Biochimica et Biophysica Acta 1818 (2012) 718–729

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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

ARTICLE INFO

Article history:
Received 2 April 2011
Received in revised form 25 September 2011
Accepted 17 October 2011
Available online 22 October 2011

Keywords:
Molecular chaperone
RIC-3
α7
Receptor trafficking

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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doi:10.1016/j.bbamem.2011.10.012

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].