

Review

Autoimmune attack of the neuromuscular junction in myasthenia gravis: nicotinic acetylcholine receptors and other targets

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8 **Autoimmune attack of the neuromuscular junction in myasthenia gravis:**
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10 **nicotinic acetylcholine receptors and other targets**
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45 The abbreviations used are: Ab, antibody; BTX, α -bungarotoxin; mAb monoclonal antibody;
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47 nAChR, nicotinic acetylcholine receptor; MG, myasthenia gravis; MuSK, muscle-specific
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49 kinase.
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Abstract

The nicotinic acetylcholine receptor (nAChR) family, the archetype member of the pentameric ligand-gated ion channels, is ubiquitously distributed in the central and peripheral nervous systems and its members are the targets for both genetic and acquired forms of neurological disorders. In the central nervous system nAChRs contribute to the pathological mechanisms of neurodegenerative disorders, such as Alzheimer and Parkinson diseases. In the peripheral nerve-muscle synapse, the vertebrate neuromuscular junction, “classical” myasthenia gravis (MG) and other forms of neuromuscular transmission disorders are antibody-mediated autoimmune diseases. In MG, antibodies to the nAChR bind to the postsynaptic receptors and activate the classical complement pathway culminating in the formation of the membrane attack complex, with the subsequent destruction of the postsynaptic apparatus. Divalent nAChR-antibodies also cause internalization and loss of the nAChRs. Loss of receptors by either mechanism results in the muscle weakness and fatigability that typify the clinical manifestations of the disease. Other targets for antibodies, in a minority of patients, include muscle specific kinase (MuSK) and low-density lipoprotein related protein 4 (LRP4). This brief review analyzes the current status of muscle-type nAChR in relation to the pathogenesis of autoimmune diseases affecting the peripheral cholinergic synapse.

Keywords: myasthenia gravis; neuromuscular junction; endplate; nicotinic acetylcholine receptor; autoimmune diseases; anti-receptor antibody.

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4
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Introduction

The superfamily of pentameric ligand-gated ion channels (pLGIC) is comprised of neurotransmitter receptor proteins which, during the course of evolution, acquired and perfected the ability to transduce chemical into electrical signals and thus mediate fast communication¹. These receptors share a common architecture of five subunits pseudo-symmetrically arranged around a central pore, the ionic channel. Their structural similarity extends to sharing a relatively large extracellular domain, which carries the ligand recognition site (the canonical or orthosteric agonist binding site) at the subunit interfaces. They also have in common four α -helical transmembrane segments (M1-M4) in each subunit making a total of 20 membrane-embedded polypeptide segments in the transmembrane domain, whose elements are connected by extra- and intra-cellular links. The transmembrane segments, and the five M2 helices in particular, conform the walls of the ion channel proper and the functional domain determining the gating of the channel. The transmembrane domain is also involved in allosteric modulation of channel function. Finally, the intracellular cytoplasmic domain, much smaller than the extracellular domain, carries sites for channel regulation via posttranslational modifications and is indirectly involved in channel conductance.

The pLGIC superfamily includes the nicotinic acetylcholine receptor (nAChR) families, γ -aminobutyric acid type A or C (GABA_{A/C}) receptors, glycine receptors (GlyR), the subtype 3 of the serotonin (5-HT₃) receptor families and the glutamate-gated chloride channel (GluCl)² family. In terms of their functional properties, these receptors can be divided into two categories: cation-selective channels (nAChR and 5-HT₃ receptors) and anion-selective

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3 channels (glycine and GABA_{A/C} receptors). They are widely distributed throughout diverse
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5 phyla in the animal kingdom ¹⁻² and unique combinations of ionic selectivity and
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7 cellular/tissue distribution determine their specialized functions in brain and elsewhere.
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11 Dysfunction of pLGICs results in several disorders of the central (CNS) and peripheral (PNS)
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13 nervous systems ². In many instances, these pathologies involve the ion conduction
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15 pathway of the macromolecules in question, falling into the category of
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17 “channelopathies”, a term coined more than two decades ago ³. Several CNS neurological
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19 and neuropsychiatric disorders may be called channelopathies. For instance, nAChRs have
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21 been reported to function abnormally in schizophrenia spectrum disorders, bipolar
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23 disorders, eating disorders, stress, addictions and in particular nicotine addiction and
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25 alcohol use disorders, cognitive and memory disorders, autism spectrum disorders,
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27 psychoses, and certain forms of epilepsy ^{4,5}. Alzheimer and Parkinson diseases, both likely
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29 of multifactorial origin ⁶, stand out among the disorders involving CNS nAChRs, and their
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31 commonalities -such as cognitive impairment evolving more frequently than not into
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33 terminal dementia- are tightly linked to nAChR dysfunction. In the CNS, the nAChR
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35 molecule is expressed in various combinations of subunits in different brain regions, not
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37 only in neuronal cells, but also in astroglia, microglia, oligodendroglia and endothelial cells
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39 ⁷. It is thus not surprising that the pathologies associated with nAChR dysfunction are so
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41 pleomorphic and exhibit such a wide repertoire of signs and symptoms.
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51 In addition to the wide distribution of the nAChR in the CNS and PNS, its widespread
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53 occurrence in non-neural tissues ⁷ has gained relevance in connection with the anti-
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55 inflammatory role it plays in pathological conditions such as ischemia, rheumatoid arthritis
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3 and pancreatitis, among others. Its expression is also of medical importance in lung and
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5 breast due to the involvement of these receptors in carcinomas of these organs ^{8,9}: e.g. an
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7 oncogenic role is attributed to the nAChR ¹⁰, thus making it a possible therapeutic target
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13 In the PNS, myasthenia gravis (MG) ¹² is the best characterized disease related to nAChR
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15 dysfunction. The pathogenic effectors as well as the biomarkers of MG are a group of
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17 autoantibodies mainly directed at the nAChR¹³. Other proteins present at the
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19 neuromuscular junction (NMJ) ¹⁴ have been reported to also elicit an autoimmune
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21 response similar to that observed with the nAChR, although rarer, and their pathogenic
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23 mechanisms are of additional interest.
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29 Several comprehensive reviews on nAChR and MG have appeared during recent decades
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31 (see e.g. refs. ^{13,15-19}). This short review attempts to summarize the current state of
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33 knowledge on nAChR structure, the molecular cascades of the clustering mechanisms
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35 involved in NMJ development, and the pathogenesis of the autoimmune disease MG
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37 affecting the muscle end-plate nAChR. In addition to the discussion of the “classical”
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39 target of the autoimmune attack extensively studied during the last few decades, the
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41 nAChR macromolecule, other membrane proteins present at the NMJ have been
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43 identified as immune targets in recent years and are also analyzed in this review.
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49 **The muscle-type nAChR**

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52 The nAChR is a pentameric macromolecule of roughly a quarter of a million Daltons made
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54 up of 5 subunits. So far, 17 nAChR different subunits have been found in the animal
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3 kingdom. There are two main types: α and non- α . The muscle-type α 1-subunit containing
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5 nAChR occurs at the NMJ of vertebrates^{14,20} and the electroplax of elasmobranch and
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7 teleost electric fish like the fresh water eel *Electrophorus electricus* and salt water
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9 *Torpedinidae*, respectively. The two latter constitute faithful model systems for which
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11 most of the molecular properties of the muscle-type receptor have been characterized
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13 over the course of the last five decades. The muscle-type nAChR is made up of 2 α and one
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15 β , δ , and γ each in its fetal form and 2 α , β , δ , and ϵ in the adult stage^{21,22}. In the muscle-
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17 type receptor, the acetylcholine (ACh) binding sites are located at the interface of the α - δ
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19 and the α - γ (ϵ) subunits²³. Three loops contributed by each α -subunit, termed A, B and C,
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21 form one wall of the canonical agonist recognition site; loops D, E and F, provided by the
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23 non- α subunits, constitute the complementary wall of the binding site²⁴. Figure 1 shows
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25 the overall architecture of the muscle-type nAChR.
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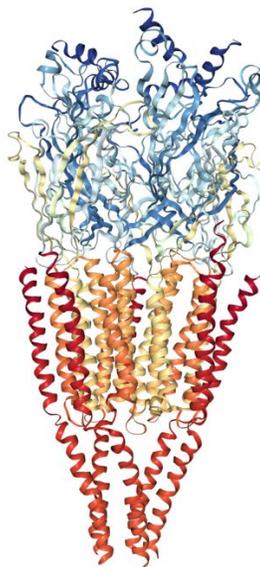


Figure 1. The nAChR molecule. Three domains are apparent: a large extracellular domain with abundant β -strand secondary structure (upper portion), the all-helical transmembrane region

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3 (middle portion), comprising 20 helices, 4 from each subunit, and the smaller cytoplasmic region
4 with a single helix from each subunit (bottom portion). The molecular model was drawn using the
5 molecular coordinates from the density maps deposited in the Protein Data Bank in Europe of the
6 PDB file with the accession number 4BOR (Zuber and Unwin, 2013)²⁵, <http://www.ebi.ac.uk/pdbe/>
7 and the software MolSoft Browser vers. 3.8.
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13 The muscle-type nAChR was the first neurotransmitter receptor system to be explored at
14 the single-molecule level with the single-channel patch-clamp recording technique^{26–28}.

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17 The single-channel activity of the muscle-type receptor consists of bursts of individual
18 openings originating from the same channel, separated by relatively long inter-burst silent
19 periods of channel inactivity in which the nAChR is in the desensitized state. It has been
20 possible to ascertain that the much shorter silent periods within a burst (the intra-burst
21 closed time) correspond to the transitions between unliganded, closed states and the bi-
22 liganded, full open channel state of the receptor.
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32 33 **Pathogenesis and clinical manifestations of MG**

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37 In MG, most of the autoantibodies are directed at the nAChR. This receptor has a set of
38 antibody epitopes which are mainly located around a loop of amino acids 66–76 on the $\alpha 1$
39 subunit that has been called the main immunogenic region (MIR); many but not all
40 autoantibodies of MG patients bind to this region²². When this loop interacts with the N-
41 terminal amino acids 1–14 of the nAChR $\alpha 1$ subunit, the receptor adopts a change in its
42 three-dimensional configuration that enhances antibody binding to the MIR²⁹.
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53 MG is an organ-specific autoimmune disease that meets all Witebsky's postulates of
54 autoimmunity: it is an auto-antibody-mediated pathology, the corresponding antigens
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3 have been identified, and an analogous autoimmune response can be induced by the
4
5 purified protein in experimental animal models. The disease in animals can be elicited
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7 either by immunization or by passive transference of human MG Abs ³⁰.
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10 Historically, MG was originally described as a disease affecting the NMJ and caused by
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12 anti-nAChR autoantibodies ^{12,15,31}. The clinical manifestations of the disease result from
13
14 the alteration of the transmission of impulses between the nerve and the muscle. The
15
16 muscular weakness associated with fatigability constitutes the cardinal symptom.
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19 Autoimmune MG currently has a reported worldwide prevalence of 40–180 per million,
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21 and an annual incidence of 4–12 per million individuals. The fact that these figures are
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23 considerably higher than earlier reports is mainly due to the increased longevity of many
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25 populations ^{32–34}
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29 Acute exacerbation of the disease is manifest in the appearance of potentially life-
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31 compromising swallowing disorders and respiratory failure ³⁵. Patients with MG have a 10
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33 to 20% risk of developing a myasthenic crisis or exacerbations throughout their life, mainly
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35 during the first year of evolution ³⁶.
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39 It is increasingly accepted that MG is a disease with high variability in its clinical expression
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41 and that it is currently possible to recognize different MG subtypes according to the
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43 autoantibody profile, age of onset, haplotype, distribution of muscle commitment and
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45 associated thymic pathology, when present ^{16,37} (Table 1).
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Table 1. Clinical Subtypes of MG and their antibodies

MG subtype	Age at onset	Thymic status	nAChR Ab / isotype (incidence)	Other Abs	Refs
Generalized					
Early onset	<40 years	hyperplasia	+ / IgG1-3 (80%)	---	37, 39, 40, 45, 46, 47, 65
Late onset	>40 years	normal	+ / IgG1-3 (80%)	anti-titin, anti-ryanodine (50%)	37, 49, 50, 51, 52, 53, 54, 65, 83
Ocular	variable	unknown	+ / IgG1-3 (50%)	---	33, 34, 37, 38, 65
Musk	<40 years	normal	- / IgG4	Musk (40-70%)	41, 42, 43, 44, 80, 81
Others forms					
SNMG	variable	hyperplasia in some	- Some present antibodies against clustered nAChR/ IgG1 (66%)		106, 108, 109, 110

nAChR=nicotinic acetylcholine receptor. MG=myasthenia gravis. MuSK=muscle-specific receptor tyrosine kinase. SNMG= seronegative MG

Antibody titer appears not to be correlated with disease severity; the lack of correlation can be attributed to differences in the effector function of the different types of Abs.

Nonetheless, monitoring the titer of MG Abs is likely to provide useful information on the course of the disease at the individual patient level. At the population level, MG patients exhibit a wide variety of Abs, and in 80-90% of cases it is possible to detect Abs directed towards the nAChR (nAChR-Ab)³⁸. These circulating Abs are responsible for the blockage

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3 and reduction of the number of receptors in the postsynaptic membrane by endocytic
4 mechanisms ¹². Within the group of nAChR-Ab patients, 20% show weakness restricted to
5 the eye muscles (ocular MG) and the other 80% have extraocular muscle weakness, with a
6 multiplicity of skeletal muscles affected (generalized MG) ³⁴. Thymic abnormalities are
7 frequently found in generalized MG patients, and the highest antibody titers are observed
8 in patients with thymic follicular hyperplasia ^{39,40} (see Table 1). In 40-70% of patients
9 negative for nAChR-Ab, Abs can be detected against another postsynaptic protein, MuSK
10 (muscle-specific tyrosine kinase) ^{41,42}. These patients usually exhibit generalized MG, and
11 are mostly young adults who do not show any thymic abnormalities ^{43,44}.

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13 For quite some time MG has been regarded as a disorder affecting young women and
14 older men. A large body of studies has demonstrated that nAChR-Ab MG patients can
15 currently be divided into early-onset (EOMG) and late-onset (LOMG) disease groups, each
16 showing differential characteristics ^{33,37} (see Table 1). EOMG is defined as onset of the first
17 symptom before the age of 40-50, these patients often being female, with thymic
18 hyperplasia ⁴⁵⁻⁴⁷. Thymectomy is recommended in most such cases and patients respond
19 favorably to the surgery, improving their condition. An association with human leukocyte
20 antigen (HLA) haplotypes has been reported, where EOMG patients are likely to have the
21 HLA-B8-DR3 haplotype ⁴⁸.

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23 The first symptoms in patients with LOMG appear after the age of 40-50. Initially this form
24 of the disease was thought to more frequently affect male individuals, though now it has
25 been established that the sex ratio is nearer unity ⁴⁹. LOMG have normal or atrophic
26 thymus glands, therefore thymectomy is not recommended for this group. These patients

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3 often present auto-antibodies to striated muscle proteins, such as titin or the ryanodine
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5 receptor ⁵⁰, and only a weak association with the *HLA-B7-DR2* haplotype has been found
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7 ^{51,52}. There is a steep rise in the incidence of MG in older patients ⁵³, with a peak between
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9 70 and 80 years of age ⁵⁴. Some patients as old as 100 years have been diagnosed in
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11 recent times ⁵⁵. This may be the result of both improved prognoses and more advanced
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13 diagnostic procedures such as the widespread use of autoantibody testing, which lead to
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15 the recognition that many older patients acquire the disease ⁵¹ (see Table 1).
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21 Regarding association with genotypes other than *HLA*, it has been shown that the *APOE4*
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23 allele present in some individuals with potentially relevant disorders of cholesterol
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25 metabolism might affect the seropositive status of MG patients ⁵⁶. It has also been
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27 reported that the intensity of the immune response may be affected in MG patients who
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29 are *APOE2/4* carriers ⁵⁷. In this regard, Sostarko and coworkers ⁵⁷ showed a positive
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31 correlation between a group of patients diagnosed with the severe form of MG (all of
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33 whom presented high nAChR-Ab titres), and the presence of the *APOE2/4* genotype,
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35 which was in fact the most frequent genotype among MG patients. The *APOE3/3*
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37 genotype was the most frequent among the healthy controls. The latter was not
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39 confirmed in a subsequent study by Suhail et al. ⁵⁶, but these authors did confirm the
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41 association between the presence of the *APOE4* allele with nAChR-Ab positive patients.
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49 In addition to the MG forms described above and their genetic associations, there is
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51 another group of myasthenic pathologies termed congenital myasthenia gravis or
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53 congenital myasthenic syndromes (CMS), which are characterized by a genetic defect,
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55 typically inherited from both parents, rather than an autoimmune disease with immune
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3 attack to the NMJ ^{58,59}. These genetic disorders are also associated with impaired
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5 neuromuscular transmission and the main molecules involved, among others, are
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7 presynaptic enzymes and transporters (ChAT, ChT, MUNC13-1, MYO9, PREPL, SYT2,
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9 VAcHT, and VAMP1), synaptic basal lamina proteins (COLQ and COL13A1), and also
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11 postsynaptic proteins (nAChR subunits: α , β , δ and ϵ , AGRN, DOK7, MUSK, LRP4, and
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13 rapsyn) ⁶⁰. Other genes encoding for ubiquitous molecules (GFPT1, DPAGT1, ALG2, ALG14,
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15 and GMPPB) related to post-translational modifications of postsynaptic proteins (such as
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17 N-glycosylation) have also been implicated in CMS. There are more than 30 CMS causative
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19 genes identified up to date, reviewed in detail by Rodriguez-Cruz and coworkers ⁶¹.
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26 **Anti-nAChR antibodies and the immune attack of the NMJ**

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28 Pathogenic nAChR-Abs bind to functional nAChRs, reducing their number at the motor
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30 endplate and resulting in a characteristic pattern of muscle weakness ^{62,63}. There are three
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32 main mechanisms underlying the loss of functional nAChRs, all of which are Ab-
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34 dependent ⁶⁴: i) complement-mediated lysis of the NMJ, resulting in simplification of the
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36 postsynaptic muscle membrane; ii) accelerated internalization and degradation of nAChRs
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38 caused by cross-linkage of nAChRs by specific IgG; and to a lesser extent iii) blockade of
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40 the nAChR by Abs which bind specifically to acetylcholine binding sites.
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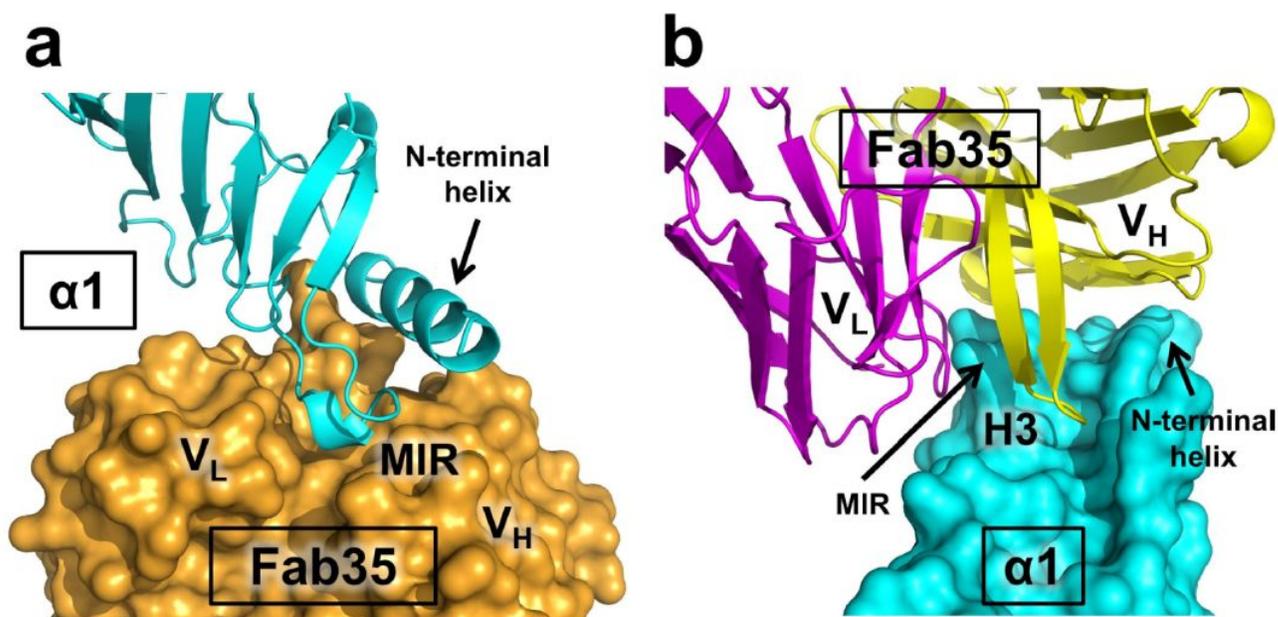
45 The most widely studied and physiopathologically relevant of these harmful mechanisms
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47 is complement-mediated lysis of the NMJ. The fact that nAChR-Abs belong mainly to the
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49 IgG1 and IgG3 subclasses makes for highly efficient activation of the complement system
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51 ⁶⁵. The classical complement pathway is initiated by specific Abs binding at very high
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53 densities to the tightly packed nAChRs at the NMJ, resulting in C3 activation and cleavage
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3 of the downstream components of the complement cascade. The final step of the
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5 pathway consists of the formation and deposit of the membrane attack complex (MAC),
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7 with the subsequent destruction of postsynaptic folds ⁶⁶. There have been several reports
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9 of complement-system activation at endplates, the strongest evidence being the
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11 identification of Abs, C3 ⁶⁷ and MAC ⁶⁸ deposition at NMJ in MG patients, demonstrated by
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13 Andrew Engel's laboratory in the late 1970's. Correspondingly, serological consumption of
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15 complement components such as C3 and C4 ⁶⁹ and the presence of terminal components
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17 in plasma has also been proven in these patients ⁷⁰.

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23 IgG1 and IgG3 anti-nAChR antibodies can recognize different epitopes on the receptor
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25 macromolecule. The majority of the nAChR-Abs in MG patients bind to the MIR
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27 extracellular region of the α -subunit ⁷¹. Anti-MIR Abs have been associated mostly with
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29 the generalized rather than with the ocular form of MG. Epitope mapping revealed that
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31 nAChR-Abs can also bind to immunogenic parts of the β and γ subunits ⁷². Moreover, a
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33 study in an animal model proved that after immunization of animals with a short peptide
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35 from the α -subunit, the immune response extended to the whole α -chain, accounting for
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37 the spreading of the autoimmune response ⁷³. This 'determinant spreading' occurs at the
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39 beginning of the autoimmune response, when the epitope targets scatter to adjacent
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41 subunits of the same molecule in MG patients ⁷⁴.

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49 Recently, Noridomi and colleagues applied X-ray crystallography to solve the structure of
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51 the nAChR α 1-subunit at 2.6 Å resolution in complex with the Fab fragment of mAb35, an
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53 extensively studied monoclonal antibody that causes experimentally-induced MG (EAMG)
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3 and competes with around 65% of human antibodies found in MG patients.^{12,22,75}. The α 1-
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5 subunit residues involved in the binding with the Fab are conserved across a wide range of
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7 species, a finding consistent with the cross-reactivity of autoantibodies in MG patients and
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9 EAMG animal models^{74,72}. Figure 2 illustrates in fine detail the close contacts between the
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11 nAChR MIR and the Fab fragment of mAb35^{75,76}. The crystallographic studies not only
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13 disclose the intimate contact between the antigen-antibody contact domains, identifying
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15 the specific amino acid residues involved, but also give rise to new hypotheses concerning
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17 the phylogenetic conservation of the Ab recognition site in vertebrates. The X-ray
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19 structures also provide insights into the functional domains involved, opening possibilities
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21 for diagnostic and therapeutic (drug design) developments in MG.
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49 **Figure 2. The key main immunogenic region (MIR) of the nAChR and its subtle but tight contact**
50 **with the antibody.** a) The MIR loop of the nAChR α 1-subunit inserts into a pocket of the Fab35
51 antibody fragment in the crystal structure. The pocket lies between the variable domains of the
52 heavy (V_H) and light (V_L) chains of Fab35 (surface-rendered in orange color). The N-term helix of
53 the nAChR α 1-subunit sits on a groove of the V_H . In b) the CDR-H3 (yellow β strand labeled H3) of
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3 the Fab35 V_H region inserts into a pocket between the MIR loop and the N-term helix of the nAChR
4 α 1-subunit extracellular domain. From Noridomi et al. ⁷⁵.
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8 **MG induced by proteins other than the nAChR**

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10 A small proportion of MG patients are negative for nAChR-Ab. In 2001, Hoch and
11
12 colleagues discovered that antibodies to MuSK were responsible for producing MG in
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14 about 70% of these myasthenic patients lacking nAChR-Ab ⁴¹ (Table 1). MuSK is involved
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16 in the physiological maturation of nAChR aggregates at the NMJ ⁷⁷. Although the MIR for
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18 MuSK is located on the most protruding portion of the first Ig-like domain of its
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20 extracellular moiety, Abs against multiple other epitopes have also been reported ^{17,78}.
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22 Importantly, MuSK-Abs are predominantly of the IgG4 subtype ⁴², which are not capable
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24 of inducing complement activation. Furthermore, they are also bispecific Abs, i.e. they
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26 have the potential to exchange one of their Fab arms with any IgG4 molecule ⁷⁹, so they
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28 are considered as functionally monovalent. Therefore, IgG4 Abs can only block function of
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30 MuSK signaling and lead to de-clustering of nAChRs, thereby destabilizing the NMJ. IgG4
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32 Abs have no direct effect on MuSK dimerization or MuSK internalization; they bind to
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34 extracellular epitopes of MuSK, thus preventing the binding between MuSK and Lrp4, and
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36 consequently inhibit Agrin-stimulated MuSK phosphorylation. This leads to failure in the
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38 activation of the MuSK kinase domain, essential for the signaling which ends in nAChR
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40 clustering and maintenance of the NMJ ^{80,81}.
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52 The distinct types of immunoglobulins involved may be partly responsible for the
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54 differences observed in the clinical evolution of patients affected by MuSK-Ab MG ^{43,44,82}.
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3 Although other minor subtypes of Abs have been identified in patients with MG, which
4 recognize mostly cytoplasmic muscle proteins (e.g. titin, myosin, actin and ryanodine
5 receptor)⁸³ and other post-synaptic membrane or intracellular proteins (cortactin, LRP4,
6 rapsyn)^{82,84-86}, only anti-nAChR and anti-MuSK Abs have a well-characterized pathogenic
7 role. However, anti-titin Abs have been strongly associated with the development of
8 thymomas⁸³.
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19 Patients without nAChR-Abs or MuSK-Abs represent less than 10% of the total and are
20 referred to as "seronegative MG" (SNMG). These patients have a clinical symptomatology
21 and evolution similar to that of nAChR-Ab MG patients, and also a similar thymic
22 pathology and responses to immunosuppressive treatment^{42,44}. SNMG patients are
23 considered to have low-titer, low-affinity nAChR-Ab that cannot be detected by the usual
24 diagnostic techniques, mainly using radiolabeled soluble nAChR.
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33 34 35 **Clustered synaptic receptors**

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38 The NMJ is undoubtedly a large synapse, enormous in comparison to cholinergic or any
39 other synapse in the CNS. nAChR clustering at the end-plate is a tightly regulated
40 developmental phenomenon culminating in the formation of a relatively stable synapse,
41 with more than 10 million receptor molecules tightly packed in an anatomically well-
42 defined area of the muscle cell surface. Outside the synaptic area proper, the density of
43 receptors drops dramatically¹⁴. The mechanisms that regulate the density of nAChRs and
44 other molecules at the vertebrate NMJ are now relatively well characterized, although
45 there are still several aspects of this complex synapse that remain to be elucidated. One of
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3 the main outcomes of various decades of research into this area is the extensive
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5 knowledge on the interplay of multiple cascades and individual molecules involved in the
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7 anchoring, stabilization and maintenance of the end-plate synapse.
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11 Before innervation, Wnt proteins together with the muscle-specific kinase (MuSK) are
12
13 involved in the formation of micron-sized aggregates of nAChRs (“micro-clusters”), in a
14
15 process known as pre-patterning ⁸⁷. This occurs at the central belt of the muscle cell,
16
17 where the axonal nerve endings make their attempt to establish contact with the target
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19 cell. The small GTPase Rac1 is also involved at these early stages. At the innervated stage,
20
21 another signaling pathway takes the leading role: agrin together with MuSK
22
23 immunoglobulin-like domains 1 and 2 converge to produce the fusion of the micro-
24
25 clusters into larger size aggregates, with the participation of another small GTPase: Rho1.
26
27 The scaffolding cytoplasmic protein rapsyn also plays a key role in both pre- and post-
28
29 innervation stages, crosslinking nAChRs ⁷⁵. A reciprocal relationship exists between agrin
30
31 and ACh in controlling nAChRs clustering: whereas agrin promotes clustering, ACh
32
33 disperses the clusters. These opposing signals may allow stabilization of nAChRs at the
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35 NMJ, leading to dispersal of extrasynaptic nAChR-rich sites and formation of receptor
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37 clusters at the postjunctional membrane ⁸⁸.
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46 There is evidence confirming the involvement of the Wnt/ β -catenin signaling pathway in
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48 neurodevelopment as well as in neurodegenerative diseases. Wnt/ β -catenin signaling
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50 occurs during critical periods in early development of the brain. Defects in this cascade
51
52 have been hypothesized to occur in disorders such as autism ^{89,90} and schizophrenia ^{91,92}.
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56 Later in life, signaling pathways are essential for maintaining proper communication
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3 between neuronal and non-neuronal cells, and factors that disrupt this balance may result
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5 in disorders like Alzheimer disease ⁹³. Other developmental disorders have also been
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7 linked to Rac-1 ⁹⁴ and also Rho1 ⁹⁵.
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11 Research in this area has disclosed a plethora of other postsynaptic proteins whose direct
12
13 role in receptor supramolecular organization in the postsynaptic muscle cell is in many
14
15 cases well established; in other cases, the involvement is of a subsidiary nature, either
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17 acting as co-receptors or stabilizing the receptor assemblies. Heat-shock proteins, which
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19 comprise tumorous imaginal disc 1 Short (Tid1S), play subsidiary roles in stabilizing
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21 nAChRs. MuSK activity is exerted at its transmembrane tyrosine kinase domain, acting
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23 directly in the formation of the NMJ ^{96,97}. The so-called low-density lipoprotein receptor-
24
25 related protein-4 (Lrp4) plays the role of receptor for signals that activate both the agrin
26
27 and Wnt cascades providing “anterograde” signals for the clustering phenomenon ^{98,99}.
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29 Retrograde signals mediated by the cysteine-rich domain (CRD) and Disheveled protein
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31 signaling cascade operate from the muscle cell to the nerve cell, with the participation of
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33 β -catenin ^{100,101}. The laminin network is also recruited into the retrograde stabilization of
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35 the extracellular post-synaptic moiety of the end-plate, together with collagen Q, and
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37 various connective tissue glycans ¹⁰². The muscle cell proper also provides glycans such as
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39 heparan sulfate and glycosaminoglycans, which might contribute to nAChR clustering by
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41 acting as muscle glycan agrin co-receptors ^{103,104}. Muscle dystroglycans traverse the
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43 membrane and link the extracellular n network, aiding in actin polymerization through
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45 Arp2/3, an actin-related complex ¹⁰⁵.
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3 In 2008 Vincent and coworkers discovered Abs against clustered nAChR in up to 66% of
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6 SNMG patients, using a cell-based assay ¹⁰⁶. This employs HEK cells co-transfected with
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9 human nAChR subunits and rapsyn to induce nAChR aggregation at the cell surface ^{107,108}.
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12 Under these conditions, the in vitro assay can detect Abs of either low affinity or low
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15 concentration that the classical radioimmunological technique cannot. This is due to the
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18 fact that although, to the best of our knowledge, anti-clustered nAChR Abs are the same
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21 as those identified using solubilized receptor as the antigen source, anti-clustered nAChR
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24 Abs are able to bind much more strongly to the nAChR when the latter is presented in
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27 clustered form at the cell surface rather than free in solution as in the radioactive assay.
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30 This is possibly because they can conveniently cross-link the nAChRs through divalent
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33 binding, which is not likely to occur in solution. Abs against clustered nAChR belong to the
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36 IgG1-3 subclass, and are therefore able to activate the complement system and produce
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39 NMJ damage ¹⁰⁶. Anti-clustered nAChRs have been reported to occur in both ocular and
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42 generalized forms of the disease and their pathogenic nature was demonstrated by
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45 transferring patient IgG antibodies to mice ¹⁰⁹. Patients with these “clustered-nAChR”
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48 antibodies generally resemble those with generalized MG (see Table 1) with typical RIA
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51 positive AChR antibodies, but their disease tends to be less severe. ^{108,110}.
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55 **Abs to clustered receptors in the CNS**

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3 Over the last ten or so years, awareness of antibody-mediated diseases has expanded
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5 considerably. Autoantibodies directed specifically at neurotransmitter receptors are not
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7 exclusively found in the peripheral cholinergic synapse. They have also been reported to
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9 occur in the CNS. Several types of excitatory ion channel forming receptors (*N*-methyl-*D*-
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11 aspartate receptor (NMDA-R), AMPAR, GABA_AR), one metabotropic receptor (GABA_BR),
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13 one inhibitory receptor (GlyR) and several proteins (LGI1, CASPR2, DPPX) that regulate the
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15 expression and localization of different forms of potassium channel have been identified
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17 in rare forms of brain encephalitis. The GlyR and the NMDA-R display selective clustering
18
19 at the postsynaptic neuronal membrane ^{111,112}. Abs that bind to the GlyR are associated
20
21 with PERM (progressive encephalomyelitis with rigidity and myoclonus) and stiff-person
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23 syndrome ¹¹³ and Abs against the NMDAR are found mainly in younger women with a
24
25 complex form of encephalitis ¹¹⁴. Although both antibodies can bind divalently to their
26
27 targets on the cell surface, and cause internalization and degradation of the receptors,
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29 anti-GlyR-Abs can also activate the complement system ^{112,115}. Finally, another channel
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31 protein, the water channel aquaporin-4 (AQP4), which is a glial surface membrane protein
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33 expressed at the end feet of astrocytes in the CNS, also displays clustered forms¹¹⁶, and is
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35 the target for antibodies that act via complement-mediated and also complement-
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37 independent pathways in neuromyelitis optica spectrum disorders ¹¹⁷.
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48 **Conclusions**

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51 The main factor determining the strength of the synapse is the number of receptors,
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53 which depends on the homeostatic equilibrium between two phenomena: i) lateral
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55 diffusion into and out of the synaptic region from/to non-synaptic (extrasynaptic) areas,
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3 and ii) the trafficking and turnover of receptors, determined by the rate and extent of
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5 biosynthetic and exocytic delivery to the plasmalemma on the one hand, and removal
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7 from the cell surface by internalization (endocytosis) on the other ¹¹⁸. This is a highly
8
9 dynamic scenario in which receptor supramolecular association and dissociation
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11 contribute to the efficacy of synaptic transmission, both in central and peripheral
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13 synapses. The main pathophysiological finding of deficient neurotransmission in the
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15 peripheral autoimmune synaptopathies -MG and related diseases- is an epiphenomenon
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17 of the loss of synaptic receptors. Such loss has become more widely appreciated in
18
19 several neurological conditions and brain pathologies. The detailed knowledge acquired
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21 on the physiopathology of the peripheral cholinergic synapse provides a solid basis on
22
23 which to build testable hypotheses to understand some of these CNS diseases.
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Autoimmune attack of the neuromuscular junction

