Advances in the understanding of disease mechanisms of autoimmune neuromuscular junction disorders

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Autoimmune Neuromuscular Junction Disorders 1

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Muscle weakness and fatigue are the hallmarks of autoimmune neuromuscular junction disorders. Although a plethora of immunosuppressive treatments exist, no cure is available to date and many patients are left with debilitating muscle weakness. Recent advances in the understanding of the structure and function of the neuromuscular junction, and the development of novel in vitro and in vivo models, have been instrumental in unravelling the pathophysiology of these autoimmune diseases. These advances are providing the rationale for the development of new therapeutic strategies. Restoration of the immune imbalance in these diseases, in parallel with symptomatic therapeutic approaches at the neuromuscular junction, will be crucial to obtain long-term remission or even cure.

Lancet Neurol 2022; 21: 163–75

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This is the first in a **Series** of three papers about autoimmune neuromuscular junction disorders

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Introduction

Autoimmunity at the neuromuscular junction can be classified into three categories on the basis of the location of the antigenic targets. Autoantibodies can be directed at: 1) postsynaptic components of the neuromuscular junction, defined as myasthenia gravis; 2) a presynaptic component of the neuromuscular junction, defined as Lambert-Eaton myasthenic syndrome; and 3) an unknown component of the neuromuscular junction, classified as seronegative myasthenia gravis or seronegative Lambert-Eaton myasthenic syndrome. Early recognition in the 1970s of acetylcholine receptors at the neuromuscular junction as the major autoantigen in myasthenia gravis, together with the easy experimental accessibility of the neuromuscular junction, made the serological subgroup of acetylcholine receptor antibody-positive myasthenia gravis a model area for study of antibody-mediated autoimmunity. In the past three decades, insights into the causes and pathophysiological mechanisms of autoimmune neuromuscular junction disorders, such as the development of novel in vitro cellular model systems for human neuromuscular junctions and single-cell analyses of pathogenic autoantibody producing lymphocytes, have improved their diagnosis and stimulated the development of novel therapeutics.

Here, we review current understanding of the physiology of the neuromuscular junction and the latest advances around mechanisms of autoimmune neuromuscular junction disease. This paper is the first in a Series of three. The accompanying papers discuss epidemiology, biomarkers, and diagnostic procedures,[1] and the latest findings on the treatment of autoimmune neuromuscular junction disorders.[2]

Structure, function, and maintenance of the neuromuscular junction

Skeletal muscle function is essential for body posture, movement, and respiration. Each motor neuron controls the contraction of multiple muscle fibres, together forming a motor unit (figure 1A). The neuromuscular junction is the synapse through which a motor axon interacts with the muscle fibre (figure 1B). Neuromuscular transmission depends upon a sequence of essential processes, including the opening of presynaptic voltage-gated calcium channels (VGCCs), subsequent release of acetylcholine, and its binding to the closely packed postsynaptic acetylcholine receptors, causing their opening (figure 1B). Spontaneous release of single acetylcholine quanta produces brief depolarisations of approximately 0·5–1 mV—ie, miniature endplate potentials, which by themselves have no known physiological function. Approximately 30 quanta are released simultaneously in response to a nerve action potential. This amount of acetylcholine produces an endplate potential of approximately 20–30 mV, which amply exceeds the firing threshold, triggering a postsynaptic action potential that induces muscle fibre contraction.[3] The postsynaptic action of acetylcholine is terminated by its hydrolytic breakdown by acetylcholinesterase in the synaptic cleft. In myasthenic diseases of the neuromuscular junction, the safety factor for neuromuscular transmission (the degree by which the endplate potential exceeds the muscle fibre's firing threshold[4]) becomes reduced. During tetanic contractions, acetylcholine release is subject to some degree of physiological rundown. In myasthenia gravis, the combination of a low safety factor and endplate potential amplitude rundown results in progressive failure of muscle fibre activation and contraction, and thus muscle weakness and fatigue.[3]

Two postsynaptic mechanisms help establish and maintain the integrity of the neuromuscular junction. First, signalling mediated by the muscle-specific tyrosine kinase (MuSK) is synapse-promoting, driving specialisation of both the postsynaptic (directly) and presynaptic (indirectly) structures. MuSK is a transmembrane tyrosine kinase that acts locally to stabilise

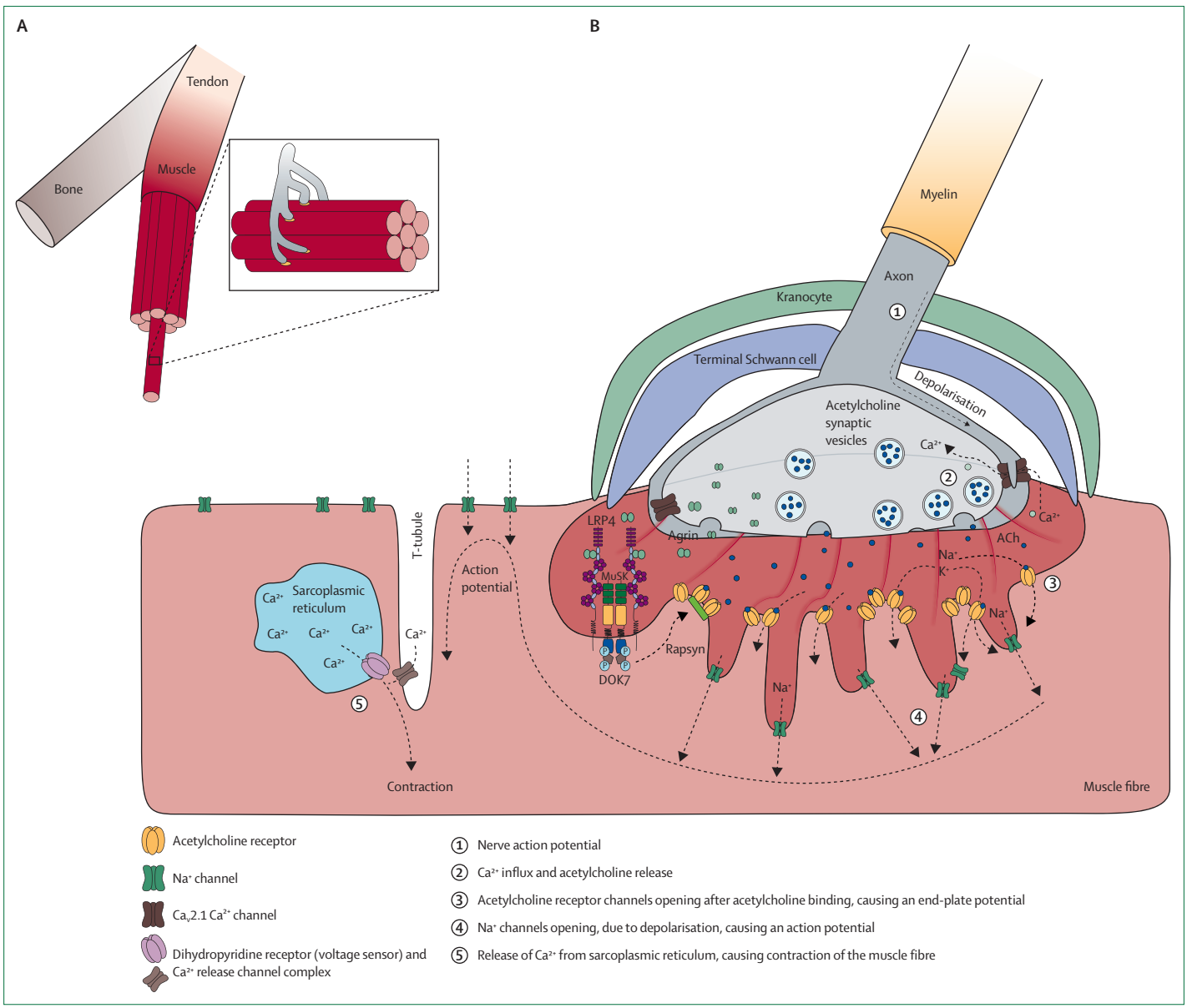

Figure 1: **Structure and physiology of the neuromuscular junction**
(A) The terminal branches of the motor axon form a spray of synaptic endings (boutons) on the midline of the muscle fibre surface. (B) Each presynaptic bouton sits in a cavity formed by the postsynaptic membrane of the muscle fibre. Specialised terminal Schwann cells cap the terminal bouton and are thought to provide structural protection and trophic support, and are involved in synaptic homoeostasis. Effective neuromuscular transmission depends upon the following sequence of essential processes: (1) the presynaptic action potential depolarises the nerve terminal membrane; (2) this process rapidly opens voltage-gated calcium channels located at vesicle release sites in the presynaptic membrane; (3) the burst of calcium ions entering the nerve terminal acts via calcium sensor proteins on the synaptic vesicle to trigger release of multiple quanta of acetylcholine; (4) the acetylcholine rapidly diffuses across the synaptic cleft to activate acetylcholine receptor cation channels that are closely packed together within the postsynaptic membrane, and when sufficient acetylcholine receptors have opened and the membrane is depolarised, an action potential starts running along the muscle membrane; (5) the action potential propagates into t-tubuli throughout the muscle fibre, and activation of L-type voltage-gated calcium channels in the t-tubular membranes then triggers release of calcium from intracellular stores, which acts on the myofibrils to cause muscle fibre contraction.

postsynaptic clusters of acetylcholine receptors.[5] Agrin, released by the motor nerve terminal, binds low-density lipoprotein receptor-related protein 4 (LRP4), which then forms a complex with MuSK (figure 1B). This complex causes dimerisation and activation of MuSK, and leads to recruitment of cytoplasmic proteins, such as downstream of tyrosine kinase 7 (DOK7) and rapsyn, ultimately inducing acetylcholine receptor clustering. The cluster site seems to be determined by a localised high-level of MuSK expression.[5] LRP4 serves an additional prosynaptic role by acting back on the motor axon to induce presynaptic differentiation.[6] Another important positive regulator of MuSK is DOK7. By binding cytoplasmic domains of MuSK, DOK7 increases the kinase activity, stimulating acetylcholine receptor clustering and thereby increasing neuromuscular junction size and endplate

	Acetylcholine receptor myasthenia gravis subtypes			MuSK myasthenia gravis	LRP4 myasthenia gravis	Agrin myasthenia gravis	Seronegative myasthenia gravis	Lambert-Eaton myasthenic syndrome subtypes		Seronegative Lambert-Eaton myasthenic syndrome
	Early-onset acetylcholine receptor antibody-positive myasthenia gravis (associated with thymic follicular hyperplasia)	Thymoma-associated myasthenia gravis	Late-onset, non-thymomatous acetylcholine receptor antibody-positive myasthenia gravis					Non-paraneoplastic (40–50%)	Paraneoplastic small-cell lung cancer (50–60%)	
Antigenic target	Acetylcholine receptor	Acetylcholine receptor	Acetylcholine receptor	MuSK	LRP4	Agrin	Unknown	$Ca_v2{\cdot}1$ VGCCs (P/Q-type)	$Ca_v2{\cdot}1$ VGCCs (P/Q-type)	Unknown
Age at onset	<50 years	Peak age 50 years	>50 years	<50 years	Early onset	Unknown	Unknown	Bimodel (young females, older males)	>50 years	Unknown
Gender bias	Yes (Female)	No	Yes (Male)	Yes (Female)	Yes (Female)	Unknown	Yes (Female)	No	Yes (Male)	Unknown
Dominant IgG isotype	IgG1–IgG3	IgG1–IgG3	IgG1–IgG3	IgG4	IgG1–IgG3	Unknown	Unknown	Potentially IgG1–IgG3	Potentially IgG1–IgG3	Unknown
Role of complement	Yes	Yes	Yes	No	Yes	Unknown	Unknown	Potentially yes	Potentially yes	Unknown
HLA association	DR3–B8	No	No consistent association	DR14–DQ5	Unknown	Unknown	Unknown	DR3-B8 and DQ2	No	Unknown
Tumour association	No	Thymoma	No	No	Unknown	Unknown	Unknown	No	Small cell lung cancer	Unknown
Class level of evidence for pathogenicity	I, II	I, II	I, II	I, II	II	III	NA	I, II	I, II	NA

Most (about 85%) patients with myasthenia gravis have acetylcholine receptor autoantibodies. MuSK, LRP4, and agrin antibody-positive myasthenia gravis variants are much less prevalent (about 5%, 2%, and <1% of patients with myasthenia gravis, respectively). About 8% of patients with myasthenia gravis are seronegative for all these antibodies. About 15% of patients with Lambert-Eaton myasthenia gravis are seronegative (ie, they have no detectable VGCC antibodies). Class I direct evidence: patient antibodies are pathogenic upon passive transfer to animals or upon application in in vitro models. Class II indirect evidence: active immunisation with the antigen causes a myasthenia gravis phenotype in animals or transplacental transfer causes a temporary phenotype in children. Class III circumstantial evidence: pathogenicity is expected based on the biological role of the antigen or positive response to immunosuppressive treatments, although direct experimental evidence is lacking. Gender bias refers to overrepresentation of the syndrome in one of two sexes. LRP4=low-density lipoprotein receptor-related protein 4. MuSK=muscle-specific tyrosine kinase. NA=not applicable. VGCCs=voltage-gated calcium channels.

Table: **Pathophysiological characteristics of the myasthenia gravis subtypes**

potential amplitude.[7] A second developmental and maintenance system opposes the MuSK signalling pathway and is driven by acetylcholine, which induces a weak calcium signal entering via acetylcholine receptors. This signal is amplified by calcium release mediated by inositol tris-phosphate receptors located immediately beneath the postsynaptic membrane.[8] Activated caspase-3 then acts on the MuSK complex to destabilise acetylcholine receptor clusters and suppress neuromuscular junction function. Interference with any of these crucial processes can result in myasthenia. Autoimmunity against postsynaptic factors causes myasthenia gravis, whereas autoimmunity targeting presynaptic VGCCs causes Lambert-Eaton myasthenic syndrome.

Immunological dysregulation

Myasthenia gravis and Lambert-Eaton myasthenic syndrome are multifactorial diseases with subtypes based on their clinical presentation and antibody status (table). Development of myasthenia gravis might depend on genetic predisposition found on HLA genes and other genes,[9] epigenetic factors including miRNA dysregulation,[10] female gender,[9,11] and immune dysregulations[12] (table). Myasthenia gravis and Lambert-Eaton myasthenic syndrome can also be of paraneoplastic origin, typically with thymoma in myasthenia gravis and small-cell lung cancer in Lambert-Eaton myasthenic syndrome.

Role of the thymus

In early-onset acetylcholine receptor antibody-positive myasthenia gravis, the thymus shows lymphoid follicles and germinal centres, such as thymic follicular hyperplasia (table, figure 2), associated with active neoangiogenesis processes and the overexpression of chemokines, as in tertiary lymphoid organs.[13] These changes indicate abnormally active immune responses in the thymus. In myasthenia gravis, the thymus is the start site of autoimmunisation that is, the place where acetylcholine receptor antibodies are produced, and the origin of autoreactive T cells, B cells, and plasma cells spreading to the extrathymic immune system to perpetuate early-onset acetylcholine receptor antibody-positive disease there, even after thymectomy.[14,15] The

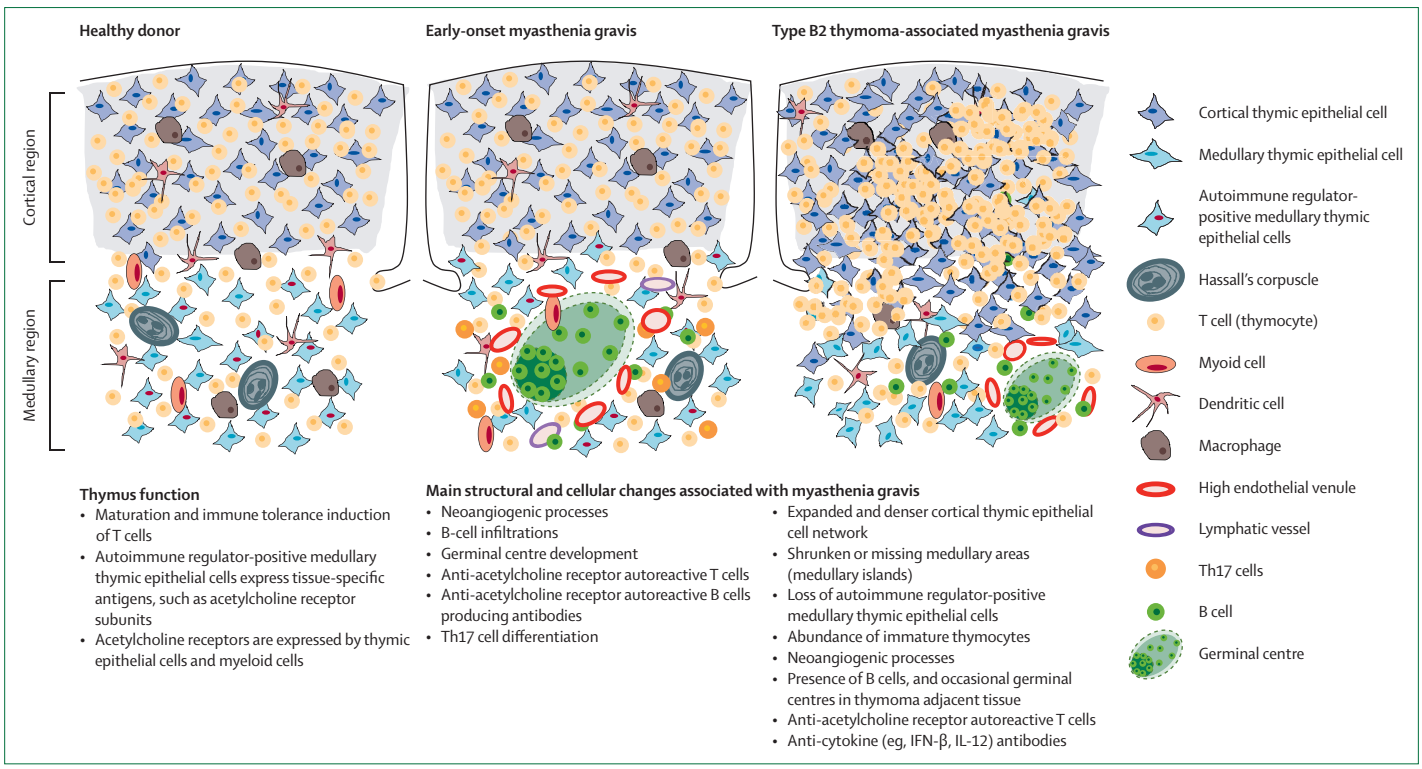

Figure 2: **Thymic changes in myasthenia gravis**
The healthy thymus has cortical regions that are mainly composed of cortical epithelial cells and abundant T cells, and medullary regions in which T cells complete their maturation process. These T cells include regulatory T cells and are accompanied by immunological tolerance-inducing dendritic and epithelial cells (including autoimmune regulator-positive epithelial cells and Hassall corpuscles) and by sparse B cells and rare myoid cells that are supposedly involved in the induction of immunological tolerance. In early-onset myasthenia gravis, the thymus shows normal cortical regions, whereas the inflamed medullary regions are expanded by abundant B cells, lymphoid follicles adjacent to myoid cells, activated T cells, and numerous blood vessels (high endothelial venules). In thymoma-associated myasthenia gravis, the cortical regions are mostly expanded through increased numbers of neoplastic epithelial cells that are still intermingled with numerous T cells. By contrast, the medullary regions show rudimentary development, only rare B cells, rare lymphoid follicles and Hassall corpuscles, and near-absence of autoimmune regulator-positive epithelial cells, regulatory T cells, and myoid cells. The autoimmune regulator is a transcription factor of medullary thymic epithelial cells that is key for the induction of immunological tolerance.[17]

2015 MGTX thymectomy trial[15] (an international, multicentre, randomised, controlled study of therapeutic thymectomy in 126 patients with myasthenia gravis aged 18–65 years) showed improved clinical outcomes (eg, lower quantitative myasthenia gravis scores and reduced need of prednisone and azathioprine). This finding further emphasised the key role of the thymus in non-thymomatous acetylcholine receptor antibody-positive myasthenia gravis.[15] Female predominance in most autoimmune diseases might be explained by oestrogens that reduce the transcription in the thymus of the *AIRE* gene and its targets, (eg, the *CHRNA1* gene encoding the acetylcholine receptor α-subunit[16]). Reduced thymus expression of such genes prevents proper tolerisation and might result in autoimmunity.

Thymoma-associated myasthenia gravis is generally a subtype of acetylcholine receptor antibody-positive myasthenia gravis.[17] Most patients have a thymoma of AB, B1, or B2 histological type, according to WHO classifications. Almost all thymomas produce and preprime acetylcholine receptor autoreactive T cells, but mature CD4 T cells leave the thymoma only in patients with thymoma-associated myasthenia gravis, supporting acetylcholine receptor-directed B cells in the extra-tumorous immune system, including the remnant thymus.[18] Three studies showed a higher proportion of T follicular helper cells,[19] B cells and CXCL13,[20] and germinal centres[18] in the tumour-adjacent thymic tissue of patients with thymoma-associated myasthenia gravis. Lambert-Eaton myasthenic syndrome is an exceptionally rare complication among the many autoimmune diseases that thymomas can be associated with.[21]

Late-onset, non-thymomatous acetylcholine receptor antibody-positive myasthenia gravis mostly occurs in males (table). The thymus shows normal-for-age atrophy, but the presence of antistriational and anticytokine autoantibodies, normally associated with thymoma-associated myasthenia gravis, strongly suggests a role of the thymus in late-onset, non-thymomatous acetylcholine receptor antibody-positive myasthenia gravis.[17] Specifically, the atrophic thymus and thymomas share paucity of muscle-like myoid cells and autoimmune regulator-positive thymic epithelial cells. Myoid cell deficiency is thought to contribute to the generation of muscle-directed autoantibodies, whereas the paucity of autoimmune regulator-positive cells

might elicit anticytokine autoantibodies.[17] The mechanisms are not understood yet.

In MuSK antibody-positive myasthenia gravis, thymic pathology is rare[22] and probably of minor relevance. In a multicentre, retrospective blinded review of rituximab treatment in 55 patients (85% of whom were women and girls) aged 8–69 years, it was observed that thymectomy did not cause clinical improvement.[23]

In LRP4-positive myasthenia gravis, involvement of the thymus remains unclear. One international, multicentre, retrospective study of clinical charts of 42 patients with myasthenia gravis with isolated anti-LRP4 autoantibodies reported that 31% had been diagnosed with thymic hyperplasia.[24] Detailed immunohistochemical analysis of four cases (2 women and 2 men aged 28–53 years) revealed no thymic pathology, but clinical improvement after thymectomy in two patients suggests that a role of the thymus in LRP4-positive myasthenia gravis cannot be excluded.[25]

Role of small cell lung cancer

Small-cell lung cancer, and rarely other cancers, can elicit Lambert-Eaton myasthenic syndrome, and in exceptional cases, acetylcholine receptor antibody-positive myasthenia gravis.[26] This is due to immune responses against tumour proteins that cross-react with neuromuscular autoantigens. For example, small-cell lung cancer cells from a patient with myasthenia gravis expressed native acetylcholine receptor, and the major histocompatibility complex I and acetylcholine receptor-peptide complex, whereas small-cell lung cancer from patients without myasthenia gravis did not.[27]

Immune checkpoint inhibitors exacerbated pre-existing acetylcholine receptor antibody-positive myasthenia gravis and Lambert-Eaton myasthenic syndrome, showing the importance of the PD-1 and PD-L1 immune checkpoint for the prevention of these disorders.[28,29] Moreover, in neurologically asymptomatic patients with small-cell lung cancer (with or without pre-existing VGCC autoantibodies), the treatment with immune checkpoint inhibitors elicited de novo acetylcholine receptor antibody-positive myasthenia gravis[29] or Lambert-Eaton myasthenic syndrome.[28] Whether failure of tolerance in myasthenia gravis induced by immune checkpoint inhibitors and Lambert-Eaton myasthenic syndrome occurs at the thymic level remains uncertain.

Immune cell dysregulation

Insights into immune dysregulations in myasthenia gravis have expanded in the past 5 years. Concentrations of circulating molecules (eg, IL-17, IL-21, BAFF, APRIL) and microRNAs (miRNAs) have been proven as biomarkers for myasthenia gravis.[1] Some of these dysregulations reflect differences in the proportions or activation states of lymphocyte subpopulations (appendix p 4).[30,31] Reduced immunosuppressive function of regulatory T cells in myasthenia gravis and Lambert-Eaton myasthenic syndrome has been suggested.[32,33] In acetylcholine receptor antibody-positive myasthenia gravis, the functional impairment is more pronounced in thymic than peripheral regulatory T cells, and these regulatory T-cell subpopulations are phenotypically different.[33] Decreased functionality of thymic regulatory T cells is partly due to myasthenia gravis thymic epithelial cells.[33]

Increased proportions of Th17 cells and IL-17 are observed in acetylcholine receptor antibody-positive myasthenia gravis thymi due to a higher than normal secretion of IL-23 by myasthenia gravis thymic epithelial cells.[34] In acetylcholine receptor antibody-positive myasthenia gravis, abnormal development of T cells, leading to an imbalance between regulatory and pathogenic cells, originates in the inflamed thymus. IFN-β might have an upstream role here.[34] The proportion of thymic and circulating T follicular helper cells is also increased.[19] They might promote thymic germinal centre development, but also B-cell activation and antibody production.[19] T follicular helper cells have also been claimed to be increased in myasthenia gravis-associated thymomas.[35] In MuSK antibody-positive myasthenia gravis, among T follicular helper cell subsets, a specific increase in Tfh17 cells is observed.[36]

B cells produce the autoantibodies in myasthenia gravis, but regulatory B cells possess immunosuppressive functions. A decreased number and altered functionality of regulatory B cells is observed in patients with untreated acetylcholine receptor antibody-positive myasthenia gravis.[37] Regulatory B cells seem sequestered in the thymus, because their number is restored after thymectomy.[37] Activation of innate signalling pathways and an IFN-I signature are clearly detected in early-onset acetylcholine receptor antibody-positive myasthenia gravis and thymoma-associated myasthenia gravis thymi.[38,39] Viral infections that induce IFN-I expression have long been suspected as myasthenia gravis triggers, but so far no specific pathogen has been linked.[40] Immunological dysregulation is a clear hallmark of autoimmunity, but the exact trigger and progression factors in myasthenia gravis and Lambert-Eaton myasthenic syndrome are yet to be discovered.

Pathophysiology of acetylcholine receptor antibody-positive myasthenia gravis

Most patients (80–85%) with myasthenia gravis have antibodies against the neuromuscular junction acetylcholine receptor, an ion channel composed of two α1 subunits, one δ1, one β1, and one ε (adult) or γ (fetal) subunit. Most antibodies bind to an extracellular domain of the α1 subunit and are of the IgG1 or IgG3 subtypes. They reduce the number and function of acetylcholine receptors via three main mechanisms (figure 3A). Insight into the pathophysiology of myasthenia gravis has come from animal models,

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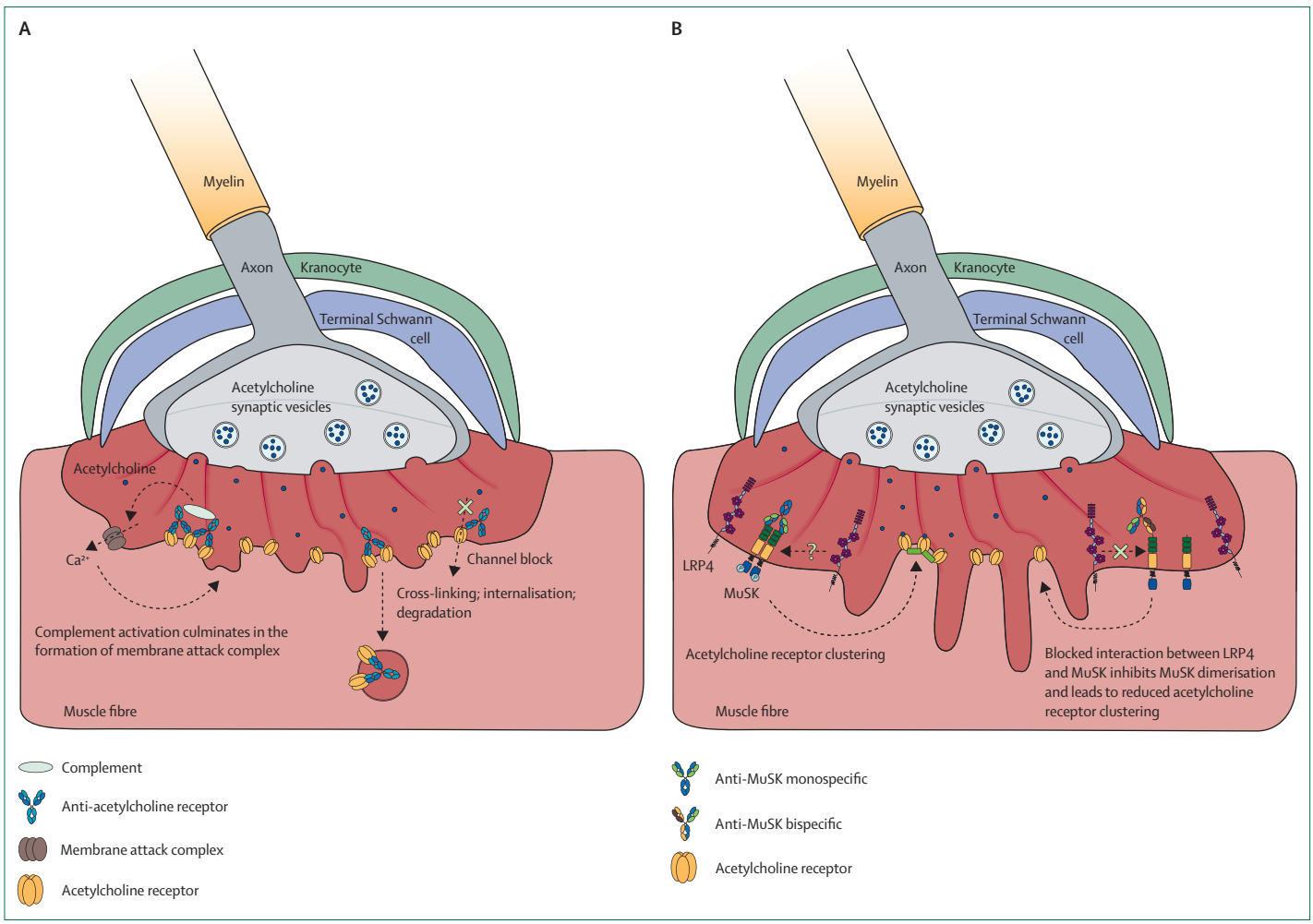

Figure 3: **Pathophysiology at the neuromuscular junction in myasthenia gravis patients**
(A) In acetylcholine receptor antibody-positive myasthenia gravis, IgG1 and IgG3 acetylcholine receptor autoantibodies activate complement with the consequent assembly of destructive cation channels of the membrane attack complex and loss of neuromuscular junction structure. These autoantibodies also cross-link acetylcholine receptors, causing their accelerated lysosomal degradation and thereby loss of signal transduction. Some patients have acetylcholine receptor autoantibodies that block the acetylcholine-binding site preventing acetylcholine receptor channel opening. (B) In MuSK-positive myasthenia gravis, IgG4 MuSK autoantibodies block LRP4 from activating the MuSK tyrosine kinase, which results in a loss of postsynaptic acetylcholine receptor clustering and synaptic fragmentation. The question mark indicates whether or not LRP4 interacts with MuSK in this pathological situation.

involving either active immunisation or passive transfer of acetylcholine receptor antibodies (appendix p 1).

Antigenic modulation of acetylcholine receptor degradation

Bivalent IgG1 and IgG3 acetylcholine receptor antibodies deplete acetylcholine receptors by cross-linking adjacent receptors, accelerating their endocytosis and lysosomal proteolysis.[41] Most acetylcholine receptor antibodies bind the main immunogenic region on the α1 subunit, as does the mab35 antibody (a rat monoclonal IgG). When passively transferred into rats, mab35 causes acute myasthenia gravis via a combination of antigenic modulation and complement-mediated damage.[42] X-ray crystallography showed that the mab35 Fab fragment forms a relatively rigid interface with the main immunogenic region and N-terminus of the α1 acetylcholine receptor subunit, perhaps stimulating endocytosis.[43]

Complement-mediated damage to the muscle membrane

Bound acetylcholine receptor antibodies activate the complement cascade that eventually forms the membrane attack complex. Rodent models of acetylcholine receptor antibody-positive myasthenia gravis proved a central role for complement and membrane attack complex in causing neuromuscular transmission failure. Mice that do not have complement factors (C3, C4, C5, or C6) are resistant to experimental autoimmune myasthenia gravis, whereas mice that do not have protective regulators, DAF1 and CD59a, are more prone to experimental autoimmune myasthenia gravis.[44] The membrane attack complex causes loss of acetylcholine

receptors and widening of the synaptic cleft, reducing endplate potential amplitudes. Moreover, the damaged postsynaptic membrane infoldings have fewer voltage-gated sodium channels, increasing the postsynaptic firing threshold.[45] Both changes reduce the safety factor of neuromuscular transmission.

Acetylcholine receptor-channel block

Some myasthenia gravis acetylcholine receptor antibodies bind at or near the acetylcholine binding site and thereby block the acetylcholine receptor channel opening. However, their clinical relevance remains elusive. A 2020 electrophysiological study assessed the prevalence of channel-blocking antibodies in the sera of 11 patients with myasthenia gravis.[46] Six of these sera samples caused an immediate reduction of acetylcholine-induced whole-cell current in cells expressing human acetylcholine receptors, apparently due to acetylcholine receptor-channel blockade. The channel blocking effects of these six sera were more pronounced in cells with rapsyn-clustered acetylcholine receptors. These results were from only 11 patients with myasthenia gravis, but hint that channel blocking antibodies might contribute more to the pathogenesis than previously thought.

Pathophysiology of MuSK antibody-positive myasthenia gravis

About 5–8% of patients with myasthenia gravis have autoantibodies against MuSK.[47] Although sharing many features with acetylcholine receptor antibody-positive myasthenia gravis, MuSK antibody-positive myasthenia gravis seems a separate disease entity on three different levels. First, MuSK antibody-positive myasthenia gravis is hallmarked by ocular, facial, and prominent bulbar muscle weakness,[48] with great interindividual variety in severity, timing, and muscles affected. Typically, ocular muscle weakness diminishes with time, and substantial bulbar weakness or generalised weakness remain. Second, MuSK antibody-positive myasthenia gravis generally responds poorly to acetylcholinesterase inhibitor therapy.[48] Third, the disease is thought to be caused predominantly by IgG4 autoantibodies, which are anti-inflammatory and do not activate complement.[49] Instead, IgG4 MuSK antibodies cause neuromuscular junction failure by blocking MuSK function, leading to the loss of acetylcholine receptor clusters (figure 3B).

The role of IgG4

Evidence of pathogenicity of MuSK autoantibodies first came from active immunisation of rabbits with MuSK, causing myasthenia gravis symptoms.[50] In humans, disease severity correlates with plasma concentrations of IgG4 MuSK antibodies.[51] Some patients have low titres of MuSK antibodies of other isotypes, but their pathogenic contribution remains unclear. IgG1, IgG2, and IgG3 fractions from patients with MuSK antibody-positive myasthenia gravis impaired acetylcholine receptor clustering in myotube cultures but did not induce a phenotype in mice.[49,52] IgG4 from the same patients clearly induced myasthenic weakness, proving the direct pathogenic nature of IgG4 MuSK antibodies.[49] IgG4 cannot bind C1q and is thereby unable to activate complement. IgG4 has low affinity for activating Fc receptors on immune cells and is generally considered anti-inflammatory.[53] Thus, the pathogenicity of MuSK antibodies must be related to binding and influencing the function of MuSK. The main immunogenic region of MuSK is the N-terminal immunoglobulin-like 1 domain,[54,55] which is crucial for interaction with LRP4 and the dimerisation of MuSK.[56] Patient antibodies block the interaction between LRP4 and MuSK and thereby the activation and phosphorylation of MuSK.[52,57] Prolonged loss of MuSK signalling results in fragmentation of postsynaptic acetylcholine receptor clusters and synaptic disintegration, which causes impaired neurotransmission and thus muscle weakness.[58] Eventually, muscle atrophy might occur. MuSK antibodies might also block the interaction between MuSK and collagen Q, which is important for anchoring acetylcholinesterase.[59] Delocalisation of acetylcholinesterase perhaps explains the hypersensitivity to acetylcholinesterase inhibitor therapy of some patients with MuSK myasthenia gravis.

IgG4 undergoes Fab-arm exchange with IgG4s of other specificities, which is thought to occur stochastically and continuously in the blood. The resulting IgG4 molecule is bispecific and interacts with its antigen in a monovalent fashion. More than 99% of MuSK autoantibodies are functionally monovalent.[60] Recombinant monoclonal MuSK antibodies have so far been derived from four patients. The scarce number of clones most likely reflects the relatively low number of circulating MuSK-specific B cells.[61,62] These antibodies bind either the immunoglobulin-like 1 domain or immunoglobulin-like 2 domain and are encoded by several *VDJ* genes. The immune response is thought to require affinity maturation to become fully pathogenic because the germlined antibody clone displayed lower binding than the affinity-matured antibodies and pathogenic effects in myotube cultures.[63] Experiments with patient-derived monoclonal antibodies revealed that monovalent (Fab-arm exchanged) MuSK antibodies blocked acetylcholine receptor clustering in myotube cultures, whereas bivalent antibodies partly activated MuSK signalling and acetylcholine receptor clustering.[62] In mice, monovalent MuSK antibodies induced myasthenia, whereas bivalent MuSK antibodies either had no effect or induced delayed and milder muscle weakness.[64] This finding suggests that MuSK antibody valency (and thus isotype) influences their pathogenicity. Whether other isotypes (ie, monospecific IgG1 or IgG3) MuSK antibodies contribute to the disease (eg, via complement activation) remains unclear.

Pathophysiology of Lambert-Eaton myasthenic syndrome

Antibodies against $Ca_V2.1$ VGCCs (also called P/Q-type calcium channels) are detected in more than 90% of patients with Lambert-Eaton myasthenic syndrome.[65,66] Their pathogenic actions at the neuromuscular junction are less well understood than those of acetylcholine receptor and MuSK antibodies in myasthenia gravis. The tiny dimensions of motor nerve terminals preclude direct measurement of $Ca_V2.1$ function. Therefore, any dysfunction must be inferred from secondary parameters, particularly from postsynaptic electrophysiological measurement of endplate potentials and miniature endplate potentials.[3] Neuromuscular junction studies in muscle biopsies and muscles from passive transfer mouse models showed endplate potentials that are too small, failing to reach the firing threshold of the muscle fibres, thereby inducing muscle weakness.[67–69] This synaptic transmission deficit is caused by a presynaptic defect, quantified as a severe reduction of quantal content (the number of acetylcholine quanta released per nerve impulse).

Lambert-Eaton myasthenic syndrome IgG binds to presynaptic active zones, where it disrupts intramembrane particles, presumably VGCCs.[70,71] More direct evidence for $Ca_V2.1$ VGCC as the autoantibody target came from studies in which Lambert-Eaton myasthenic syndrome IgG reduced $Ca_V2.1$-mediated currents in small-cell lung cancer or neuronal cell lines and in $Ca_V2.1$-transfected human embryonic kidney cells.[72–75] Furthermore, the IgG of patients with Lambert-Eaton myasthenic syndrome reduced neurotransmitter release from cultured neurons, but did not if they were genetically deficient for $Ca_V2.1$.[76] Several cellular and serological studies identified coexisting antibodies against other active zone proteins, and against $Ca_V2.2$ (N-type) VGCCs.[65,66,77] In Lambert-Eaton myasthenic syndrome patients with small-cell lung cancer, these antibodies probably result from an immunological response to the small-cell lung cancer, expressing these antigens in addition to $Ca_V2.1$.[78,79] However, such antibodies are thought to have a pathogenic role in only a minority of patients.[77]

$Ca_V2.1$ VGCCs are the predominant autoantigens in Lambert-Eaton myasthenic syndrome, but the mechanism by which the autoantibodies affect them is unclear. Their subclasses are unknown but are assumed to be IgG1 and IgG3 (as in acetylcholine receptor antibody-positive myasthenia gravis), capable of cross-linking and complement activation. Similar to acetylcholine receptor antibody-positive myasthenia gravis, three conceivable modes of action can be considered. First, direct block or functional change of $Ca_V2.1$ channels. However, no indications for such effects exist.[72,73] Second, cross-linking and reshuffling or depletion of channels. Ultrastructural studies indeed form strong evidence for such a mechanism.[70,71] Third, there is no direct evidence for local presynaptic neuromuscular junction damage through complement activation. Lambert-Eaton myasthenic syndrome biopsy neuromuscular junctions do not display complement deposits.[80] Furthermore, Lambert-Eaton myasthenic syndrome neuromuscular junction phenotypes can be induced by passive transfer of Lambert-Eaton myasthenic syndrome patient IgG to complement C5-deficient mice, and by transfer of F(ab)2 fragments of Lambert-Eaton myasthenic syndrome patient IgG (ie, incapable to activate complement) into healthy mice.[68,70,81] More detailed information on the molecular pathogenic mechanisms of $Ca_V2.1$ VGCC antibodies awaits further study.

Pathophysiology of non-acetylcholine receptor antibody-positive and non-MuSK antibody-positive myasthenia gravis

Roughly 5–10% of patients clinically diagnosed with myasthenia gravis have no detectable antibodies against either acetylcholine receptor or MuSK and are termed double seronegative. However, autoimmune causes in these patients are most likely and their sera probably harbour pathogenic antibodies against other neuromuscular junction proteins.[82]

Antibodies to LRP4

Within the double seronegative group, antibodies against LRP4 have been detected in highly variable proportions (1–50%), depending upon the geographic origin of cohorts and assay used.[83–86] LRP4 antibodies can coexist with antibodies against agrin.[87] Furthermore, LRP4 antibodies are present in a small proportion of patients with acetylcholine receptor antibody-positive myasthenia gravis or MuSK antibody-positive myasthenia gravis.[24,86] LRP4 myasthenia gravis antibodies are of the IgG1 subclass, but many patients have additional IgG2 and IgG3 reactivity.[24,83]

In view of the crucial participation of LRP4 and agrin in the acetylcholine receptor clustering pathway at the neuromuscular junction, antibodies to these antigens are hypothesised to eventually reduce the postsynaptic acetylcholine receptor density, explaining the myasthenic muscle weakness. Indeed, LRP4 myasthenia gravis sera and IgGs obstruct the interaction between agrin and LRP4 in ELISAs,[83,85] and inhibit agrin-induced acetylcholine receptor clustering in the C2C12 myotube assay.[84,85] The first in vivo evidence of pathogenicity of LRP4 antibodies came from mice actively immunised with the extracellular domain of rat LRP4.[88] They developed myasthenic weakness and neuromuscular junctions showed fragmented acetylcholine receptor clusters with myasthenic electrophysiological features (ie, small miniature endplate potentials and endplate potentials). Direct pathogenicity of LRP4 antibodies was further shown through passive transfer of IgG from LRP4-immunised rabbits to mice, which developed myasthenic weakness with associated fragmentation of

acetylcholine receptor clusters at their neuromuscular junctions.[89] No compensatory increase in acetylcholine release was present at neuromuscular junctions of LRP4 myasthenia gravis mice. Rather, quantal content was decreased,[88,90] suggesting primary or secondary presynaptic effects of LRP4 antibodies and a possible role of LRP4 in synaptic homoeostasis. Complement activation by bound LRP4 antibodies might be one of the contributing mechanisms,[88,89] but is not absolutely required for induction of experimental myasthenia gravis.[90] No passive transfer studies with human IgG from patients with LRP4 myasthenia gravis have been published to date.

Antibodies to agrin

Antibodies to agrin are detected in the sera of 10–15% of patients with myasthenia gravis, often in coexistence with acetylcholine receptor, MuSK, or LRP4 antibodies.[87,91,92] In vivo pathogenicity of agrin antibodies has so far been experimentally explored in only one study, in which mice were actively immunised with either the neural or muscle variant of agrin.[93] Mice injected with neural agrin developed myasthenic muscle weakness, with an associated neuromuscular junction phenotype of acetylcholine receptor cluster fragmentation and small miniature endplate potentials. Mice immunised with muscle agrin developed antibodies, but did not have muscle weakness or neuromuscular junction deficits.

Autoantibodies directed against other antigens

In addition to autoantibodies against acetylcholine receptor, MuSK, LRP4, and agrin in myasthenia gravis variants, several types of other autoantibodies can be present that target other neuromuscular junction proteins with extracellular epitopes. Whether these autoantibodies contribute to myasthenia gravis symptoms or have diagnostic value are unclear, and their mechanisms of emergence are unknown. Additionally, autoantibodies to intracellular muscular proteins can be present in a proportion of patients. These autoantibodies do not cause myasthenia gravis, but some of them are of diagnostic value and associated with more severe disease. Further details on these other antibodies to extracellular and intracellular proteins are provided in the appendix (p 1).

Muscle weakness in myasthenic disorders

Not all muscles are equally affected in patients with myasthenic disorders. Within the acetylcholine receptor antibody-positive myasthenia gravis population, there is high variability, ranging from restricted extraocular to generalised weakness.[94] Furthermore, frequent shifting of the affected muscle groups occurs during the disease course, with a tendency to progress in a craniocaudal direction. In Lambert-Eaton myasthenic syndrome, this direction is opposite, with proximal leg muscles being affected first. Extraocular muscles are often spared but can also be affected occasionally.[95] MuSK myasthenia gravis differs from acetylcholine receptor myasthenia gravis, with extraocular muscle weakness being less prominent, and presence instead of distinct bulbar weakness with frequent progression to respiratory muscles.[48]

The pathophysiological basis of weakness heterogeneity between muscle groups, patients, and myasthenia gravis subtypes is a longstanding and yet largely unresolved question. Several subtle physiological and molecular differences between muscle groups and their neuromuscular junctions might underlie several factors. First, the access of pathogenic IgG to neuromuscular junctions via the interstitial fluid of muscle and its lymphatic removal, which depends on the level of vascularisation, FcRn-mediated endothelial transcytosis, and usage intensity of a muscle.[96] Second, the density, characteristics, and turnover rate of antigenic proteins at neuromuscular junctions. Third, the magnitude of the safety factor of neuromuscular transmission at the neuromuscular junction; neuromuscular junctions on slow and fast skeletal muscle fibre types have been shown to differ in morphological and transmission characteristics.[97] Fourth, the level of protection against complement activation;[98] for instance, extraocular muscles contain relatively low levels of complement protective molecules. Fifth, intermuscular and interpatient differences in muscle regeneration processes, which are possibly influenced by impaired satellite cells in myasthenia gravis.[99] Neuromuscular transmission in myasthenic disorders is in a critical state, with perithreshold endplate potentials. Thus, small variations in these five factors between muscle types and patients might have large consequences for regional distribution and weakness severity. Further study is needed to elucidate these complex relationships.

Synaptic adaptations to autoantibody attack at neuromuscular junctions

The neuromuscular junction is a highly plastic synapse, capable of structural and functional adaptation (within specific boundaries) to changing physiological conditions and pathological disturbances.[100,101] Basic studies in invertebrate neuromuscular junctions identified the involvement of several candidate molecules and pathways, and some of them might be of relevance for the mammalian neuromuscular junction.[101] An important homoeostatic aspect for the postsynaptic myasthenic disorders is that neuromuscular junctions can increase presynaptic acetylcholine release in response to loss of postsynaptic acetylcholine sensitivity.[102] This increase is regulated at the level of individual neuromuscular junctions, because in rodent and human myasthenia gravis neuromuscular junctions display an inverse relationship between acetylcholine receptor density and quantal content.[103,104] Most likely, retrograde signalling occurs from postsynapse to presynapse. The extracellular domain of LRP4 has been implicated in retrograde

signalling during neuromuscular junction development.[6] It can be shed from the postsynaptic membrane through enzymatic cleavage.[105] Furthermore, overexpression of DOK7 in muscles of mice causes enlargement of the presynaptic nerve terminal.[7] Moreover, in myasthenia gravis caused by agrin, LRP4, or MuSK antibodies, the compensatory upregulation of quantal content fails to occur at neuromuscular junctions.[49,88,93,103] Together, these findings suggest a role for LRP4 and the agrin, LRP4, MuSK, and DOK7 pathway in transsynaptic homoeostasis at the neuromuscular junction. Thus far, there are no indications that neuromuscular junctions in patients with Lambert-Eaton myasthenic syndrome or animal models develop postsynaptic compensations in response to their presynaptic defect.[102] Further detailed studies of homoeostatic mechanisms at myasthenic neuromuscular junctions might identify pharmacological targets to improve neurotransmission.

Novel tools for the study of autoimmune neuromuscular junction disorders

Our understanding of myasthenia gravis pathophysiology is increased each time a new technology allows for more in-depth investigations. Major advances in the past 4 years have been made possible due to single-cell RNA sequencing analyses and the development of novel in vitro and in vivo models.

In two 2020 studies, single-nucleus RNA-sequencing showed regional transcriptional diversity within multinucleated skeletal myofibers.[106,107] The technology enabled purification of neuromuscular junction-specific nuclei based on their unique gene expression pattern and showed novel neuromuscular junction-specific genes.[106,107] These methods could lead to the identification of novel autoantigens and therapeutic targets in myasthenia gravis and Lambert-Eaton myasthenic syndrome.

Single-cell sequencing methodologies have also been used to investigate the role of antigen-specific B cells and T cells from patients with myasthenia gravis.[14,108–110] Thymus-derived peripheral B cells from patients with acetylcholine receptor antibody-positive myasthenia gravis persisted in the circulation after thymectomy to a degree that correlated with poor symptom resolution.[14] Single-cell analyses of lymphocytes from patients with MuSK antibody-positive myasthenia gravis treated with rituximab (a B cell depleting drug) identified resistant B cell clones emerging from failed depletion of pre-existing clones. These clones are composed of plasma cells and memory B cells that express reduced levels of CD20 and increased genes associated with B-cell survival.[109] Analysis of blood and thymi from patients with myasthenia gravis with cytometry by time-of-flight identified two novel dysregulated subsets of memory T helper cells, providing insight into the immune cause of myasthenia gravis, and identifying potential therapeutic immunological targets.[110]

Additionally, the study of monoclonal antibodies from patients with MuSK antibody-positive myasthenia gravis has led to a better understanding of the mechanisms of action of MuSK antibodies. Two 2019 studies showed that such monoclonal antibodies can impair acetylcholine receptor clustering by distinct mechanisms, leading either to the induction or inhibition of MuSK phosphorylation.[61,62] These antibodies could be used as reproducible, long-acting tools to generate in vitro and in vivo myasthenia gravis models for preclinical testing of potential therapeutics.

The development of novel in vitro cellular model systems for human neuromuscular junctions is another important new advancement.[111] With the use of human pluripotent stem cells, it was shown that the cell types required for the formation of functional neuromuscular junctions can be cultured and self-organised in a three dimensional in vitro nerve-muscle model, and stimulated through use of optogenetics.[112–114] Exposure of such neuromuscular organoids to acetylcholine receptor antibodies from patients with myasthenia gravis induced severe defects of their integrity and reduced their contractile activity, recapitulating key aspects of the disease phenotype. These models could help in reducing experimental use of animals and provide clean high-throughput platforms for screening potential therapeutics in the genetic background of people with these diseases.

Conclusions and future directions

The pathophysiology of myasthenia gravis and Lambert-Eaton myasthenic syndrome depends greatly on the autoantibody subtype. Although many aspects of the pathophysiology at the neuromuscular junction have been unravelled, the primary autoimmune causes of these diseases are still little understood. These disorders remain as chronic autoimmune diseases with no definite cure. Future research should aim at identifying the triggering events leading to myasthenia gravis or Lambert-Eaton myasthenic syndrome. In the context of personalised medicine, this is of major interest to be able to develop preventive approaches or avoid environmental triggers for predisposed individuals. The pathophysiological actions of several myasthenia-associated autoantibodies have not yet been completely elucidated. Especially the pathogenic actions of antibodies against $Ca_v2.1$, LRP4, and agrin await further clarification. With respect to immunology, restoring the immune imbalance in parallel to symptomatic therapeutic approaches at the neuromuscular junction will be crucial to obtain long-term remission or even a cure. Although several useful disease models already exist, generation of faithful and clinically relevant preclinical models of all subtypes of myasthenia gravis and Lambert-Eaton myasthenic syndrome is needed to further clarify the pathophysiology and facilitate development of new therapeutics. Finally, weakness pattern heterogeneity

Search strategy and selection criteria

This Series paper covers landmark papers, as judged by our expert opinion, and publications from the NCBI PubMed database with the search terms described in the appendix (p 3). We did not apply a time limitation on our search and included papers that were in English. In short, we used key terms describing the main topics of this Series paper including myasthenia gravis, Lambert-Eaton myasthenic syndrome, and seronegative myasthenia gravis, in combination with terms describing the specific subtopic (eg, pathophysiology and neuromuscular junction). For technological advances made in the past 5 years, we focussed on publications specifically describing new models (eg, organoids or sequencing methodologies).

between patients, muscle groups, and myasthenia gravis subtypes is a long-standing issue that deserves further study. Much of what we have learnt about autoantibody pathophysiology and related treatments of myasthenic autoimmune disorders might be also translated to the rapidly expanding field of other antibody-mediated non-myasthenic neurological autoimmune diseases, and thereby speed up processes of diagnosis and development of new therapeutics.

Contributors

All authors contributed equally to the writing, revision, and approval of the manuscript.

Declaration of interests

MGH reports financial support paid to her institution from Leiden University Medical Center (OIO, 2017), Top Sector Life Sciences & Health to Samenwerkende Gezondheidsfondsen via the Target to B! consortium (LSHM18055-SGF), Prinses Beatrix Spierfonds (W.OR-17.13 and W.OR-19.13), and the Dutch Science Organization NWO (VENI 0915016181 0040); reports royalties for a diagnostic MuSK ELISA; and is a coinventor on two patent applications on MuSK-related research. AM is on the medical advisory board for the German Myasthenia Gravis Association. JJP reports research support paid to his institution from Association Française contre les Myopathies, Prinses Beatrix Spierfonds, the Dutch Science Organization NWO, and Alnylam Pharmaceuticals; and is a coinventor on two patent applications on MuSK-related research. RLP reports financial support paid to their institution from the Association Française contre les Myopathies. WDP reports financial support from the Lambert Initiative for Cannabinoid Therapeutics and Bridging Grants from the University of Sydney. The funders had no role in the writing of this paper.

Acknowledgments

We thank Roy Augustinus for help with drafting figures 1 and 3, and Rian Schopmeijer for help with designing figure 3.

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