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RIC-3, a potential target for regulating cholinergic signaling and inflammation

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ABSTRACT

The nic acetylcholine receptor (nAChR) gene family encodes for subunits of acetylcholine gated ion channels. These receptors are expressed widely and have many including an tory e ects mediated by the α7 nAChR, as part of the cholinergic an tory pathway, in immune cells, microglia and astrocytes. Matura of α7 nAChRs into ligand-gated ion channels in the plasma membrane is a complex process likely to require the RIC-3 protein. This endoplasmic re resident chaperone a ects matura of nAChRs, but its inter with these receptors and its e ects on their matura er for erent nAChRs. Moreover, these inter and e ects are regulated by mechanisms. Gene analysis has implicated RIC-3 in the neuroin tory disease Sclerosis (MS), and in the neurodegenera e Parkinson's disease (PD). Neuroin contributes to the progression of neurodegenera e diseases including PD. This informa combines to suggest that RIC-3 may contribute to progression of both MS and PD via its e ects on the α7 nAChR and the cholinergic an in tory pathway. Furthermore, we suggest that mechanisms regula RIC-3 expr y have a role in controlling in

The nicotinic acetylcholine receptor family

The nicotinic acetylcholine receptors (nAChRs) are a large and diverse family of acetylcholine-gated ion channels. In mammals this family is composed of nine alpha subunits and seven non-alpha subunits. These subunits assemble to form mostly heteromeric (α7 and α9 nAChR subunits can form homomeric receptors), cation-selective channels having diverse properties and expression patterns. In skeletal-muscles and in the autonomic nervous system nAChRs mediate excitatory synaptic transmission. However, in the central nervous system (CNS) nAChRs do not usually mediate synaptic transmission and instead play modulatory roles, including a role in regulating neurotransmitter release, reviewed by Dani and Bertrand[1]. In addition to being expressed in muscles and neurons, nAChRs are also found in multiple types of non-excitable cells where they affect migration, differentiation, proliferation, and signal transduction[2].

CNS-expressed nAChRs have been implicated in memory, cognition, addiction, and several neurodegenerative diseases. Evidence for these functions comes from the addictive effects of tobacco-derived nicotine and from epidemiological studies linking tobacco smoking to neurodegenerative diseases such as schizophrenia and Parkinson's disease (reviewed by Dani and

Bertrand[1]). In addition, decreased brain expression of nAChRs was shown in Alzheimer's disease, Parkinson's disease (PD), Lewy-body dementia, and schizophrenia[3-5].

Alpha7 nAChR and the cholinergic anti-inflammatory pathway

The α7 nAChR was first identified as the α-bungarotoxin binding receptor in the CNS[6]. The wide distribution and high calcium permeability of this receptor suggested an important role for this nAChR[7,8]. However, knockout mice are viable and show no gross abnormalities in development or behavioral phenotypes[9]. In contrast, in humans, copy number variations in the gene encoding for the α7 subunit, *CHRNA7*, are associated with brain diseases such as epilepsy or autism[10]. Understanding the role of *CHRNA7* in diseases of the nervous system is, unfortunately, hampered by the fact that most deletions encompassing this gene also cover additional genes. Nevertheless, behavioral phenotypes associated with these deletions are likely to be a result of *CHRNA7* haploinsufficiency as rare small heterozygote deletions covering *CHRNA7* alone are associated with similar phenotypes to those associated with larger deletions[10]. This conclusion, however, is complicated by findings showing that some heterozygous carriers of *CHRNA7* containing deletions are phenotypically normal, possibly a result of incomplete penetrance of these deletions[10].

CHRNA7 knockout mice show no readily discernable phenotypes[9]. Nevertheless, α7 nAChR has one well-established role, i.e., mediating the anti-inflammatory effects of acetylcholine, as part of the cholinergic anti-inflammatory pathway. This pathway enables inhibition of inflammation by vagal efferents and depends on α7 nAChR expression in immune cells[11,12]. For its anti-inflammatory effects, α7 nAChR functions via activation of signaling cascades such as MAPK and NF-kappaB and via regulation of gene expression[13-15]. This pathway, first shown to function in macrophages, is now known to function in multiple immune cells and in glial cells, micrglia, and astrocytes, having immune functions in the CNS, reviewed by Treinin et. al.[16].

Maturation of nAChRs and the RIC-3 protein

Maturation of nAChRs is a complex and inefficient process requiring assistance from cellular factors. Several proteins were shown to affect functional expression and properties of nAChRs. These proteins differ in specificity and mode of action, as reviewed by Treinin[17]. The best characterized of these proteins are LYNX1, functioning at the plasma membrane to affect properties of nAChRs[18], and RIC-3 and NACHO, endoplasmic reticulum resident chaperones affecting functional expression of multiple nAChRs[19,20].

Mutations in the *C. elegans ric-3* gene were first identified in a screen for resistance to cholinesterase inhibitors[21]. Additional mutations were found by the Treinin lab in a screen for suppressors of neuronal degeneration caused by constitutive activity of a *C. elegans* nAChR, DEG-3/DES-2[22]. Characterization of the *C. elegans* RIC-3 protein showed that it resides in the endoplasmic reticulum and is needed for maturation of multiple nAChRs. Further analysis showed conservation of *ric-3*'s sequence and function in evolution[19]. Importantly, RIC-3 enables functional expression of α7 nAChR when heterologously expressed in non-neuronal cells, even in cells that cannot normally express this receptor[23]. Additionally, brain expression patterns of mouse *ric3* mRNA and *CHRNA7* overlap, a result consistent with the suggestion that mammalian RIC-3 is required for α7 nAChR's maturation in vivo[19].

RIC-3 was shown to affect stability of unassembled nAChR subunits, assembly of subunits to form a nAChR, and trafficking of nAChRs[24-29] (Figure 1 and below). In *C. elegans* loss of *ric-3* gene function led to reduced plasma membrane expression of multiple nAChRs, demonstrating a positive, nAChR expression promoting function for RIC-3[22]. In contrast, RIC-3's effects on mammalian nAChRs are diverse. First, positive or negative effects on expression of functional receptors at the surface were demonstrated, depending on the identity of the nAChR and on the expression system

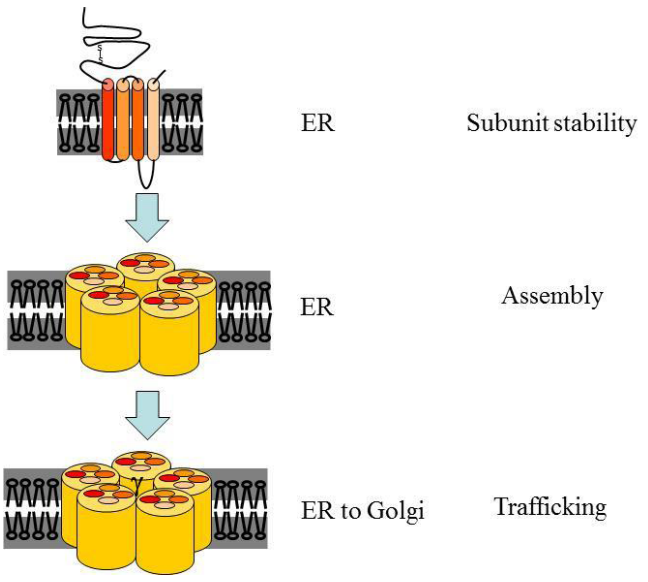

Figure 1. Steps in the maturation of nAChRs affected by RIC-3. Transla subunit folding, and assembly of nAChRs occur in the endoplasmic re (ER). Several aspects of this matura process are a ected by RIC-3. First, some unassembled subunits are stabilized by RIC-3. Second, RIC-3 increases assembly of some receptors and a ects the subunit stoichiometry within the DEG-3/DES-2 receptor. Third, RIC-3 a ects tra of fully assembled receptors from the ER to the Golgi on their way to the plasma membrane. Notably, e ects on tra include both e and nega e e ects.

used[19,30]. Recently we showed that these opposite effects on nAChR expression can be achieved using different RIC-3-to-receptor ratios, and that sensitivity of nAChR maturation to this ratio depends on the identity of the nAChR and on the specific RIC-3 isoform (different isoforms are produced by alternative splicing, see below)[31]. Second, RIC-3 was shown to affect different aspects of nAChR maturation depending on identity of the receptor's subunits. Specifically, RIC-3 increased assembly and trafficking to the plasma membrane of α7 receptors, but not the quantity (likely to indicate stability) of α7 nAChR subunits; in contrast, RIC-3 increased quantity of α4 and β2 subunits while having no effect on their assembly into α4β2 receptors[29]. Thus, RIC-3 may interact with and affect different nAChRs differently.

Consistent with the suggestion that RIC-3 interacts differently with different nAChRs are results of structure-function analysis showing that mutations and deletions having no effect on one receptor strongly affect other receptors[25,32]. Moreover, RIC-3 was shown to affect properties (kinetics of desensitization and affinity to acetylcholine) of the *C. elegans* DEG-3/DES-2 nAChR. These effects were attributed to a RIC-3-dependent increase in the DEG-3 to DES-2 stoichiometry of this receptor, likely to be the result of preferential interaction between RIC-3 and the DEG-3 subunit, leading to its preferential insertion into the mature receptor[24,25].

Mechanisms regulating RIC-3 expression and activity and their implications

As described above the nAChR family is large and diverse. Nevertheless, RIC-3 was shown to interact with and affect multiple nAChRs, differing in sequence and function[19,30]. But, these interactions and their outcome are variable as described above. This variability in function and interaction, combined with bioinformatics analysis, suggested that RIC-3 functions as an intrinsically disordered protein[25] – proteins found to elicit opposing (inhibiting or activating) effects, even on the same partner molecule[33]. The variable and flexible interaction of intrinsically disordered proteins with their targets, and the extended protein interface dedicated to these interactions, favor regulation by alternative splicing and post-translational modifications, as these regulatory mechanisms can modify the interaction between the intrinsically disordered protein and its target thus regulating the outcome of the interaction[34]. Indeed, in *C. elegans* and mammals *ric*-3 expression, splicing, and activity are regulated, as described below and in Figure 2.

First, RIC-3 was shown to have either positive or negative effects on co-expressed receptors depending on the RIC-3-to-receptor ratio[31]. Thus mechanisms regulating RIC-3 expression or stability are likely to regulate cholinergic signaling via nAChRs. Indeed, our work identified mechanisms regulating RIC-3 quantity: In *C.* elegans BATH-42, an adaptor for CUL-3-dependent ubiquitin ligase, was shown to interact with RIC-3, an interaction leading to CUL-3-dependent degradation of RIC-3[35]. And in murine macrophages we have shown that immune activation leads to a transient reduction in *ric*-3 mRNA[31]. Last, *ric-3* expression was upregulated in postmortem brains of patients with Schizophrenia or Bipolar disorder[36].

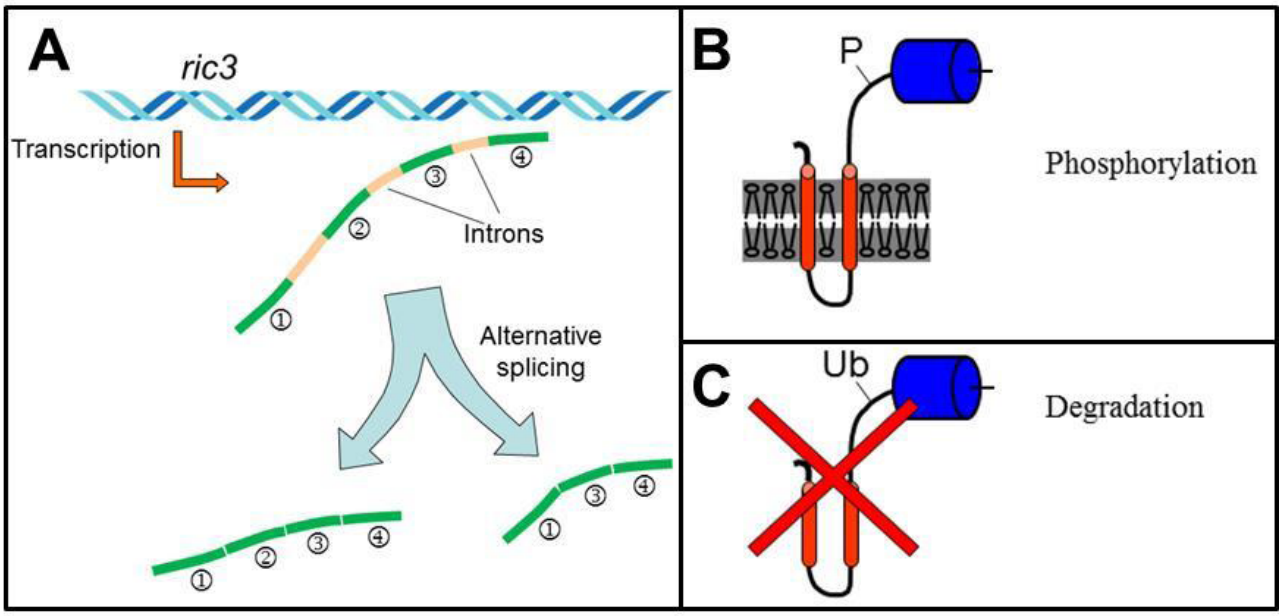

Figure 2. Mechanisms regulating RIC-3 expression and activity. A) In mammals *ric-3* transcrip and alterna e splicing are regulated during in with the gene's transcrip being upregulated in Schizophrenia and Bipolar disorder pa ts. B) In *C. elegans* phosphoryla and dephosphoryla of RIC-3 a ect its inter with receptors. C) In *C. elegans* of RIC-3 is controlled y the CUL-3 adaptor, BATH-42, to protect cells for the deleterious e ects of RIC-3 overexpression.

Second, phosphoproteomic analysis showed that in *C. elegans* RIC-3 is phosphorylated at multiple sites[37]. Analysis of one of these sites identified a Calcineurin-dependent signaling pathway regulating RIC-3 dephosphorylation and muscle excitability. Calcineurin is a calcium regulated phosphatase, thus we hypothesized that this pathway enables activity-dependent regulation of muscle excitability.

Third, vertebrates and invertebrates express multiple *ric-3* transcripts, a result of alternative promoters, alternative splicing, and editing, reviewed by Treinin[17]. One conserved alternatively spliced RIC-3 isoform, lacking the conserved C-terminal coiled-coil domain, is defective for either the positive or negative effects of RIC-3 in a nAChR subtype dependent manner[31].

RIC-3 and diseases of the Central Nervous System

RIC-3 was implicated in CNS diseases via expression studies and genetics. Expression analysis showed increased expression of *ric3* in postmortem brains of patients with Schizophrenia and Bipolar disorder[36]. Genome-wide association studies demonstrated an association between single nucleotide polymorphisms upstream to the *ric3* gene, which may affect its promoter, and MS or specific types of MS lesions[38,39]. And, RIC-3 was recently implicated in PD, as two different mutations affecting this protein were identified in patients. One mutation, P57T, was identified in a family with several members having late-onset PD with non-motor phenotypes; in this family all members carrying the RIC-3 mutation showed some disease phenotypes, with younger carriers showing weaker symptoms. The second mutation, V168L, was identified in an unrelated early-onset case[40].

Multiple sclerosis (MS) is an inflammatory, demyelinating, immune-mediated disease of the CNS leading to axonal injury, axonal loss, and neurological deficits. Experimental Autoimmune Encephalomyelitis (EAE) serves as an animal model for the study of MS. The cholinergic anti-inflammatory pathway was suggested to influence MS progression. Indeed, cholinergic upregulation by acetylcholine esterase inhibitors or by nicotine reduced disease severity as well as causing immune-modulation, an effect mediated by the α7 nAChR[41,42]. Moreover, the cholinergic anti-inflammatory pathway was implicated in MS by studies comparing anti-inflammatory effects of nicotine on peripheral blood mononuclear cells (PBMCs) from patients vs. healthy donors[43]. This together with the known role of RIC-3 in α7 nAChR maturation suggests that *RIC3* variants may affect MS via their effects on α7 nAChR maturation in immune cells. Importantly, we demonstrated that *ric3* expression in immune cells is dynamically regulated following immune activation[31]. Moreover, recent unpublished results show that *ric3* expression is transiently reduced in splenocytes and is gradually reduced in spinal cords during EAE progression (Yael Ben-David, unpublished). This suggests that regulation of RIC-3 expression is a means for regulating inflammation during EAE and MS.

PD is a neurodegenerative disease mainly affecting the motor system due to loss of dopaminergic neurons in the substantia nigra. Inflammation involving reactive astrocytes is known to contribute to progression of PD[44]. Involvement of nAChRs in PD was suggested by epidemiological studies showing reduced occurrence of this disease in smokers and altered expression of nAChRs in patients and disease models[4,45]. But, the mechanisms of action of nicotine and nAChRs in reducing PD are, as yet, unknown. Suggested mechanisms include neuroprotection[46,47], effects on dopamine release[48], and reduced neuro-inflammation resulting from activation of astrocytes or microglia[49]. Thus, the mechanisms of action of PD-associated RIC-3 mutations in this disease need to be identified. But, the demonstrated effects of these mutations on α7 nAChR surface expression[40], the known role of this nAChR in regulating inflammation (above), and contribution of inflammation to neurodegenerative processes occurring in PD[44,50], suggest a mechanism involving decreased activity of the cholinergic anti-inflammatory pathway as a mechanism explaining effects of both PD-associated RIC-3 mutations and nicotine on PD progression.

Conclusions

RIC-3 is an ER resident chaperone affecting maturation of multiple nAChRs including strong effects on functional expression of α7 nAChR. Several mechanisms regulate activity and expression of RIC-3, and the effects on nAChR maturation of these regulatory mechanisms depend on the nAChR subtype. RIC-3 has been implicated in progression of CNS diseases to which neuroinflammation and the cholinergic anti-inflammatory pathway are likely to contribute. Future work, therefore, should examine effects of genetic variants and regulatory mechanisms affecting RIC-3 expression and activity on the cholinergic anti-inflammatory pathway and neuroinflammation occurring in these diseases.

Conflict of interest statement

No conflict of interest to declare

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