes bind ^{3}H-epibatine, a nicotinic ligand that interacts with the interface of assembled nAChR subunits and quantifies the assembled receptors, separate in vitro expression systems have been created for each putative α6* receptor combination in order to fully explore differences in their pharmacology. However, unlike in the case of α4β2 receptors, it has been difficult to generate heterologous cell lines that express α6* receptors [46].

3.2.1. NACHO

It has recently been found in heterologous cells that, by mediating the early steps in the assembly of immature intra-cellular nAChR complexes, NACHO, promotes the surface expression of different classes of functional nAChRs (including α4β2, α3β2, and α3β4 receptors), but not that of α6-containing receptors in HEK cells transfected with α6, β2, and β3 subunits [28]. However, NACHO does enhance the binding of ^{3}H-epibatidine to the membranes of cells transfected with α6 and β2 or α6, β2 and β3 subunits (binding that was competed for by α-conotoxin MII, the selective ligand of α6-containing receptor), thus indicating that NACHO can enhance intracellular receptor assembly, but is not sufficient for the expression of surface receptors [28].

3.2.2. BARP, LAMP5 and SULT2B1

Gu *et al.* [47] have used the high-throughput genomic screening of cDNA libraries to identify possible proteins that can enable the functional expression of recombinant α6β2β3 receptors, and found three proteins (β-anchoring and -regulatory protein (BARP), lysosomal-associated membrane protein 5 (LAMP5), and sulfotransferase 2B1 (SULT2B1) that complement NACHO in the functional reconstitution of α6β2β3 receptors in heterologous systems. BARP is a neuronal single-pass transmembrane protein that binds calcium channels [48], enhances α6β2β3 receptor assembly, and enhances channel activation by decreasing the desensitisation and deactivation of α6β2β3 channels, whereas LAMP5 (a vesicular protein present in axon terminals) [49] and SULT2B1 (a cytosolic sulfotransferase) [50] both enhance α6β2β3 surface trafficking. These studies highlight the complexity of the biogenesis of α6β2* receptors, and explain why their functional expression in heterologous systems failed until the discovery and co-expression of NACHO, BARP, LAMP5 and SULT2B1 proteins.

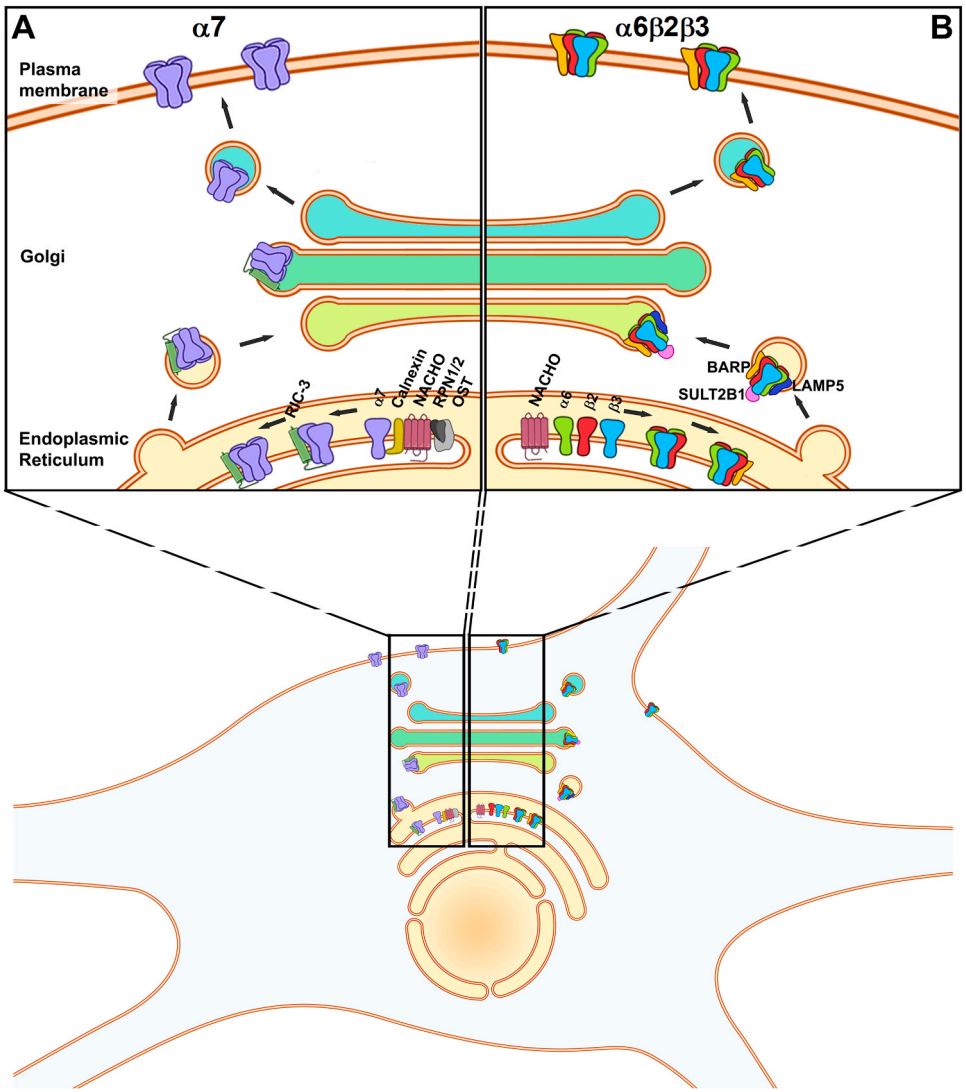

Fig. 1. A) Regulation of α7 subtype assembly and trafficking by chaperone and associated proteins. NACHO, calnexin and the ribophorin-1 and ribophorin-2 (RPN1/2) components of the N-glycan oligosaccharyltransferase (OST) proteins promote the folding of α7 subunits in the ER [27,28]. After initial folding, calnexin dissociates from the α7 subunits, which then interact with RIC3 [27,31,32] to complete the folding of the subunits and their assembly into the pentameric receptors that progress through the Golgi to the plasma membrane. These later steps are also facilitated by Bcl-2 family proteins [32]. B) Regulation of α6β2β3 subtype assembly and trafficking by chaperone and associated proteins. NACHO promotes the assembly of α6, β2 and β3 subunits into pentameric receptors in the ER [28]. Sulfotransferase 2B1 (SULT2B1) and lysosomal-associated membrane protein 5 (LAMP5) proteins associate with the α6β2β3 subtype in the vesicles going from the ER to the Golgi apparatus [47], but it is unclear whether they are also present in the vesicles going from the Golgi to the plasma membrane. At the plasma membrane, only BARP is associated with the α6β2β3 subtype [47].

3.2.3. *IRE1α protein*

Recent studies have revealed the presence of α6β4 receptors in animal and human sensory dorsal root ganglia (DRG) neurons as well as retina, locus coeruleus and adrenal chromaffin cells, which are involved, e.g., in analgesia and the control of catecholamine secretion. However, as in the case of α6β2* receptors, the expression of human α6β4 nAChRs has not yet been extensively characterised because of technical difficulties. It was not until 2020 that genome-wide cDNA screening for proteins enabling the robust functional expression of human α6β4 receptors by Knowland *et al.* [51] demonstrated that their expression is enhanced by BARP and inositol requiring enzyme-1α (IRE1α), but unaffected by NACHO. As mentioned above, BARP is an accessory component that is important for α6β2β3 nAChR expression, whereas IRE1α is a constituent of the unfolded protein response (UPR) that promotes protein folding in the ER during cell stress [52]. The expression of α6 and β4 subunits in *Xenopus* oocytes or HEK293T cells fails to induce ACh-evoked currents, but co-expression of the subunits with BARP generates large currents [53]. BARP also increases α6β4 plasma membrane localisation on HEK293T cells, but does not induce any changes in ^{3}H-epibatidine binding, whereas the co-expression of IRE1α with α6 and β4 subunits does not induce ACh-evoked currents or a change in α6β4 plasma membrane expression, but increases ^{3}H-epibatidine binding [51]. These findings indicate that IRE1α enhances receptor assembly but not α6β4 surface expression, whereas BARP directly binds to α6β4 and promotes trafficking to the plasma membrane, but has no effect on α6β4 desensitisation or deactivation, thus supporting the fact that the two proteins affect α6β4 function by means of distinct mechanisms. BARP also enhances α6-containing nAChRs by means of distinct mechanisms: it enhances α6β4 function in HEK cells, oocytes and neurons by regulating their surface expression, but regulates the gating of α6β2β3 receptors by decreasing their desensitisation and deactivation kinetics.

Fig. 1B and Table 1 summarise the chaperone and accessory proteins involved in the assembly, trafficking and activation of α6β2β3 receptors.

Table 1
Effects of accessory/ auxiliary proteins on nAChR subtypes.

PROTEIN	nAChR subtype and effect	Ref.
NACHO	**Nicotinic Acetylcholine Receptor Regulator, neuronal ER-localised chaperone**	[27]
	Not a surface-expressed auxiliary subunit, but a chaperone for α7 and heteromeric nAChRs	[27] [28]
	When co-transfected with α7, ↑ ACh-evoked currents and ↑ the number of receptors at the plasma membrane.	[27] [28]
	In heterologous systems, when co-transfected with:	[27]
	α4β2 ↑ assembly, ↑ surface localisation, ↑ ACh-evoked currents	[27, 28]
	α3β2 ↑ assembly, ↑ surface localisation, ↑ ACh-evoked currents	[28] [28]
	α3β4 ↑ assembly, ↑ surface localisation, ↑ ACh-evoked currents	[28]
	α6β2β3 ↑ assembly	
	Its knockdown in mouse brain:	[28]
	abolishes ^{125}Iα-Bgtx binding and α7-mediated currents in hippocampal neurons	[29]
	↓ the binding of ^{3}H epibatidine to heteromeric receptors	
	Interacts with the molecular machinery involved in the early stage of α7 nAChR insertion into the ER membrane	[30]
	Works synergistically with RIC3 to promote α7 receptor assembly and function	[27] [28]
	Promotes the plasma membrane expression of the $(\alpha4)_2(\beta2)_3$ stoichiometry	[65]
RIC3	**Resistance to Inhibitors of Cholinesterase 3, ER resident protein**	[31]
	Assists later in the folding of α7 subunits before their assembly. Its action requires the cytosolic M3-M4 intracellular loop of the α7 subunit	[30]
	↑ arrival and functional expression of α7 nAChRs at the plasma membrane	[31]
	Allows the interaction of α7 with the 39 different proteins involved in intracellular trafficking.	[95]
	RIC-3 knockdown only causes a slight decrease in ^{125}Iα-Bgtx binding in mouse brain	[29]
Anti-apoptotic Bcl-2 proteins	**B-cell lymphoma 2 proteins, regulate apoptosis. The binding of anti-apoptotic Bcl-2 proteins to pro-apoptotic Bcl-2 proteins prevents the signalling cascade that initiates programmed cell death**	[39]
	Enhance assembly and functional expression of α7 nAChRs in the presence of NACHO and, to a lesser extent, in the absence of NACHO	[32]
	Overexpression of Bcl-2 member Mcl-1 in neurons enhances the surface expression of endogenous α7 nAChRs	[32]
	Combination of chemotherapeutic Bcl-2- inhibitors suppresses neuronal α7 receptor assembly.	
	The TM3-TM4 intracellular loop of the α7 subunit contains a BH3-like motif necessary for α7 regulation by anti-apoptotic Bcl-2 proteins.	[32]
BARP	**β-anchoring and regulatory protein, a single-pass transmembrane protein**	[48]
	An auxiliary subunit for the α6β2β3, α6β4 and α3β2 subtypes.	[47] [47]
	Regulates the gating of α6β2β3 and α3β2 channels	[51]
	↑ α6β2β3 receptor functional response, but does not affect receptor surface expression	[51]
	↑ functional response of the α3β2 subtype	
	↑ functional response of the α6β4 subtype in oocytes, HEK cells, and neurons	[51]
	↑ surface expression of the α6β4 subtype in HEK cells	
	Directly interacts with α6β4 via its transmembrane region and promotes trafficking to the membrane	[51]
	BARP-KO mice,show a 25% reduction in striatal	[47]
	α6β2 * receptors and decreased α6β4 receptor surface expression in DRG	[51]
SULT2B1	**Sulfotransferase, a cytosolic protein**	[50]
	When expressed alone with α6β2β3, only slightly ↑ subtype functional expression	[47] [47]
	Combined with NACHO, BARP and LAMP5, greatly ↑ α6β2β3 functional expression	
	When expressed alone with α6β4, ↑ nicotine-induced functional response	[51]
LAMP5	**Lysosomal-associated membrane protein 5**	[49]
	LAMP5 alone does not affect the functional activity of α6β2β3 receptors	[47] [47]
	Co-transfection of LAMP5 and SULT2B1 with α6β2β3 is sufficient to promote α6-containing nAChR surface trafficking, reconstitute functional α6 * receptors, and ↑ peak cell currents	[47]
	LAMP5 and SULT2B1 act synergistically with BARP and NACHO and, when combined with them, induce robust responses from α6-containing receptors	
IRE1α	**Inositol-requiring enzyme-1α, a constituent of the unfolded protein response (UPR)**	[52]
	Moderately enhances the functional response of the α7, α4β2, and α6β2β3 * subtypes	[51]
	Enhances α6β4 subtype assembly and ^{3}H-epibatidine binding, but not its surface expression	[51]
TMIE	**Transmembrane inner ear protein, a product of a human deafness gene. A single-pass transmembrane protein and a subunit of the mechanotransduction channel of cochlear hair cells**	[59]
	An auxiliary subunit of the α9α10 subtype	[58]
	Has no effect on α9α10 receptor assembly or trafficking, but	
	↑ α9α10 functional activity	
	Associates stably with the α9α10 subtype on the cell surface, and modulates channel gating	
	Acts synergistically with ChAT to enhance α9α10 receptor function	[58]
TMEM132e	**A single-pass transmembrane protein, product of a human deafness gene**	
	Acts synergistically with ChAT to enhance α9α10 receptor function	[58]
14-3-3η	**Cytosolic molecular chaperone protein**	[63]
	↑ α4β2 expression and ↓ α4β2 sensitivity to ACh	[65]
	Promotes plasma membrane expression of $(\alpha4)_3(\beta2)_2$ stoichiometry	
LY6 prototoxin family	**Small modulatory proteins characterised by five disulphide bonds and three finger motifs**	[22]
Ly6h	**Endogenous GPI-anchored prototoxin**	
	Binds α7 nAChRs	[110]
	↓ the trafficking of α7 to the plasma membrane and ↓ agonist receptor-induced currents.	
	↑ epibatidine sensitivity of α4β2 receptors.	[110]
	Inhibits assembly of α7 nAChRs during biogenesis	[112]
	↓ Ly6h levels ↑ α7 nAChRs at the plasma membrane, ↑ cell Ca^{2+} currents that may be excitotoxic	[112]
SLURP1	**Secreted prototoxin**	
	An allosteric antagonist of α7 that ↓ its response to ACh by means of non-competitive inhibition	[117]
	Inhibits growth of various carcinoma and glioma cells in vitro in 2D and 3D tumour models	[118]
	Diminishes lung adenocarcinoma A549 cell proliferation and abolishes nicotine-induced growth stimulation	[118]
	Has antiproliferative activity and suppresses metastatic growth in a mouse model of epidermoid carcinoma	[120]

Abbreviations: ↑increases, ↓decreases, ACh acetylcholine, ChAT choline acetyltransferase, nAChR neuronal nicotinic receptor.

3.3. α9-α10 receptors

On the basis of their sequence similarity to nAChRs, the α9 and α10 subunits were initially classified as neuronal subunits, but homomeric α9 and heteromeric α9α10 receptors are not functionally present in the brain [54]. The α9α10 subtype is only endogenously expressed in cochlear and vestibular hair cells in the inner ear, and in extra-neuronal tissues. In the auditory system, outer hair cells (OHCs) express α9α10 nAChRs and receive cholinergic efferent innervation from medial

olivocochlear (MOC) neurons. The ACh released by efferent MOC terminals, activates the calcium permeable α9α10 nAChRs of OHCs, and this is coupled with the subsequent activation of Ca^{2+}-activated SK2 K^+ channels and hair cell hyperpolarisation that supress OHC electromotility [55]. The olivocochlear network improves signal discrimination amid background noise, protects against sound-induced hearing loss, and modulates synapse formation. Studies of α9 [56] and α10 knockout (KO) mice [57] have demonstrated that targeted disruption of the α9 or α10 subunits abolishes ACh-mediated synaptic currents in both inner and outer hair cells, and KO mice show a number of auditory processing deficits.

3.3.1. TMIE and ChAT

The α9α10 subtype is not functional when expressed in transfected cell lines, but genome-wide screening has shown that the ACh biosynthetic enzyme choline acetyltransferase (ChAT) and the Transmembrane inner ear (TMIE) protein product of the deafness gene [58], are factors enabling the expression of functional α9α10 nAChRs.TMIE is also a subunit of the mechanotransduction channel of cochlear hair cells [59]. When transfected separately, ChAT and TMIE increase ACh-evoked α9α10 responses but, when transfected together, they have powerful synergistic effects on α9α10 function. Moreover, analysis of hemagglutinin (HA)-tagged α9α10 receptors has shown that the transfection of α9 or α10 subunits alone does not lead to detectable cell surface labelling of α9α10 receptors, whereas ChAT transfection induces clear surface α9α10 immunofluorescence that is not increased by adding TMIE.

These findings indicate that ChAT enables α9α10 assembly and surface expression, and that this is a prerequisite for the robust functional enhancement of channel activity induced by TMIE. The increased assembly and surface expression of α9α10 is abolished if acetylcolinesterase (the enzyme that metabolises ACh) is co-transfected with α9α10+ TMIE + ChAT or when in the binding site of the α9α10 subtype contains mutations that prevent agonist (ACh) or antagonist (αBgtx) binding.

As in the case of other nAChR subtypes, the presence of intra- or extra-cellular orthosteric ligands enhances the surface expression of α9α10 nAChRs possibly as a result of a chemical chaperone mechanism. However, the effect of ACh is not due to cell membrane permeabilisation but stabilises assembled α9α10 receptors on the cell surface, and this effect also occurs in the presence of the peptidic αBgtx antagonist that cannot cross the cell membrane. Furthermore, the studies also show that TMIE protein has no effect on α9α10 receptor assembly or trafficking, but it is important for their correct functioning. TMIE acts as an auxiliary subunit of cell surface α9α10 receptors and promotes their function by directly interacting with them [58].

Although the studies described above have provided new information regarding the role of accessory proteins in the trafficking of α9-α10 receptors, it is still unclear whether mutations and/or changes in the expression of these accessory proteins contribute to the deregulation of α9-α10 receptors in the auditory system.

3.4. α4β2 receptors

α4β2, the most abundant nAChR subtype in the brain has two stoichiometries - $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ that have different pharmacological and functional properties [60,61]. In addition to the two orthosteric binding sites at the α4/β2 interface, the $(\alpha4)_3(\beta2)_2$ stoichiometry has an unorthodox binding site at the α4α4 interface [62]. The two forms respond differently to chronic nicotine administration, differ dramatically in their sensitivity to ACh and its NS9283 potentiator, and have different regional distributions. In the trafficking of this subtype, very important roles are played by specific amino acid sequences present in the ICD of the α4 and β2 subunits such the ER retention/retrieval sequences and signals that promote ER export and chaperone protein binding (reviewed in [13,17]).

3.4.1. NACHO and 14-3-3η protein

Using the large cytoplasmic domain of the α4 subunit as a bait in the yeast two-hybrid system, Jenclos *et al.* [63] isolated the 14–3-3η cytosolic protein that directly interacts with native α4β2 receptors. In vitro, 14–3-3η increases the expression of α4β2 nAChRs and decreases the ACh sensitivity of the resulting nAChRs [64]. It has also very recently been shown that, by mediating the early steps in the assembly of immature intra-cellular nAChR complexes, the chaperone NACHO promotes the functional surface expression of various classes of nAChRs, including α4β2 receptors. Mazzaferro *et al.* [65] have used their heterologous expression in mammalian cells, single-channel patch-clamp electrophysiology, and calcium imaging to show that although both NACHO and 14–3-3η increase the trafficking of α4β2 nAChRs to the plasma membrane, they have opposing effects on the expression of the two stoichiometric forms: NACHO selectively promotes the expression of $(\alpha4)_2(\beta2)_3$, and 14–3-3η selectively promotes the expression of the $(\alpha4)_3(\beta2)_2$. The fact that 14–3-3η enriches cell surface receptors with the $(\alpha4)_3(\beta2)_2$ stoichiometry suggests that it either slows the degradation of the α4 subunit (whose greater availability increases the frequency of the $(\alpha4)_3(\beta2)_2$ stoichiometry), or enhances the ability of the α4 subunit to oligomerise with individual subunits or higher-order assembly intermediates. Similarly, the fact that NACHO promotes the $(\alpha4)_2(\beta2)_3$ stoichiometry suggests that it either stabilises the β2 subunit within the ER or enhances its ability to oligomerise with other subunits or assembly intermediates.

3.5. α3β4 receptors

α3β4 nAChRs are the predominant subtype expressed in the sensory and autonomic ganglia, and in a few sub-populations of brain neurons [4]. They can also have an extra-neuronal localisation as α3 and β4 mRNAs are significantly over-expressed in small-cell lung carcinoma cells, an aggressive form of lung cancer [66].

In vivo and in heterologous systems, the α3 subunit forms receptors with various combinations of β2, β4, and α5 subunits. The α3 and β4 subunits readily form functional receptors without any additional subunits, although it has recently been shown that the co-expression of NACHO greatly increases their surface expression [28].

3.5.1. LFM export motif

We initially found that, when expressed in heterologous cells, the α3β4 subtype is correctly assembled and the pentamers are retained in the ER. However, chronic nicotine treatment favours the assembly of receptors with the $(\alpha3)_2(\beta4)_3$ stoichiometry and, under this condition, the total number of receptors is up-regulated, and more pentamers are transported to the cell surface. In line with the role of the β4 subunit in facilitating transport, we identified an export motif (LFM) in the cytoplasmic loop between TM3 and TM4 of the β4 subunit but not in the corresponding loop of the α3 subunit, and demonstrated that this motif facilitates export of the assembled pentamer from the ER [67].

Subsequently, a new experimental approach was used to investigate whether the presence of three LFM motifs determines the more efficient transport of the $(\alpha3)_2(\beta4)_3$ subtype to the cell surface. We generated a dimeric construct (β4-α3) that, when co-transfected with a monomeric subunit (α3 or β4), allowed us to study a specific population of receptors with a fixed stoichiometry. Morphological, biochemical and functional assays showed that, 24 h after transfection, the receptors with either stoichiometry were equally efficiently assembled, but only those with three β4 subunits could efficiently reach the cell surface, whereas those with the $(\alpha3)_3(\beta4)_2$ stoichiometry were mainly retained intracellularly, thus demonstrating that the LFM motif plays no role in trafficking when it is in the β4-α3 dimer, and only facilitates export when it is present in the β4 subunit in the fifth position [68].

4. Modulation of nAChR assembly, trafficking and kinetics by pharmacological chaperones

In addition to endogenous proteins, there are also target-specific and non-target-specific pharmacological compounds that are cell-permeable or endogenously present in the cells that can facilitate the biogenesis of nAChRs, prevent or correct subunit misfolding, and enhance receptor assembly in the ER and their trafficking through the Golgi to the plasma membrane. Permanent and transient protein interactions and the presence of pharmacological compounds are not mutually exclusive, and can combine to generate more complex patterns of receptor modulation. We here describe a series of substances that affect receptor function via interactions that do not always involve non-canonical receptor binding sites.

4.1. Nicotine

is a tertiary amine and highly lipophilic compound that can easily penetrate the blood–brain barrier and concentrate in the brain, where it has many psychoactive effects. Exposure to nicotine increases nAChR levels in vitro and in vivo in a subtype- and region-selective manner [17,69]. Various mechanisms may underlie this upregulation, but the assembly and trafficking of nAChR subtypes and their cell surface expression are the key elements. By acting intra-cellularly, nicotine facilitates specific receptor subtype assembly, enhances the export of assembled pentamers from the ER and/or stabilises assembled receptors, and thus enables more receptors to enter the plasma membrane. In particular, it is the most widely known pharmacological chaperone of nAChRs and acts on α4β2 and α7-, α3- and α6-containing receptors (see [17,23,69,70] for recent exhaustive reviews), although similar effects have been reported in the case of other nicotinic ligands (see [71] for more information).

Prolonged exposure to nicotine increases the number of α4β2 nAChR binding sites as a result of increased α4 and β2 subunit protein levels without any change in the levels of mRNA. This up-regulation is brain region-dependent as it is more substantial in the cerebral cortex and hippocampus, but less robust or absent in other regions [72–74]. Moreover, the upregulated cortical receptors include a greater proportion of those with $(\alpha4)_2(\beta2)_3$ stoichiometry [74]. The α4 and β2 subunits can also combine with the α5 subunit to form the $(\alpha4)_2(\beta2)_2\alpha5$ subtype, but its expression is not affected by nicotine exposure [75].

The large intracellular loop between TM3 and TM4 of the α4 subunit is involved in regulating nAChR assembly, trafficking and function [76], and the serine amino acids in the intracellular loop are critical for the nicotine-induced up-regulation of α4β2 nAChRs. Brain region-specific, chronic nicotine-induced up-regulation can be replicated in primary neuronal cultures, and Zambrano *et al.* [77] have infected primary neurons from the cortex, hippocampus and subcortex of α4 KO mice with adeno-associated virus containing cDNA for the WT α4 nAChR subunit or α4 nAChRs with the S336A, S470A or S530A point mutations. After chronic nicotine exposure, they found that the neurons in the three brain areas expressing α4 nAChR subunits containing serine to alanine mutations showed an increase in the total number of α4β2 receptors, but the neurons in the hippocampus and subcortex did not show any increase in the number of α4β2* receptors in the plasma membrane, thus indicating that, even in the brain regions showing the upregulation of the total number of binding sites, there may be multiple, potentially neuron type-specific mechanisms contributing to upregulated nicotine-induced surface α4β2* expression.

Chronic nicotine exposure may modulate membrane nAChRs as a result of its effects on other genes expressed by the cells (reviewed in [78]). Quantitative proteomics of immunopurified β2-containing receptors and associated proteins taken from nicotine-treated mouse cortex or from the *post mortem* human temporal cortex tissue of tobacco-exposed subjects has shown that, in addition to upregulating α4β2 subunits, chronic nicotine exposure increases the expression of specific nAChR interacting proteins, including Na/K ATPases, syntaxins, SNAP25, synaptotagmin, and the 14–3-3 chaperone protein, thus revealing the effect of nicotine on the expression of intra-cellular proteins involved in nAChR regulation [73].

4.2. Cotinine

is a minor alkaloid but the major metabolite of nicotine, and can act as a weak agonist of nAChRs. It is commonly used as a biomarker of nicotine exposure [79,80] as it has a longer half-life and reaches higher blood levels than nicotine itself, and can also cross the blood-brain barrier. Accumulating evidence indicates that cotinine binds the α4β2, α7 and α6β2 subtypes, but its binding affinity is much less than that of nicotine (reviewed in [81]) and may only contribute to the effects of nicotine. As in the case of nicotine, not all of the biological effects of cotinine depend on nAChRs [81].

Fox *et al.* [82] used super-ecliptic pHluorin-based fluorescence imaging to show that low cotinine concentrations of up to 1 μM favour the trafficking of α4β2 receptors to the plasma membrane, whereas higher concentrations (>5 μM) do not increase their presence on the cell surface. This suggests that low cotinine concentrations may participate in the up-regulation of α4β2 receptors, whereas high concentrations may cause endocytosis and lead to a reduction in the number of α4β2 receptors on the plasma membrane.

Treatment with 500 nM nicotine or 1 μM cotinine induces the up-regulation of α4β2 receptors in N2A cells, and both treatments increase the presence of receptors with the high-sensitivity stoichiometry of $(\alpha4)_2(\beta2)_3$ [82].

4.3. Menthol

is a common additive in tobacco cigarettes and e-cigarette vaping solutions [83], and recent studies have identified its pharmacological effects on various members of the Cys-loop receptor family, including nAChR subtypes, GabaA, Glycine, and 5HT3 receptors [84–87].

Earlier studies showed that menthol inhibits nicotine metabolism [88] and that menthol cigarette smoking leads to high serum nicotine and cotinine levels. Smokers of mentholated cigarettes attending standardised treatment programmes have lower cessation rates than smokers of non-mentholated cigarettes [89,90], and Brody *et al.* [91] have shown that they have higher levels of β2-containing nAChRs. It has also been reported that menthol enhances the effects of nicotine on the brain, and can make tobacco products even more addictive [92].

Chronic 10-day treatment with menthol alone selectively up-regulates α4 and α6 nAChR subunits in mid-brain dopaminergic neurons [25], whereas nicotine alone up-regulates the number of α4β2 receptors in GABAergic cell bodies of the substantia nigra pars reticulata and dopaminergic terminals of the striatum [93,94]. Chronic menthol treatment alone favours the formation of the low-sensitivity $(\alpha4)_3(\beta2)_2$ stoichiometry, prevents nicotine reward-related behaviour [25], and the co-administration of menthol and nicotine significantly up-regulates α4α6β2 and α4β2 subtypes in the ventral tegmental area, substantia nigra, and hypothalamus in comparison with nicotine alone [84,95].

Mentholated tobacco products contain (+)menthol and (-)menthol in varying proportions, and a recent study [96] has shown that long-term treatment with (-)menthol but not (+)menthol, upregulates α4β2 nAChRs, and decreases dopamine neuron baseline firing and excitability, thus suggesting that long-term exposure to (-)menthol may contribute to increasing tobacco addiction. Moreover, immunoblotting and ^{3}H-epibatidine binding analyses of 14 brain areas of mice chronically exposed to (-)menthol have shown a reduced level of β2-containing receptors in the habenula [97]. These data indicate that, in addition to having different effects on the function of α4β2 nAChRs, different menthol preparations may have brain region-specific differences in the regulation of β2-containing receptor levels.

Neuronal N2a cells transfected with nicotinic subunits and treated

for 24 h with (-)menthol alone show an increased number of α4β2 receptors at the plasma membrane but chronic (-)menthol treatment does not change α3β4 nAChR subunit stoichiometry or the ACh response of plasma membrane α3β4 receptors [98], probably because the β4 subunit has an ER export motif (LXM) but no ER retention motif (RRQR) and therefore α3β4 nAChRs efficiently exit the ER and enter the plasma membrane [67,99] without a need for additional support.

4.4. Polyamines

such as spermidine and spermine, are abundant endogenous polycations that play multiple roles in cell growth, differentiation and survival [100]. In neurons, they play an important role in synaptic transmission by inwardly rectifying certain potassium channels [101], AMPA receptors, and nAChRs [102].

One recent study [103] has shown that, by acting at the intracellular loop of α7 and α4β2 nAChRs, polyamines negatively regulate their assembly and cell surface localisation. Genome-wide cDNA screening identified a clone encoding spermidine/spermine acetyltransferase (SAT1), a rate-limiting polyamine catabolism enzyme [103] and, when SAT1 is co-expressed with NACHO, it up-regulates α7 and α4β2 receptor assembly, surface expression, and functional activity. This effect is due to its enzyme activity as SAT1 mutants devoid of enzymatic activity or the inhibitor of ornithine decarboxylase 1 (the rate-limiting polyamine synthesis enzyme) did not change the surface expression of α4β2 and α7 in the presence or absence of NACHO. To identify the region responsible for the effect of polyamine, the authors prepared a number of subunit chimeras and identified negatively charged residues within the TM3-TM4 cytosolic loop that can mediate polyamine regulation of nAChR assembly and surface expression.

5. The lack of nAChR regulation by accessory proteins contributes to diseases

It is known that nAChRs are dysregulated in patients suffering from a number of neurological and psychiatric disorders: for example, the reinforcing and withdrawal effects of nicotine seem to be involved in the up-regulation of α4-, α6- and β2-containing nAChRs [104,105], and Alzheimer's disease (AD) is associated with changes in the levels of α4β2 and α7 nAChRs (reviewed in [106,107]). As the discovery and characterisation of nAChR accessory proteins has raised the possibility that these subunits may become druggable targets for treatment, we will now move on to discuss recent studies showing the role that some of them play in neuronal and extra-neuronal diseases.

5.1. α7 receptors

Aberrant α7 nAChR signalling has been associated with neuropsychiatric and neurodegenerative disorders such as schizophrenia and AD (reviewed in [108]), and it is known that the α7 receptors expressed in some non-neuronal tissues play an important pathogenic role in immunological and oncological diseases (reviewed in [40,41,109]).

The trafficking of α7 receptors -ìn neurons, is controlled by the endogenous GPI anchored prototoxin Ly6h, which acts as a bi-functional inhibitor of α7 nAChRs by delaying their subunit oligomerisation and reducing their cell-surface expression and calcium signalling [110], and by acutely antagonising the function of cell surface receptors [111]. Wu *et al.* [112] have found that the unbalanced regulation of α7 by Ly6h and NACHO, the chaperone protein that enhances the assembly and delivery of α7 receptors to the plasma membrane (see Section 3.1.1), may contribute to AD. Ly6h and NACHO co-exist in the same hippocampal pyramidal neurons and compete for access to the α7 subunits involved in α7 assembly; this causes proportionate changes in the surface expression and ultimately the amplitude of Ca^{2+} signalling by functional α7 nAChRs. Sustained activation of α7 nAChRs by the positive allosteric compound PNU-120596 increases neuronal cell death, an effect that is enhanced by the knock-down of Ly6h and suppressed by the knockdown of NACHO: i.e. Ly6h protects against the neurotoxicity caused by sustained α7 receptor activation, and NACHO contributes to it. In particular, it has been found that decreasing the Ly6h/NACHO ratio is neurotoxic, and increasing it is neuroprotective, thus supporting the hypothesis that a balanced ratio is crucial for the assembly of α7 receptors and keeps ACh-induced Ca^{2+} influx within a narrow physiological range, whereas its dysregulation has deleterious consequences [112].

The neuronal tissues in AD patients and animal models of the disease show an accumulation of amyloid β (Aβ), protein plaques [113]. Treating hippocampal neurons with the pathogenic fragment of Aβ (Aβ1–42) a high affinity ligand of α7 receptors, for seven days decreases Ly6h protein levels by 50% but has no effect on other membrane proteins, an effect that requires α7 receptor activation as the simultaneous addition of the α7 antagonist methyllycaconitine (MLA) completely blocks the decrease in Ly6h levels [112]. Moreover, the reduction in Ly6h up-regulates phosphorylated tau (a histopathological marker and correlate of neuronal loss in AD) and causes the degeneration of hippocampal neurons, an early hallmark of disease pathogenesis. In comparison with age-matched controls, a reduction in Ly6h levels has also been observed in temporal cortex tissue samples taken from AD patients, and Ly6h levels inversely correlate with AD severity [112].

Ly6h and NACHO therefore act antagonistically to maintain the amplitude of α7 nAChR signalling within an optimal range, and the inverse correlation between Ly6h levels and AD severity in human temporal cortex suggests that there is a close link between Ly6h function and the pathogenesis of AD.

As noted above, NACHO is important for the functional expression of homomeric α7 receptors and the assembly of heteromeric α3-, α4-, and α6-containing nAChRs. NACHO KO mice show thermal hyperalgesia and mechanical allodynia in response to hot and cold stimuli, a phenotype that is associated with the loss of α7 or a reduction in the neuronal activity of α3-, α4-, and α6-containing nAChR subtypes, and accompanied by molecular changes in the spinal cord indicative of neuroinflammation [114]. The intrathecal administration of nicotine or the α7-specific agonist PHA543613 to NACHO KO mice respectively induces analgesic responses to noxious heat and mechanical stimuli, thus suggesting the residual expression of these receptors or off-target effects. As NACHO is only expressed in neurons, these findings indicate that neuronal α7 nAChRs in the spinal cord contribute to heat nociception [114].

The α7 subtype is also involved in tumour proliferation, apoptosis, metastatic growth and chemotherapy resistance, and various studies have shown that it is the main nAChR subtype mediating the proliferative and angiogenic effects of nicotine in lung cancer cells (reviewed in [109,115]). The activation of cell-surface α7 nAChRs in non-neuronal cells leads to two main types of response: an ionotropic response that is associated with Ca^{2+} influx into the cytosol [116], and a metabotropic response that activates mitogenic intracellular signalling pathways without opening the nAChR channel [15].

Ly6/urokinase type plasminogen activator receptor (uPAR)-related protein 1 (SLURP-1), a selective negative allosteric modulator of the α7 subtype [117], inhibits the in vitro growth of different carcinoma and glioma cells in 2D and 3D tumour models, and abolishes nicotine-induced cell proliferation [118]. It also controls the growth and migration of lung adenocarcinoma A549 cells by interacting with α7 nAChR and epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) heterocomplexes and modulating the PI3K/AKT/mTOR and inositol-1,4,5-trisphosphate (IP3) pathways [118,119].

In a xenograft mouse model of epidermoid carcinoma, SLURP1 has shown strong antiproliferative activity and suppressed metastatic growth [120]: tumour volume began to decrease only on the fifth day after the end of the 10-day treatment period probably because SLURP-1 reprogrammed the tumour cells, which stopped their proliferation and stimulated apoptosis or necrosis. Analysis of the proteins interacting

with SLURP-1 in the xenografted A431/NanoLuc tumours showed that α7 nAChR and EGFR both bind SLURP-1 and, given this dual targeting, SLURP-1 triggers both pro- and anti-oncogenic signalling, thus indicating that its overall antiproliferative activity is due to an interplay of various pro- and anti-oncogenic intracellular signalling pathways.

5.2. α6β4 receptors

Animal and human studies have shown the involvement of DRG -enriched α6β4 receptors in chronic pain states. Wieskopf *et al.* [121] used the genomic screening of DRG tissue from 25 in-bred mouse strains to show that the α6 nAChR subunit is the main phenotypic contributor to mechanical allodynia, a prominent symptom of chronic pain, and that greater expression of *Chrna6* was associated with lower allodynia levels. Furthermore, KO mouse studies showed that α6- but not α4-containing receptors are required for peripheral and spinal nicotine-induced analgesia [121], and that α6* receptors can improve chronic pain via cross-inhibition with P2X2/3 receptors involving direct between-protein contact.

As mentioned above, BARP is an important accessory protein involved in the membrane trafficking of the α6β4 subtype, and studies of BARP KO mice have shown that they are characterised by a reduction in the expression of α6* receptors on the surface of DRG neurons, but the total number of α6*-containing, α4β2 and α7 receptors is similar to that of controls [51].

When tested in the spared nerve injury (SNI) model of neuropathic allodynia, it has been found that the intraperitoneal administration of nicotine reduces post-injury mechanical allodynia in WT but not BART KO mice. Control SNI surgery on NACHO KO mice, which have substantially reduced levels of nAChR expression except in the case of α6β4 receptors, showed that the anti-allodynic effect of nicotine was comparable in WT and NACHO KO mice [51]. These findings are in line with the hypothesis that the deficits seen in BARP KO mice are predominantly mediated by the α6β4 subtype, which also mediates the anti-allodynic effect of nicotine.

The identification of the accessory BARP required for the activity and localisation of the α6β4 subtype has allowed successful investigations of the functional expression of this important subtype in heterologous systems, the in-depth characterisation of its pharmacology, and the screening of new subtype-specific compounds [53].

5.3. α3β4 receptors

The role of the many rare missense variants of the human β4 subunit [122] has been investigated, particularly in the case of variants R349C (the most frequently encountered mutation in patients with sporadic amyotrophic lateral sclerosis) [123], and D444Y. When expressed with the WT α3 subunit in heterologous systems, these variants elicit significantly different nicotine-induced currents from those elicited by WT α3β4 receptors: R349C β4 subunits significantly reduce nicotine-induced currents, and D444Y β4 subunits significantly increase them [122,124].

We have analysed the effects of these mutations in the accessory β4 subunit in the $(\alpha3)_2(\beta4)_3$ stoichiometry by co-expressing the WT β4α3 dimer with a monomeric WT or mutated β4 subunit, and found that α3β4 nAChRs with an R349C β4 subunit were retained intracellularly whereas those with a D444Y β4 subunit reached the plasma membrane, suggesting that D444Y facilitates plasma membrane trafficking.

The amyotrophic lateral sclerosis (ALS)-linked R349C mutation in the β4 subunit is adjacent to the LFM motif and, as the LXM motif in the β4 subunit is important for outward trafficking through the secretory pathway, it is possible that a local disruption in the amino acid sequence may affect LFM function by interfering with its interaction with Sec24D and consequent receptor recruitment at ER exit sites. In the case of the D444Y β4 mutation, a charged aspartic acid is substituted by a non-polar tyrosine, and it is possible that the change in amino acids in α3-D444Yβ4 receptors affects the conformation of the subunit/receptor or that the presence of tyrosine and its phosphorylation allows for the binding of a chaperone protein that increases the receptor's surface expression [125].

6. Conclusions and perspectives

This review of the most recent findings concerning auxiliary proteins shows that some control receptor biogenesis and others regulate channel activation and pharmacology; some are subtype selective and others regulate various subtypes; and some not only modulate nAChRs, but also target other receptors and signalling pathways. In most cases, these interactions are hugely significant in vivo, underlining the importance of auxiliary proteins to normal receptor functioning: in some cases, their modulation of channel properties is far more significant than subunit composition alone.

However, little or nothing is known about the subcellular distribution patterns of most of these proteins patterns in neurons or extraneuronal cells, which is the molecular basis of their interactions with nAChRs. It is not known whether they may be affected by post-translational modifications that influence their expression, localisation or interactions with nAChRs, or whether their interactions with nAChRs are subject to activity-dependent regulation, although the complexity of the regulation of the different nAChR subtypes by the various proteins may be due to the need to control the different subtypes in different cell types, different locations, and/or different physiological conditions.

We have also shown that, nAChRs play a pathogenic role in various diseases and medical conditions, and that this may be at least partially due to alterations in the expression of their auxiliary/ accessory proteins. Future studies further characterising the mechanistic and physiological roles of auxiliary/ accessory proteins or pharmaceutical compounds will not only advance our understanding of nAChR biology, but may also lead to the development of more efficacious nAChR pharmacology.

CRediT authorship contribution statement

Zoli Michele: Writing – review & editing. **Gotti Cecilia:** Writing – review & editing. **Clementi Francesco:** Writing – original draft.

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During the preparation of this work the authors did not used any AI and AI-assisted technologies in the writing process.

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