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Review

Chaperoning α7 neuronal nicotinic acetylcholine receptors

Ana S. Vallés [a], Francisco J. Barrantes [a,b,*]

[a] Instituto de Investigaciones Bioquímicas and UNESCO Chair of Biophysics and Molecular Neurobiology, BZW800 Bahía Blanca, Argentina
[b] Laboratory of Molecular Neurobiology, PIB UCA-CONICET, Faculty of Medicine, Catholic University of Argentina, C1107AFF Buenos Aires, Argentina

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ABSTRACT

The α7 subtype of nicotinic acetylcholine receptors (AChRs) is one of the most abundant members of the Cys-loop family of receptors present in the central nervous system. It participates in various physiological processes and has received much attention as a potential therapeutic target for a variety of pathologies. The importance of understanding the mechanisms controlling AChR assembly and cell-surface delivery lies in the fact that these two processes are key to determining the functional pool of receptors actively engaged in synaptic transmission. Here we review recent studies showing that RIC-3, a protein originally identified in the worm *Caenorhabditis elegans*, modulates the expression of α7 AChRs in a subtype-specific manner. Potentiation of AChR expression by post-transcriptional events is also critically assessed.

1. Introduction

AChRs are members of the Cys-loop family within the ligand-gated ion channel (LGIC) superfamily and are assembled from a diverse collection of subunits forming pentameric transmembrane receptors having different properties and functions (for reviews see [1–10]). All subunits comprise a large N-terminal extracellular domain, four α-helical transmembrane segments and a small C-terminal extracellular domain. In vertebrate species, 17 subunits (α1–α10, β1–β4, γ, δ and ε) have been identified to date, α1, β1, γ, δ and ε being expressed at the neuromuscular junction and the electromotor synapse and the remaining subunits (α2–α10 and β1–β4) at the central and peripheral nervous system (reviewed in [1,6,11–15]).

The homomeric α7 AChR is one of the most abundant AChR subtypes in the mammalian central nervous system [15–17]. The highest levels of expression of this subtype of receptor are found in the hippocampus, an area of the brain involved in various aspects of learning and memory [18–22]. α7 AChRs can act at the presynaptic, postsynaptic or perisynaptic levels to facilitate the liberation of neurotransmitters, mediate synaptic transmission, or modulate the connections of different neurons by activating diverse second messenger routes [1,19,23–31]. The α7 AChR transcripts are also found in non-neural, peripheral tissues, ranging from vascular endothelium to skin, T-cells, macrophages, or lung epithelial cells, and high levels of expression of α7 transcripts are present in mammalian sperm cells, implicating a role for α7 AChR in the reproductive system [32–46].

AChRs have been linked to many neurodegenerative disorders [13,47–60]. A complete characterization of AChR synthesis, assembly and trafficking constitutes a fundamental step in understanding the physiological mechanisms that may contribute to the development of drugs for treating these diseases. In this review we describe the complex processes and actors enabling proper mature AChR formation as well as the several steps required for AChRs to reach the plasma membrane and become functional ion channels.

2. Synthesis, folding and assembly of α7 AChRs

The efficacy of synaptic transmission depends largely on the population of active AChRs at the synapse. However, the assembly of ion channels such as the AChR is a slow and inefficient process, with only 30% of newly synthesized subunits forming functional receptors upon adopting the correct transmembrane topology and undergoing critical post-translational modifications [8,61–65]. Biosynthesis of α7 AChR in various mammalian cells has been reported, but functional heterologous expression has been very hard to attain [66–70].

Most AChR subunits appear to be incapable of forming functional AChRs unless they co-assemble with at least one other type of subunit in a heteromeric complex. However, the neuronal α7 and the α8 subunits are the only ones which appear to preferentially form homomeric, rather than heteromeric, receptors in heterologous expression systems [71–74].

Each subunit of the AChR is a separate gene product with a processed signal sequence and one to three N-linked glycosylation sites [75,76]. As they are synthesized, the subunits are inserted into the lumen of endoplasmic reticulum (ER) membranes which contain the proteins required for efficient protein folding and post-translational modification [63,77,78]. The latter is well documented for the AChR,

* Corresponding author at: Laboratory of Molecular Neurobiology, PIB UCA-CONICET, Pontificia Universidad Católica Argentina, C1107AFF Buenos Aires, Argentina. Tel.: +54 291 4861201; fax: +54 291 4861200.
E-mail address: rtfjb1@criba.edu.ar (F.J. Barrantes).

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which undergoes glycosylation [76], disulphide-bond formation, [79–81], palmitoylation [82,83], proline isomerization [63] and proteolytic cleavage of the N-terminus signal sequence in the ER [63].

Previous studies performed on the α7 AChR suggest that inappropriate disulphide-bond formation precludes correct subunit folding, as observed in some mammalian cell lines [84]. Additionally, the prolyl isomerase enzyme cyclophilin has been shown to be necessary for efficient folding of the α7 subunit in *Xenopus* oocytes [85–88].

Recent studies [89,90] have highlighted the importance of the α-helix present at the N-terminus of the α7 AChR subunits as well as the interaction of the α-helix with loop 3 between β-strands β2 and β3 in the expression of functional channels. Evidence shows that the latter interaction is relevant during receptor biogenesis, most likely by favoring or initiating the correct global folding required for receptor assembly.

After subunit synthesis, properly folded and oligomerized AChRs are transported from the ER along the exocytic pathway [1,61,91].

3. The role of chaperone proteins

There is evidence that AChR folding, assembly and trafficking are influenced by several chaperone proteins, such as the 14-3-3 protein [92,93], BiP [94–96] or calnexin [97–99]. Rapsyn is essential for AChR clustering in muscle [100] and has also been detected in non-muscle cells, including neurons of the ciliary ganglia [101,102], fibroblasts [103], myocardial cells, and Leydig cells [104].

Rapsyn has more recently joined the above chaperone club, as it "escorts" the AChR from the ER to the plasmalemma when heterologously expressed in mammalian cells [105]. All these proteins have been shown not only to influence the trafficking of the AChR subunits but also to interact with a diverse range of target proteins [93,106]. More recently, the transmembrane protein resistant to inhibitors of cholinesterase (RIC-3), originally identified in *Caenorhabditis elegans*, has been classed as a much more selective chaperone of the AChR [71,107–112].

Regulation of receptor subunits by the proteasome, the large protein complex that proteolytically degrades unneeded proteins, has also been demonstrated [113,114]. Furthermore, the proteasome indirectly regulates synaptic transmission mediated by AChRs via regulation of RIC-3 [113].

3.1. RIC-3 is a selective AChR chaperone

The RIC-3 protein was first identified in 1995 in the nematode *C. elegans* as a protein encoded by the gene ric-3 [71,77,110,115,116]. In C. *elegans*, RIC-3 is necessary for synaptic transmission mediated by neuronal AChRs but not by other LGICs [71,77,109]. RIC-3 is a highly charged protein containing no less than 8% aspartic acid, 13% glutamic acid, 10% lysine and 8% arginine [77,110,112,115] residues. It comprises an N-terminal domain, a membrane-spanning domain separated by a proline-rich spacer, and a cytosolic C-terminal domain having two coiled-coil domains. The position of the former is still unclear as in some vertebrates the first transmembrane domain may function as a signal peptide (Fig. 1) [77,78,110,112,115,117–120].

Homologs of the gene ric-3 have been identified in a great number of species such as the invertebrates *Ostertagia ostertagi* and *Drosophila melanogaster*, and the vertebrates *Xenopus laevis, Danio rerio*, *Mus musculus*, and *Homo sapiens* [109]. The *H. sapiens* ric-3 homolog shares 22% of the sequence with the *C. elegans* ric-3 gene and its structure differs merely in having a shorter N-terminal domain and only one coiled-coil domain (Fig. 1) [71,109,112,115,121]. Five distinct transcripts of human ric-3 (a, b, c, d and e) have been found [109,117,119]. The ric-3a isoform is encoded by clones AY326435 and BC022455 (GenBank® nucleotide sequence database accession numbers). These human ric-3 homologs are 2.9 kb in length. The BC022455 clone shares sequence identity with AY326435 except for a single serine. Transcript AK021670 is 1.5 kb long and corresponds to isoform ric-3b, which encodes only for a soluble coiled-coil domain [109]. Transcript AL832601 is 5.2 kb in length and corresponds to isoform ric-3c. The latter isoform encodes for the first membrane-spanning domain spliced directly to the C-terminus. The isoform ric-3d, corresponding to clones AY326436/AY358475 and BI832705, codes for the two trans-membrane domains only [118]. Finally, ric-3e (GenBank® nucleotide sequence database accession number AM422214) encodes a RIC-3 protein of 288 amino acids which lacks the coiled-coil domain and part of the C-terminal sequence [119].

3.2. Regulation of AChR by RIC-3

The degree of AChR regulation depends on various factors such as receptor and cell type (see Table 1) [67,71,111]. The neuronal receptor DEG-3/DES-2 is one of the four well-characterized AChRs in *C. elegans* [121]. Co-expression with RIC-3 was shown to be required for AChR activity in *C. elegans* body muscles and for enhanced AChR activity in *Xenopus* oocytes [110,112]. Co-immunoprecipitation studies have also provided proof of an interaction between RIC-3 and the α7 AChR subunit [71,111] and the α3, α4, β2 and β4 AChR subunits [71]. In contrast, RIC-3 caused a marked inhibition of functional responses with heteromeric α3β4 and α4β2 AChRs in *Xenopus* oocytes [109]. Co-expression of RIC-3 and chick α8 subunits in heterologous cell lines enhances AChR functional expression [71]. Co-expression of RIC-3 apparently has no effect on α9 or α10 AChR expression in cultured mammalian cell lines [71] and functional expression of α9α10 AChR in cultured mammalian cells is rare [71,122–124] Nevertheless, recent studies suggest that although co-expression of RIC-3 has no effect on the binding of ^{125}I-α-bungarotoxin to either homomeric α9 or α9α10 heteromeric receptors [122], such co-expression might enhance the effect of rapsyn on AChR clustering at the cell surface. Osman et al. [122] find that RIC-3 expression increases the total amount of α9 AChR in CL4 cells, supporting the view that RIC-3 regulates AChR trafficking by increasing the number of mature or correctly folded receptor subunits reaching the cell surface. Alternatively, RIC-3 might affect α9 levels in CL4 cells by regulating the turnover of the α9 receptor subunits. With the exception of the 5-HT$_3$ receptor, RIC-3 appears to have little or no effect upon other LGICs, including those activated by GABA and glutamate [71,85,109,112,118].

Additional host cell factors appear to be involved in modulating the chaperone activity of RIC-3 on AChRs [71,85,108–111,118,119,125]. Co-expression of RIC-3 with the 5-HT$_3$ receptor in *X. laevis* oocytes totally abolishes 5-HT$_3$ surface expression [108,109]. In contrast, RIC-3 was reported to enhance functional expression of 5-HT$_3$ receptors in a human kidney cell line [85,118,126]. It is noteworthy that RIC-3 has been shown to increase α7 AChR heterologous expression both in *X. laevis* oocytes and in HEK-293, CHO and SHE-P1 mammalian cell lines [66,71,77,107–112,119,125]. The evidence therefore suggests that RIC-3 is required for the correct folding of the α7 AChR and for it to attain functional expression in all cell systems tested so far.

Since all published results concur that RIC-3 interacts with α7 AChR, it is likely that α7 AChR distribution correlates with that of the RIC-3 protein. In general this is the case, although α7 AChR labeling is low [117]. Purkinje cells in the cerebellum appear to be an exception; these cells express α7 protein but, as previously stated, exhibit no detectable levels of RIC-3 [117]. There are also discrepancies in the functionality of the α7 AChRs expressed in these cells [127]. Such discrepancies may apply as well to cell lines that lack RIC-3 expression and upon transfection with α7 cDNA are incapable of expressing functional α7 AChRs at their cell membrane [111]. Interestingly, RIC-3 is also detected in some areas of the brain (corpus callosum, pituitary gland and the cerebellum) which express relatively low levels of α7 AChR (see below), suggesting that alternative mechanisms are operative in these loci to catalyze maturation of α7 AChR [119].

Ric-3 transcripts and RIC-3 protein are not confined to brain areas. In *C. elegans*, RIC-3 is also required for maturation of the levamisole-sensitive vulval muscle AChR and for maturation of the EAT-2 AChR that enables pharyngeal pumping [112,113].

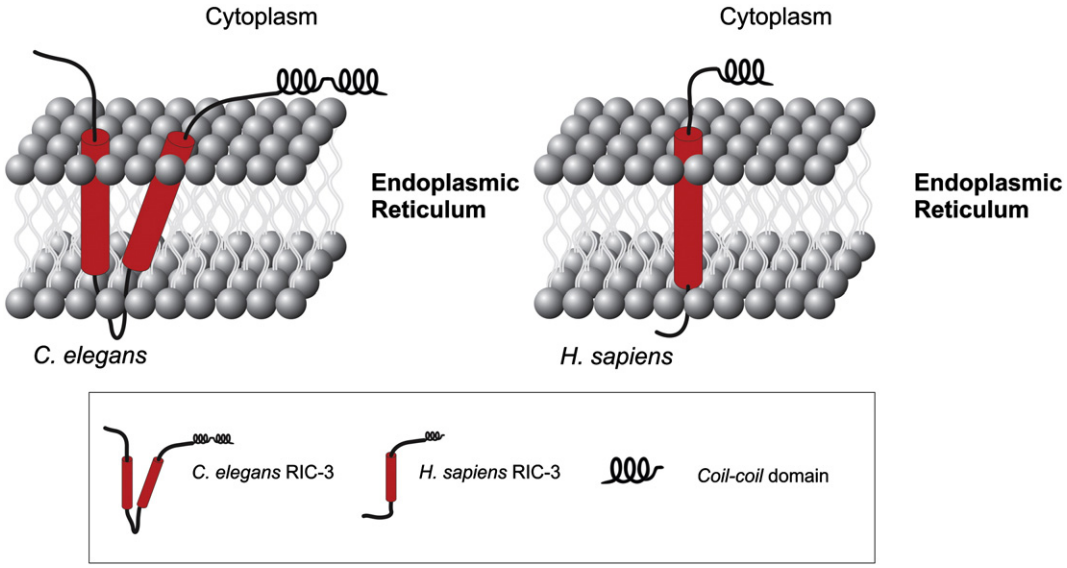

Fig. 1. The RIC-3 protein. In C. *elegans*, the RIC-3 protein (left) comprises an N-terminal domain (the exact position of which has not yet been clearly established), a membrane-spanning domain separated by a proline-rich spacer, and a C-terminal domain exposed to the cytoplasmic compartment, which possesses two coiled-coil domains. The suggested topology of the H. *sapiens* Ric-3 homolog (right) differs structurally simply in having a shorter N-terminal domain and only one coiled-coil domain [78].

3.3. Localization of RIC-3

Analysis of the distribution of ric-3 transcripts and RIC-3 protein facilitates identification of their in vivo targets. The presence of ric-3 transcripts has been demonstrated in most regions of mouse brain [109,111]. The areas with the highest ric-3 transcript signal were the CA1–CA3 region of the hippocampus, the deep nuclei and the Purkinje cell layer in the cerebellum, and the superior colliculus. In a more recent study [117] focusing on the distribution of the RIC-3 protein in rat brain, the authors found that it was broadly distributed with a moderate labeling intensity, in parallel with the localization of the corresponding mRNA in mouse brain [109]. Different levels of labeling were described: no signal was detected in the Purkinje cell layer and the molecular layer of the cerebellum; weak labeling was found in the granular layer and in pyramidal cells from different cortical areas; and moderate labeling was observed in the dentate gyrus and the CA1 and CA3 neurons of the hippocampus. Except for the medial habenula, where neurons were intensely labeled, all neurons from the thalamus as well as the striatum, the globus pallidum, the hypothalamus (primarily in the mammillary nuclei) and the substantia nigra neurons also displayed labeling of moderate intensity. The highest intensities of immunoreactivity to RIC-3 were found in the inferior olive and also in the dorsal cochlear nucleus, in the solitary complex, and in motor nuclei, such as trigeminal (V), abducens (VI), facial (VII) and hypoglossal (XII) nuclei in the brainstem. Interestingly neurons in the deep cerebellar nuclei appeared intensely labeled. No ric-3 transcripts were found in the dentate gyrus, although RIC-3 protein was detected. The opposite was found in the Purkinje cell layer, where ric-3 transcripts were found and no labeling for RIC-3 was observed. This lack of correspondence between the distribution of ric-3 transcripts and RIC-3 protein could be due to low levels of either protein or RNA, which would prevent their detection. Ric-3 transcripts were detected in some neuronal cell lines such as human neuroblastoma SH-SY5Y and pheochromocytoma PC12 [111,117] cells; RIC-3 protein was found in these cells. RIC-3 levels have been shown to increase during differentiation of SH-SY5Y and PC12 cells. The mechanism that activates RIC-3 expression upon cell differentiation has still not been clarified.

Table 1
Regulation of the level of expression of the nicotinic receptor by RIC-3 depends on the LGIC and cell type.

Receptor	Cell type	Effect	Reference
DEG-3/DES-2	*C. elegans*	Enhanced expression	[109,112,121]
DEG-3/DES-2	X. *laevis* oocytes	Enhanced expression	[109,112,121]
α7 AchR	X. *laevis* oocytes	Enhanced expression	[108,112,202]
α7 AChR	Mammalian cells	Enhanced expression	[66,71,108,111,125,202]
α8 AchR	Mammalian cells	Enhanced expression	[71]
α9 AChR	Mammalian cells	Unaffected	[71]
α9 AChR	Mammalian cells	Enhanced expression	[122]
α9α10 AChR	Mammalian cells	Unaffected	[71]
α3β4 AChR	Mammalian cells	Enhanced expression	[71]
α4β2 AChR	X. *laevis* oocytes	Inhibited expression	[107,109]
α3β2 AChR	Mammalian cells	Enhanced expression	[71]
α4β2 AChR	Mammalian cells	Enhanced expression	[71]
α4β4 AChR	Mammalian cells	Enhanced expression	[71]
5-HT_{3A}	Mammalian cells	Enhanced expression	[85,118,126]
α7/5-HT_{3A}	Mammalian cells	Inhibited expression	[108]
α7/5-HT_{3A}	X. *laevis* oocytes	Inhibited expression	[108]
GABA	X. *laevis* oocytes	Unaffected	[77,109,112]
GABA	Mammalian cells	Unaffected	[71,85]
Glutamate	X. *laevis* oocytes	Unaffected	[71,77,109,112]
Glycine	X. *laevis* oocytes	Unaffected	[71,109,112]

3.4. The endoplasmic reticulum is the arena for the chaperone action of RIC-3

Several studies support the view that RIC-3 is localized in the ER [91,126]. Since RIC-3 has been shown to interact with mutant AChR subunits that are unable to exit this organelle, it is highly plausible that the interaction between RIC-3 and AChR subunits occurs in the ER [85,91,108,111,112,118]. It is also possible that the missing chaperone in the work by Drisdel and coworkers [83] is no other than Ric-3. If this is the case, the chaperone role of RIC-3 might consist in making cysteine residues in α7 AChR accessible to palmitoylation at the ER, a necessary step for subsequent functional expression at the plasma membrane [82,83].This idea is in agreement with the fact that interaction with RIC-3 stabilizes receptors or receptor assembly intermediates [85,107].

It has been reported that both the N- and the C-terminal regions of RIC-3 are needed for enhancing AChR expression [108]. The C-terminus of all RIC-3 homologs contains one or two coiled-coil domains [77,109,110,115,119,120], known to be important for protein–protein interactions involved in the organization of molecular scaffolds, in addition to other functions [128]. However, deletion analysis of RIC-3 suggests that ablation of the coiled-coil domain does not modify the

capacity of RIC-3 to modulate the expression of AChRs or 5-HT_3 receptors [108,110]. Furthermore, the ric-3e isoform that contains deletions of the coiled-coil domain and part of the C-terminal domain, mimics the capacity of the whole RIC-3 protein to modulate α7 AChR subunits, promoting α7 AChR surface expression and functional receptor activity [119]. Similarly, no changes were found in the chaperone activity of naturally occurring RIC-3 variants lacking the coiled-coil domain in *D. melanogaster* [110]. It has been suggested that two other regions of RIC-3 are also involved in protein–protein interactions in *C. elegans*, the first involving the proline residues within the spacer between the two transmembrane domains. These amino acids may provide a structural scaffold on which to anchor interacting proteins. The second region involves the membrane-spanning domains. It is interesting that the second transmembrane domain in RIC-3 homologs shows conservation beyond that required for traversing the membrane [109]. Transmembrane interactions may therefore bring together AChR subunits and the RIC-3 homologs, the effects of RIC-3 thus being the result of more than one interaction mediated by several domains within the RIC-3 molecule.

Aside from interacting with AChR subunits or 5-HT_3 receptors, RIC-3 may be necessary for additional interactions with yet unidentified proteins to form a multiprotein maturation complex. Such machinery may be required as a scaffold for the maturation of α7 AChRs. Furthermore, proteins that interact with the RIC-3 C-terminus, such as BATH-42, a BTB- (broad-complex, Tramtrack and bric-a-brac) and MATH- (meprin-associated Traf homology), domain-containing protein, have recently been described [113]. Reduced expression of the latter protein has been shown to be detrimental to AChR function in *C. elegans* [113] (See Fig. 2).

4. Mechanism of α7 AChR modulation by phosphorylation/dephosphorylation

Protein phosphorylation and dephosphorylation are key mechanisms for regulating the activity of membrane proteins such as ion channels. For example, phosphorylation of glutamate receptors by Src-family kinases (SFKs) is associated with long-term potentiation (LTP) and spatial learning in the hippocampus [129–131]. Although the mechanisms that regulate phosphorylation of AChRs are still essentially unknown, protein tyrosine phosphorylation by the SFKs has been shown to affect peripheral AChRs in various ways, depending on the tissue, subunit type and functional role of the receptors involved. In mammalian muscle, SFKs interact with the AChR [132] and play a major role in receptor clustering and cytoskeletal anchoring of AChRs at the neuromuscular junction [133–140].

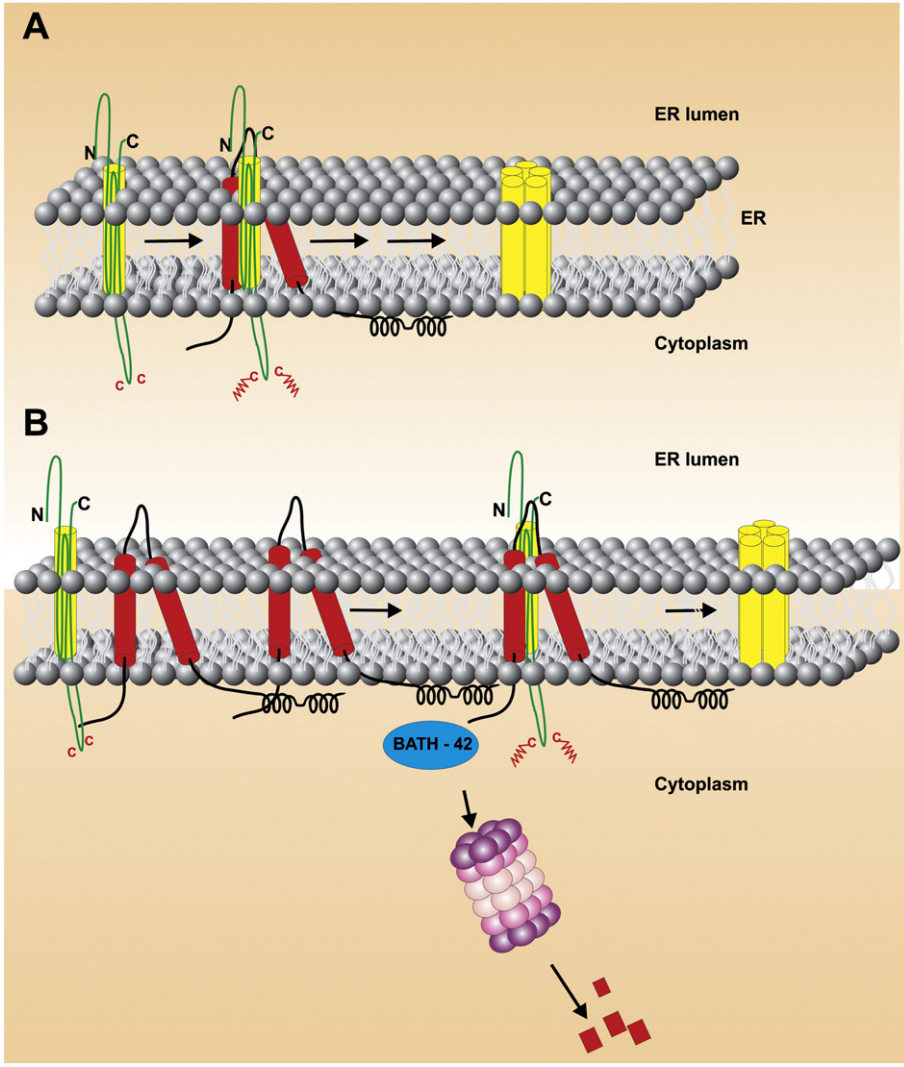

Fig. 2. Importance of a tight regulation of RIC-3 levels. BATH-42 interacts with the RIC-3 C-terminus and may form a multiprotein maturation complex required as a scaffold for the maturation of α7 AChRs at the ER [113]. A) RIC-3 interacts with α7 subunits and enhances α7 AChR assembly at the ER. B) Under physiological conditions RIC-3 is regulated by BATH-42. BATH-42 targets excess RIC-3 for degradation by the ubiquitin proteasome system, thus maintaining optimal levels of the chaperone and facilitating proper α7 AChR formation. C) Overexpression of BATH-42 is detrimental to AChR function, precluding RIC-3/AChR interaction. D) Overexpression of RIC-3 influences AChR distribution and function. Data suggest [113] that excess RIC-3 may lead to formation of RIC-3 aggregates that sequester AChR subunits away from the assembly process, thereby leading to a reduction in the formation of functional AChRs.

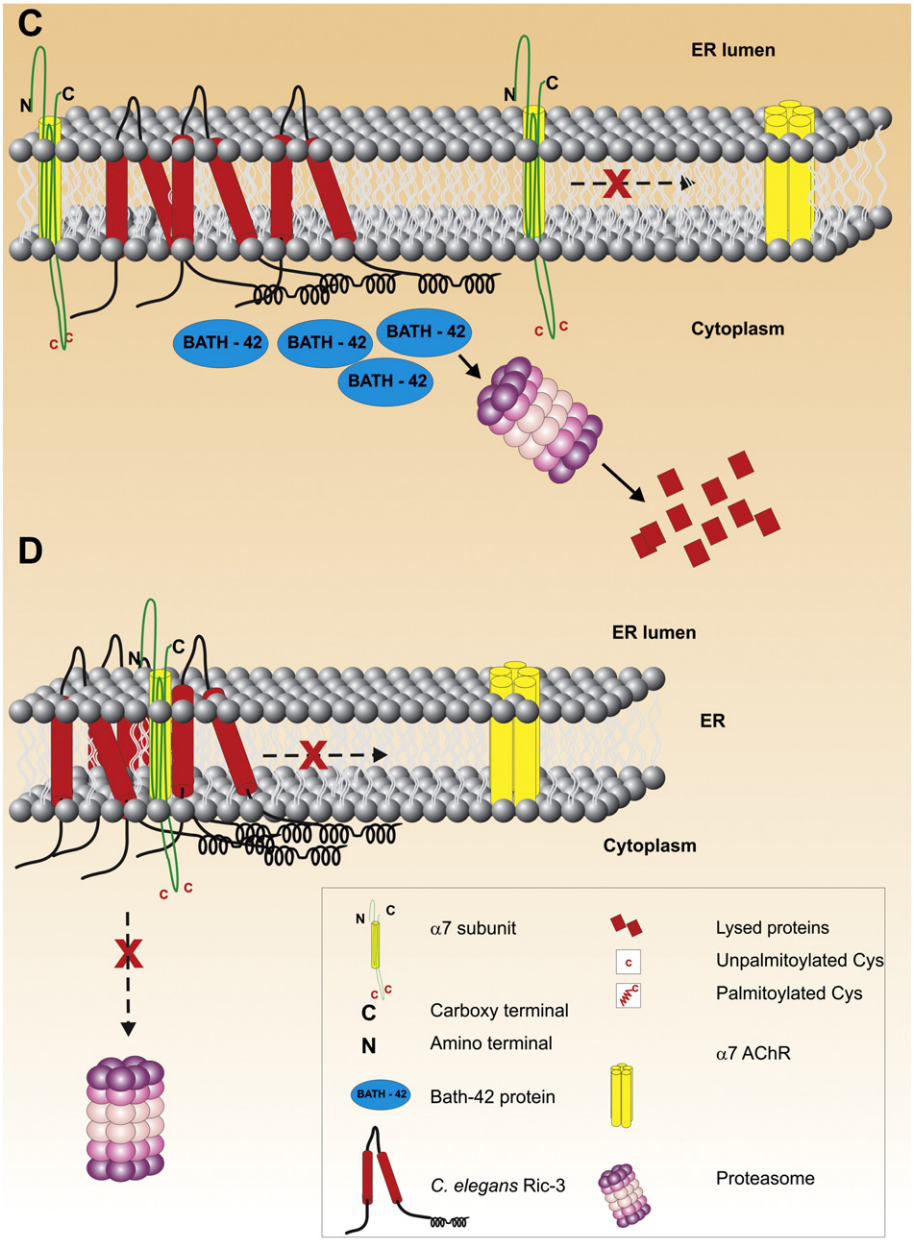

Fig. 2 (*continued*).

In *Torpedo* electric organ, phosphorylation of AChRs by SFKs causes subtle changes in desensitization kinetics but not in I_{max}, the maximal current flowing through the receptor channel [141–143]. In adrenal medulla chromaffin cells the tyrosine kinases c-SRC and FYN associate with the α3β4 receptor and are involved in the cholinergic stimulation of catecholamine secretion [141,144,145].

In cortical neurons, FYN associates with the α7 AChR [146,147]. Recent studies have also demonstrated the importance of the phosphatidylinositol 3-kinase (PI3K) pathway downstream of AChRs in protecting neurons from death and up-regulating these receptors [148]. Specifically, it has been shown that upon stimulation, α7 AChR activates PI3K via direct association with non-receptor type tyrosine kinase FYN and Janus-activated kinase 2 (JAK2), promoting the survival of neuronal cells (Fig. 3). This in turn proceeds via activation of the Akt-Bcl-2 pathway [149] since treatment with PP2, AG490, LY294002 and wortmannin-inhibitors of Fyn, JAK2 and PI3K, respectively- significantly inhibits neuroprotection by donepezil and galantamine, two acetylcholinesterase inhibitors [146].

A recent study [141] demonstrated that the balance between phosphorylation and de-phosphorylation of the α7 AChR by SFKs did indeed modify the I_{max} but not the time-course of the response in oocytes, SH-SY5Y cells, and hippocampal interneurons. The α7 subunit present in mammalian spermatozoa also associates with a member of the SFKs [32]. Tyrosine phosphorylation of α7 AChR was found to negatively regulate receptor activity in neuroblastoma cells, hippocampal CA1 interneurons, and supraoptic magnocellular neurons, whereas de-phosphorylation of α7 AChR was found to potentiate ACh-evoked currents in these cells. The mechanism of α7 modulation by phosphorylation does not involve modification of the number and clustering of receptors at the cell surface [141]. Instead, the potentiation induced by de-phosphorylation of α7 AChRs must stem from mechanisms other than insertion of additional receptors into the cell

membrane. Charpantier et al. [141] suggest that phosphorylation by SFKs affects the number of activatable receptors at the plasma membrane, such that de-phosphorylated receptors are responsive, whereas phosphorylated receptors are probably nonresponsive to agonist. Collectively, these observations suggest that modulation of the α7 AChR by SFKs constitutes an important regulatory mechanism for the activity of receptors at the cell surface.

5. Mechanism of α7 AChR modulation by brain-derived neurotrophic factor (BDNF)

BDNF regulates development of neuronal structures both in the peripheral and central nervous systems [150–155]. It has acute effects on the synapse, serving as an activity-dependent regulator of synaptic plasticity and participating in rapid synaptic transmission [150,151,156–159], in the maturation of GABAergic signaling and in the stabilization of newly formed synapses [151,160–163]. BDNF can also influence the level of α7 AChRs subunits (Fig. 4) in the hippocampus and other brain regions [160,164,165]. Recent studies using dissociated rat hippocampal neurons in culture demonstrated that BDNF increases both surface and internal α7 AChRs pools. This increment depends on glutamatergic activity and is restricted to distinct neuronal subtypes, suggesting the existence of cell-type specific regulatory mechanisms. In particular, interneurons inhibiting glutamatergic cells show large increments in α7 AChRs when exposed to BDNF, possibly due to de novo synthesis, since long exposure to BDNF is required to detect the increases in α7 AChRs [160].

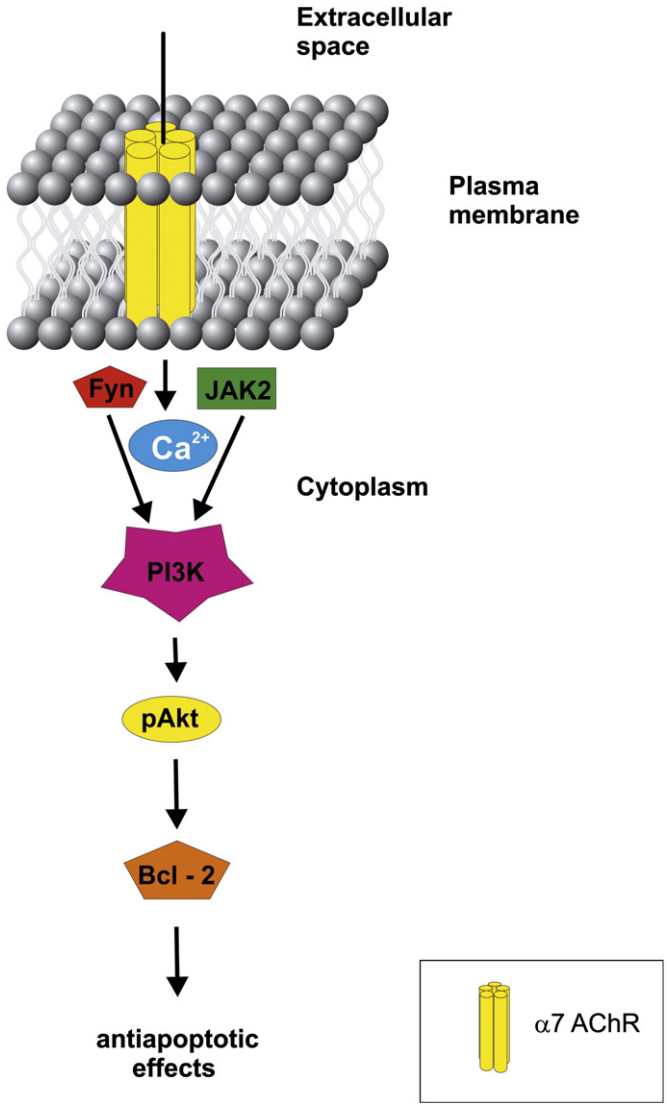

Fig. 3. α7 AChRs activate downstream signaling pathways. Co-immunoprecipitation studies have demonstrated that phosphatidylinositol 3-kinase (PI3K) and non-receptor type tyrosine kinase (Fyn) are physically associated with α7 AChR [147]. When stimulated, α7 AChRs activate PI3K and promote survival of neuronal cells via activation of Akt-Bcl-2 pathway [147]. Other studies demonstrate that janus-activated kinase 2 (JAK2) stimulates survival via the latter antiapoptotic pathway [146].

6. α7 AChR trafficking depends on soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)

Recently, α7 AChRs were found to co-distribute postsynaptically with target soluble SNAREs [166] (Fig. 5). Furthermore, nicotinic stimulation rapidly induced SNARE-dependent vesicular endocytosis accompanied by receptor internalization [166]. However, the number of surface α7 AChRs was not modified since a SNARE-dependent process also recruited receptors to the cell surface from internal pools (Fig. 5). It is interesting to note that trafficking of α7 AChRs induced by nicotine and dependent on SNARE proteins, both for receptor internalization and receptor recruitment to the cell surface, is a rapid process. This differs significantly from previously described forms of trafficking for other nicotinic receptors, which operate on slower time scales [167,168]. Furthermore, at the neuromuscular junction and for muscle AChRs expressed in CHO-K1/A5 cells, receptor blockade has been shown to accelerate the rate of nicotinic receptor removal but both the mechanism and time course differ significantly from those seen for α7 AChRs [169,170]. Additionally, SNARE-dependent trafficking was required for α7 AChRs to be capable of activating the transcription factor cAMP response element-binding protein and attendant gene expression when challenged. In other words, SNARE-dependent trafficking appears to be necessary for maintaining a functional link between α7 AChR responses and downstream signaling on somatic spines.

7. α7 AChR trafficking is influenced by the M3–M4 cytoplasmic loop

AChR subunits share a common topology, having a large N-terminal extracellular domain containing the ligand-recognition sites, four transmembrane domains (M1–M4), a large cytoplasmic domain between M3 and M4, which is highly divergent among different subtypes, and finally an extracellular C-terminal domain. Alignment of the first twenty amino acids of the M3–M4 cytoplasmic domain reveals a stretch of nine amino acids present in all AChR subunits that consists of two pairs of hydrophobic amino acids separated by five non-conserved amino acids. The cytoplasmic domain has been shown to be critical for the assembly of functional α7 AChRs [171] and for trafficking of α4β2 AChRs from the ER to the cell surface [172]. Mutations in this region of α7 subunits were found to abolish expression of mature AChRs, apparently by inhibiting conformational maturation of the subunits and consequently preventing their assembly into mature AChRs in the ER. A recent study showed that mutation of amino acids from this region (leucines 335, 336 or 343) to alanine reduced cell-surface expression of α7 AChRs [173]. Similar mutations in α4 and β2 subunits did not prevent assembly of mature α4β2 AChRs, which were capable of binding cholinergic ligands though unable to reach the cell surface [172]. These results suggest that mutations in the M3–M4 cytoplasmic domain affect α7 AChR biogenesis but not that of the α4 and β2 subunits.

The M3–M4 cytoplasmic domain of the AChR subunits has also been found to regulate targeting of neuronal AChRs (to pre- or post-synaptic sites). The influence of this region upon receptor targeting has been previously studied using expression of subunit chimeras in chick ciliary ganglion neurons by retrovirus-mediated gene transfer [174]. Mutational analysis performed in these neurons by changing the M3–M4 cytoplasmic loop of the α7 subunit for the analogous loop region of the α3 subunit resulted in alterations of α7 subunit

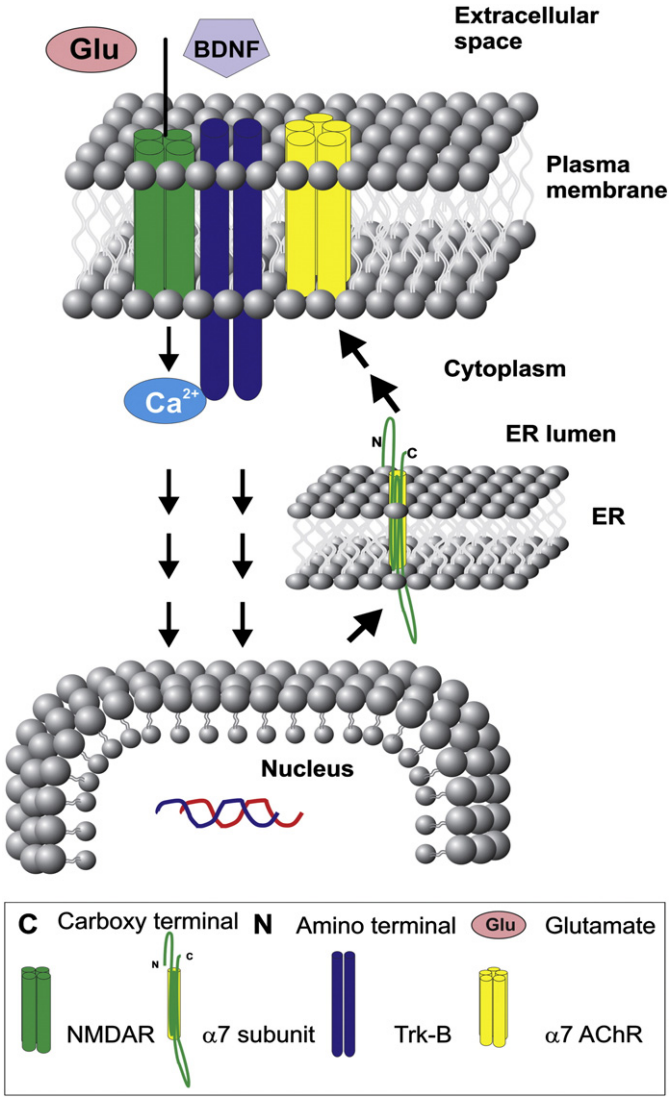

Fig. 4. α7 AChRs are modulated by brain-derived neurotrophic factor (BDNF). BDNF can influence the level of α7 AChR subunits in the hippocampus and other systems [164,165] by increasing both surface and internal pools of α7 AChRs, possibly by de novo synthesis of α7 AChRs. This increment depends on glutamatergic activity and is cell-type specific.

localization from perisynaptic to postsynaptic sites. However, when the M3–M4 cytoplasmic loop was replaced by that of either α5 or α4 subunits, no such effect was observed, implying that amino acid sequences within the M3–M4 cytoplasmic loop of the α3 subunit are involved in the targeting of receptors to postsynaptic regions in ciliary ganglion neurons [1,174,175]. Additionally, sequence motifs responsible for differential targeting to axons and dendrites have also been identified within these regions of the α4 and α7 subunits [176]. All in all, these studies highlight the importance of the M3–M4 domain and its influence on subunit folding, cell-surface expression and receptor targeting [177].

8. Pathological α7 AChR synthesis, assembly and exocytic trafficking

The ubiquitous occurrence and participation of the α7 AChRs in many cellular and physiological processes is bound to have pathological counterparts. The α7 AChR subtype has received much attention as a potential therapeutic target for a variety of pathologies [50,51,146,178–187].

Reduction of α7 AChRs in the CNS is linked with Alzheimer disease, which has been shown to lead to neuronal loss [53,188–190]. One of the salient events at early stages of this disease (usually preclinical) is the impairment in hippocampus-based episodic memory which can be improved by enhancement of cholinergic transmission [191]. Another important event that associates well with the Alzheimer disease pathology is the aggregation of the β-amyloid peptide [53]. This peptide interacts with α7 AChRs and has been reported to affect the normal functioning of the latter, causing reduced neuronal survival [146,192–194].

Decreased expression of α7 AChR has also been associated with schizophrenia [51,195–197]. Since α7 AChRs are highly permeable to calcium [198] and increased calcium permeability is required for neuronal migration [199], neurons with less α7 AChRs would fail to migrate to their correct destinations [200] and be activated by acetylcholine.

The role of RIC-3 in disease-associated cholinergic dysfunction is currently undefined. Interestingly, levels of RIC-3 mRNA are elevated in postmortem brains of individuals with bipolar disorder and schizophrenia [181], and a link has been suggested between deficient RIC-3 mediated chaperoning of an AChR subunit and individuals with bipolar disorder and psychotic symptoms [181]. Excess RIC-3 has been reported to be deleterious for AChR function and distribution, giving rise to the need for chaperone regulation [113,126]. Under physiological conditions, BATH-42 activity maintains optimal levels of RIC-3 by targeting excess RIC-3 for degradation by the proteasome (Fig. 2B). Consequently, inhibition of the proteasome leads to increased amounts of AChRs. Loss of function of BATH-42 interferes with AChR function by causing RIC-3 to increase [113]. In turn, overexpression of RIC-3 leads to formation of RIC-3 aggregates, reducing the amount of AChR subunits available for the formation of functional receptors [113] (Fig. 2D). Interestingly, overexpression of BATH-42 leads to a reduction of α7AChRs since it sends RIC-3 for degradation by the proteasome (Fig. 2C) and inhibition of the proteasome consequently leads to increased amounts of AChR. This suggests that a balance between degradation and assembly regulates the level of mature AChRs [113,114].

The α7 AChR expressed in macrophages plays an important role in the cholinergic anti-inflammatory pathway [45,180]. During acute inflammatory processes α7 AChRs attenuate renal failure induced by ischemia/reperfusion by inhibiting pro-inflammatory cytokine expression, and subsequently decreasing cell apoptosis [180,201]. Therefore a strict regulation of the levels of expression of α7 AChRs appears to be of key importance to the correct maintenance of physiological mechanisms within a wide spectrum of cells and tissues in the organism.

9. Concluding remarks

The neuronal-type α7 AChR interacts with different proteins at different stages of its biosynthesis, folding, assembly, and trafficking. The participation of the chaperone protein RIC-3 is of unique importance in the life of the α7 AChR, constituting an essential requirement for functional expression. Functional potentiation is also mediated by post-transcriptional events such as SFK phosphorylation or phosphatase activity. Additionally, SNARE protein-dependent trafficking appears to be necessary for maintaining a functional link between α7 AChR responses and downstream signaling, at least in neuronal somatic spines. Structural analysis of the α7 AChR proteins has shown that trafficking is also influenced by the M3-M4 cytoplasmic loop whereas receptor assembly requires the interaction between the N-terminal α-helix and the β-strands β2 and β3.

AChRs have been linked to many neurodegenerative diseases and numerous studies for palliative treatments are currently underway. A complete characterization of AChR synthesis, assembly and trafficking constitutes a fundamental step in understanding physiological mechanisms which may contribute to the development of therapeutic drugs for treating these diseases.

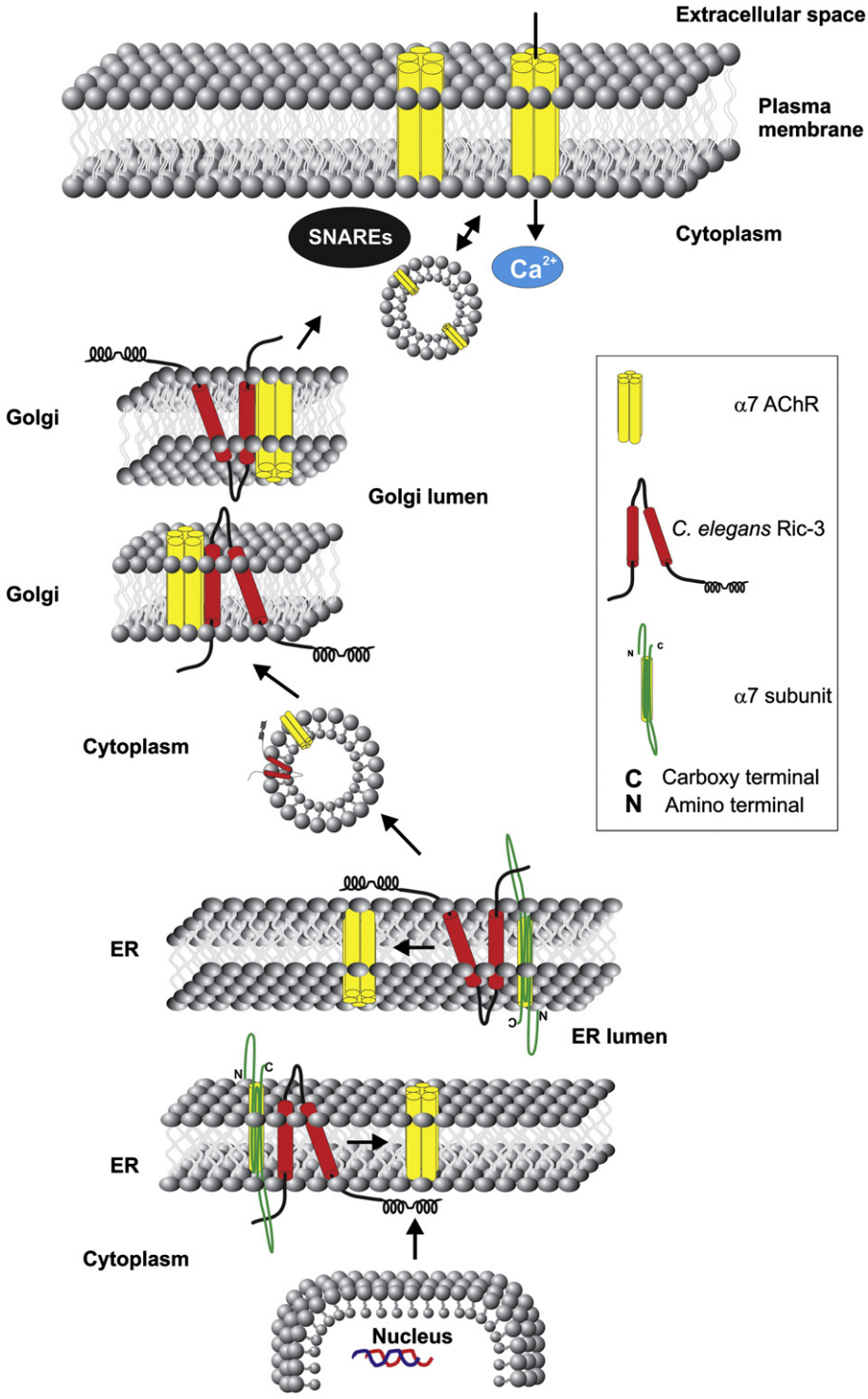

Fig. 5. α7 AChRs are modulated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE). α7 AChRs on neurons are subject to rapid SNARE dependent trafficking [166]. The latter is bidirectional, involving both internalization of activated α7 AChRs and recruitment of new receptors from intracellular pools to repopulate the plasma membrane. This process is dependent on extracellular calcium and may thus be initiated by α7 AChR activation, since the α7 AChR is extremely permeable to Ca^{2+}.

Further studies are required to fully elucidate the exact mechanisms that determine receptor maturation. Among the issues remaining to be addressed are whether different subunit domains influence assembly and trafficking; what determines the functional state of AChRs; what determines subtype specificity of chaperone proteins; and the role of scaffolding proteins in AChR targeting to the plasma membrane. These and more are just some of the pieces of a still unresolved puzzle.

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