Implications of Epigenetics in Myasthenia Gravis

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Abstract

Myasthenia gravis is an autoimmune disease characterized by muscle weakness. The concordance rate of monozygotic twins in myasthenia gravis is about 30-40%, significantly higher than that in dizygotic twins, indicating genetic factors play a role in the pathogenesis of the autoimmune disorder. On the other hand, the concordance rate of the monozygotic twin is far below 100%, supporting the notion that environmental factors also play a role in the progression of the disorder. It is widely believed that environmental factors will influence the disease progression by altering gene expression. A long standing question is how the environment alters gene expression in humans. This review summarizes our current understanding of known mechanisms by which environmental factors such as medications, pollutants, food, sunlight, bacteria and viruses alter gene expression in some common autoimmune disorders. In this review, we also provide some literature evidence that supports epigenetics is a link between environment and abnormal gene expression in myasthenia gravis.

Keywords: Myasthenia gravis; Epigenetics; Genetics; Diagnosis; Treatment; Autoimmunity; Acetylcholine; Autoantibody

Introduction

Myasthenia gravis is an autoimmune disease. Patients with myasthenia gravis generally have weakness in skeletal and extracocular muscles with fatigue. The disorder is progressive and it often starts with a mild form (ocular form), in which weakness occurs only in specific muscles, most often, around the eye. The condition may become more severe with weakness seen in the extremities (generalized form). The name, myasthenia gravis, has both Greek and Latin roots. ‘Myasthenia’, derived from Greek, represents muscle weakness while ‘gravis’ in Latin stands for heavy or grievous [1]. The condition can occur in all races, both genders and at any age.

Molecular basis of myasthenia gravis

Among a vast majority of cases characterized to date, myasthenia gravis is caused by production of autoantibodies against own proteins at the postsynaptic neuromuscular junction. The major autoantibody found in myasthenia gravis recognizes acetylcholine receptor (AChR) [2]. The other antibodies involved in this condition may target muscle specific kinase (MuSK) or lipoprotein receptor related protein 4 (LRP4) [3].

The neuromuscular junction is a chemical synapse that forms at the contact site between a motor neuron and a muscle fiber. Through the neuromuscular junction, motor neurons can transmit signals to the muscle. Often the direct outcome of the neural signal transmission is muscle contraction. Upon receiving an action potential, the presynaptic terminal of a motor neuron releases the neurotransmitter acetylcholine into the synaptic cleft (Figure 1). Acetylcholine diffuses and binds nicotinic acetylcholine receptor (AChR) on the postsynaptic membrane of the muscle cell. Nicotinic AChR is a ligand-gated ion channel that allows Na+ and K+ to pass through the membrane upon activation. The resulting depolarization opens a voltage-gated sodium channel that generates an action potential traveling along the muscle, leading to muscle contraction [4]. A key feature of the neuromuscular junction is that AChR form clusters. With AChR clusters, AChR is packed at a high density (about 10,000 molecules/µm²), which is important for rapid signal transmission between neurons and muscle fibers [5]. Muscle-specific kinase (MuSK) is a master regulator of the AChR clustering and formation of the neuromuscular junction [6]. MuSK is a receptor tyrosine kinase and, not surprisingly, it interacts with a wealth of proteins.
Among them, the secreted protein agrin is a ligand for the MuSK pathway and the membrane protein low-density lipoprotein receptor-related protein 4 (LRP4) is a co-receptor for agrin. LRP4 but not MuSK binds agrin directly. At the same time, LRP4 binds MuSK. In the cytoplasm, MuSK binds cytoskeletal regulators such as disheveled, Abl, Src homologous collagen D, Dok7 and Rapsyn (Figure 1). Through these cytoskeletal regulators, MuSK controls AChR clustering. So far, autoantibodies seen in myasthenia gravis interfere with signal transmission in the neuromuscular junction. Especially, Anti-MuSK and anti-LRP4 antibodies block AChR clustering while anti-AChR antibodies directly block the function of AChR in the muscle.

**Cellular basis of myasthenia gravis**

In a healthy individual, production of antibody is well controlled. How do patients develop autoantibodies against their own proteins?

Antibodies are produced by plasma cells that are originally derived from B cells [7]. B cells that recognize and bind a specific foreign antigen will be activated and start to proliferate. Activation of most B cells requires the signals from helper T cells. Training and selection of helper T cells occur in the thymus in two steps. In the first step, naïve T cells that recognize self-major histocompatibility complex (self-MHC) proteins will survive in the thymic cortex (positive selection) and pass to the medulla. In the second step, developing T cells that recognize the self-antigen bound to self-MHC will be selected to die (negative selection). Only those T cells that
recognize self-MHC but not self-antigens will survive and move on to blood circulation after passing capillaries (Figure 2).

![Image](https://example.com/image.png)

**Figure 2:** The thymus and the selection of lymphocytes. Naïve lymphocytes that recognize self-major histocompatibility complex survive the first selection (positive selection) in the cortex and move to the medulla. Lymphocytes that recognize self-antigens will be removed by apoptosis (negative selection) in the medulla. T cells that pass the two selections will continue to move toward the bloodstream via capillaries.

So far, it is clear that myasthenia gravis is directly caused by defects in either B cells, T cells or both. Involvement of B cells in myasthenia gravis has been observed in thymic follicular hyperplasia. Normally germinal centers where most developing B cells reside during maturation are mainly found in peripheral lymphoid organs and only very few of them are found in the thymus. In thymic follicular hyperplasia, ectopic germinal centers are seen in the thymus. Patients with thymic follicular hyperplasia frequently develop anti-AChR antibodies [8]. Significantly, B cells from the thymus of myasthenia gravis patients are already activated and do not require other factors for activation [9], indicating that some B cells in the ectopic germinal centers are no longer subject to regulation by T cells. On the other hand, involvement of T cells in myasthenia gravis has been seen in thymoma. It has been reported that 55% of thymoma patients have myasthenia gravis [10]. Thymomas are tumors of the thymic epithelial origin. Thymic stromal cells (especially thymic epithelial cells) play a central role in training and selection of T cells. It is very likely that abnormal epithelial cells in thymoma patients provide aberrant signals to developing T cells, leading to abnormal training and selection of T cells and subsequent release of autoreactive helper T cells or defective regulatory T cells to the circulation. For example, regulatory T cells from myasthenia gravis patients have severely reduced suppressive function in a co-culture assay [11,12]. A similar defect has also been seen in other conventional T cells [13].

**Genetic contribution to myasthenia gravis**

What makes a patient more likely develop autoantibody against own proteins? Genetics clearly plays a role in myasthenia gravis. Twin studies indicate that the concordance rate for myasthenia gravis in monozygotic twins is 30-40% compare with 4-5% in dizygotic twins [14], supporting the role of genetic contribution to the disorder.

The HLA alleles have been implicated in several autoimmune disorders including myasthenia gravis. For example, HLA A1-B8-DR3-DQ2 haplotype has been associated with early onset myasthenia gravis in a Caucasian population [15-17]. The HLA DQ9 haplotype increases the risk of early onset myasthenia gravis in Southern Han Chinese while DRB1(*09) allele is implicated in late onset myasthenia gravis in Northern Han Chinese [18,19]. DRB1*15:01, DQB1*05:02 and DRB1*16 have been linked to late onset myasthenia gravis in Norwegian and Italian groups and DQ5 allele to the disorder of the MuSK type in Southern and Northern European groups [20-23]. Within the HLA locus, a variant of the TNF gene is strongly linked to HLA A1-B8-DR3-DQ2 haplotype. A single nucleotide polymorphism (SNP), rs1800629, located at the position -308 within the TNF-α gene corresponds to two alleles: -308 A and -308 G. It has been shown that the TNF-α transcript level in the A allele is 2 fold of that in the G allele [24]. Not surprisingly, the A allele increases the risk of myasthenia gravis [25-27]. Another SNP, rs2233290, is located within the TNFAIP3-interacting protein 1 (TNIP1) gene.Normally TNIP1 inhibits signal transduction by cytokine receptors and nuclear receptors [28]. The SNP also has two alleles. One allele that yields Ala at the amino acid 151 is associated with early onset myasthenia gravis in a northern European population [29].

Some non-HLA loci have also been implicated in myasthenia gravis. For example, variants of ENOX1, PTPN22, CTLA4, FOXP3 and AChR-related genes are associated with myasthenia gravis. A variant of ENOX1 was linked to early onset myasthenia gravis in an Italian American kindred with parental consanguinity where 5 out of 10 siblings are affected [30]. ENOX1 encodes ecto-NADH oxidase. In those affected, the ENOX1 mRNA levels were significantly reduced [30]. PTPN22 encodes protein tyrosine phosphatase non-receptor 22, an intracellular protein tyrosine phosphatase. It binds C-terminal Src kinase (CSK). Both PTPN22 and CSK function together in mediating T cell activation [31]. In a PTPN22 variant, Arg at position 620 is replaced by Trp. The W620 variant binds CSK less efficiently than the R620 allele, leading to hyperactivation of CSK [32,33]. As a result, the W620 variant is prone to autoimmune diseases [34-37]. CTLA4 encodes a membrane-bound receptor on T cells. It represses IL-2, interferon-γ and IL-4 and CD86, ligands expressed in antigen-presenting cells [38]. CTLA4 is essential for development of regulatory T cells. SNPs located in the promoter region of the CTLA4 gene are linked to myasthenia gravis [39,40]. Likely, reduced expression level or the activity of CTLA4 is responsible for defects in regulatory T cells, leading to production of autoantibodies. FOXP3 encodes a transcription factor and it is a master regulator of regulatory T cell development and function [38]. Interestingly, a particular allele of the FOXP3 gene, FOXP3 V599G599G, reduces the risk of myasthenia gravis in a Han Chinese population [41]. It is suggested that this allele affects both the number and function of regulatory T cells, which confers beneficial effects [41].
Some genes coding for AChR are also implicated in myasthenia gravis. Different from loci described above, effects of AChR related genes on autoimmune responses are more specific to neuromuscular junctions and therefore specific to myasthenia gravis. In particular, polymorphism within the two AChR genes (CHRNA1 and CHRND) increases the risk of myasthenia gravis. CHRNA1 and CHRND encode the α- and δ-subunit of AChR, respectively. One of the CHRNA1 SNPs, rs16862847, is located within the promoter region of CHRNA1. A particular allele of this SNP is found to be twice more frequent in myasthenia gravis patients [42]. Further analyses show that the allele disrupts the binding site of a transcription factor [42].

Finally, sex hormones also play a role in several autoimmune diseases including myasthenia gravis. For example, among myasthenia gravis patients younger than 50 year old, 60-70% are females [43]. Many genes within the HLA locus contain estrogen response elements, suggesting that the genes related to immune responses are potentially controlled by estrogen [44]. In a mouse model of myasthenia gravis, estrogen enhances production of anti-AChR antibodies [45].

Environmental and epigenetic contribution to myasthenia gravis

The fact that the concordance rate for myasthenia gravis in monozygotic twins is less than 100% as described above indicates that environmental factors also contribute to the disorder. What environmental factors are involved and how they play a role in autoimmune responses are currently two key questions for active research. Although some evidence suggests that certain drugs such as D-penicillamine and interferon-β (IFN-β), and pollutants increase the risk of developing autoimmune diseases [46], main environmental risk factors for myasthenia gravis known to date are viruses and gut microbes.

An antiviral signature has been observed in myasthenia gravis thymus. For example, IFN-β, Toll-like receptor 4 (TLR4) and proteins involved in double-stranded RNA (dsRNA) signaling are over-expressed in the diseased thymus [47,48]. The dsRNA signaling pathway includes TLR3, protein kinase R, interferon regulatory factor 5 (IRF5) and IRF7 [48]. In addition, Epstein-Barr virus has been found in the myasthenia gravis thymus [46]. It is known that Epstein-Barr virus produces small RNA, which activates TLR3 signaling [49]. Further, when a synthetic dsRNA was injected into mice, the number of B cells was increased in the thymus and the anti-AChR antibody was found in the periphery [48]. These observations support the notion that virus plays a role in the pathogenesis of myasthenia gravis.

Microbes in the gastro-intestinal tract may also contribute to myasthenia gravis. In an animal model, differences in microbiota composition due to differences in hormones confer protection against type 1 diabetes in one gender but not in the other [50]. Since disturbance in the gut microbiota is known to cause autoimmune responses [51], it is hypothesized that the gut microbes also play a role in myasthenia gravis. Clearly more studies are needed to test this hypothesis.

How do these environmental factors affect host cell immune responses? There are potentially many ways in which environment affects host cell immunity. One emerging paradigm involves epigenetics. Mechanisms underlying epigenetics utilize DNA methylation, histone modification and microRNA to control gene expression without modifying genomic DNA sequence. Several microRNAs are associated with autoimmune diseases. For example, miR-146a, miR-155 and miR-326 promote T-cell response and inflammation while miR-145, miR-320a and let-7c have an opposite effect [52-56]. Either an elevated level in miR-146a, miR-155 or miR-326 or a reduced level in miR-320a, let-7c or miR-145 has been found in myasthenia gravis in humans or in an animal model of the disease [52,55,57]. Some targets of these microRNAs have been identified. For example, let-7c represses translation of IL-10 mRNA by targeting 3’-UTR of IL-10 at least in some cell lines [56]. Since many viruses produce dsRNA, dsRNA may provide a link between environment and genetics. In this paradigm, environmental factors such as viruses or drugs interfere with the levels of microRNAs. The aberrant pool of microRNAs in turn disturbs the balance of the immune system. Individuals with a particular genetic background may enhance the immune response, leading to a full blown autoimmune disease.

Diagnosis and treatment of myasthenia gravis

Diagnosis: Typical signs of myasthenia gravis include a drooping of the upper eyelid (ptosis), inability to hold head straight, double vision (diplopia), difficulties in speaking, swallowing or chewing and difficulties in raising the arms or breathing. Occasionally, patients with myasthenia gravis may encounter alternating ptosis from one eye to the other and wrist drop [57].

It is important to distinguish myasthenia gravis from congenital myasthenic syndrome and Lambert-Eaton myasthenic syndrome. Congenital myasthenic syndrome is an inherited neuromuscular disorder caused by genetic defects that affect proteins in the neuromuscular junction [58]. As a result, myasthenic syndrome is not an autoimmune disorder. On the other hand, Lambert–Eaton myasthenic syndrome (or Eaton–Lambert syndrome) is an autoimmune disorder characterized by muscle weakness of the limbs. However, different from myasthenia gravis, Eaton–Lambert syndrome is caused by autoantibodies against presynaptic membrane proteins, especially voltage-gated calcium channel [59]. The key sign of myasthenia gravis is muscle weakness that improves with rest.

Two tests can be performed to assist diagnosis of myasthenia gravis: Ice-pack test and edrophonium test. The ice-pack test is carried out by placing a small ice bag over the drooping eyelids for 2 to 5 minutes. If the test is positive, cooling often ameliorates the characteristic ptosis symptom [60]. Evidence suggests that by cooling the tissue, the acetylcholinesterase is less active [61]. Edrophonium test involves injection of edrophonium chloride (Tensilon)
intravenously. Edrophonium chloride blocks acetylcholinesterase activities [62]. If injection of edrophonium chloride results in a sudden, although temporary, improvement in the muscle strength, this will support the diagnosis of myasthenia gravis.

To confirm the diagnosis of myasthenia gravis, a blood test should be performed. A laboratory test for anti-AChR, anti-MuSK and anti-LRP4 antibodies should be included. In 85 to 90% of patients with generalized myasthenia gravis, anti-AChR antibodies are detected in the serum [43]. A small group of patients have either anti-MuSK (~4%) or anti-LRP4 (~2%) antibodies. In the rest of patients, these antibodies have not been detected so far and, therefore, they are classified into a seronegative group.

For patients in the seronegative group, electrophysiological tests can be useful in diagnosis.

Two tests currently are recommended: Repetitive nerve stimulation test and single-fiber electromyography.

Repetitive nerve stimulation test involves electrical stimulation delivered to a motor nerve repeatedly several times per second and recording of the muscle electrical response. The test is positive if the muscle electrical response falls below threshold by more than 10%.

Single-fiber electromyography, a more sensitive test than repetitive nerve stimulation, involves measuring the temporal variability in their firing patterns of two muscle fibers belonging to the same motor unit. Two abnormal firing patterns, “jitter” and “blocking”, are diagnostic. Jitter describes the abnormal variation in the time interval between action potentials of adjacent muscle fibers while blocking refers to the failure of nerve impulses to elicit action potentials in adjacent muscle fibers of the same motor unit. Jitter is seen in over 85% of patients with the ocular form and more than 90% of patients with the generalized form of myasthenia gravis [63].

To test whether autoantibody production is due to any abnormality in the thymus (e.g., thymoma), chest computed tomography (CT) or magnetic resonance imaging (MRI) is recommended [64].

Treatment: Current therapy for myasthenia gravis includes acetylcholinesterase (AchE) inhibitors, immunomodulating agents, intravenous immune globulin (IVIg), plasmapheresis and thymectomy.

Acetylcholinesterase inhibitors: Pyridostigmine and neostigmine are the first line medications to treat mild cases of myasthenia gravis [65]. Both of them are reversible competitive inhibitors of acetylcholinesterase at the neuromuscular junction.

Immunomodulating agents: This group of therapy includes glucocorticoids (e.g., prednisone), cyclophosphamide, azathioprine, mycophenolate mofetil, methotrexate, rituximab and cyclosporine (or ciclosporin). These drugs alone or in combination can be used to treat difficult cases of myasthenia gravis. Glucocorticoids suppress expression of IL-2 along with a few other cytokines, whereby it reduces B- and T-cell proliferation [66]. In the meantime, glucocorticoids also increase expression of lipocortin-1, which reduces production of prostaglandins by inhibiting phospholipase A2 activity [67]. Glucocorticoids can be effective in relieving the condition for a short-term. Cyclophosphamide is an alkylating agent. Azathioprine is a purine analog. Mycophenolate mofetil is a non-competitive inhibitor of inosine-5’-monophosphate dehydrogenase, a key enzyme in the de novo guanosine nucleotide synthesis. Methotrexate is a folic acid analog. A common mode of action among cyclophosphamide, azathioprine, mycophenolate mofetil and methotrexate is to inhibit DNA synthesis, whereby they inhibit proliferation of both T- and B-cells. Rituximab is a monoclonal antibody against B-cell marker CD20 [68]. Binding of CD20 by rituximab leads to death of the lymphocytes [68]. Ciclosporin is a calcineurin inhibitor that reduces expression of the pro-inflammatory factor interleukin-2 (IL-2). Taken together, all of these immunomodulating agents either block proliferation of lymphocytes and/or reduce production of pro-inflammatory factors.

Intravenous immunoglobulin (IVIg): For patients with severe myasthenia gravis, intravenous immunoglobulin may be considered. It involves delivery of immunoglobulin (IVIg) to patients via an intravenous route. IVIgs are sterile IgG products purified from pooled human plasma and typically contain more than 95% unmodified IgG. The high content of anti-idiotypes against autoantibodies in IVIg facilitates its ability to neutralize autoantibodies, as is demonstrated in patients with acquired hemophilia that is caused by autoantibodies against factor VIII [69].

Plasmapheresis: This therapy involves removal, treatment and return of plasma. The beneficial effect can be seen within the first week of the treatment. However, the improvement usually does not last more than two months. Plasmapheresis can be used for severe or rapidly worsening cases of myasthenia gravis [70].

Thymectomy: Surgical removal of the thymus is recommended for patients with thymoma and for patients aged at 10-55 years with generalized myasthenia gravis [71].

Future prospect

To date, the genome-wide association studies (GWAS) have provided several hundreds of genetic loci associated with some common complex disorders [72]. However, most variants identified so far only contribute to a small increment in risk, leaving a gap in understanding between genetic variants and the phenotypic variations. Deeper understanding of the interplay between environment and epigenetics may help us eventually close the gap. Myasthenia gravis is one of the well-characterized complex disorders. Recent work has raised the possibility that antiviral responses trigger production of autoantibodies and dsRNA may provide a link between viruses and host immunity in myasthenia gravis. Other aspects of epigenetics are known to play a role in autoimmune disorders. For example, a histone deacetylase inhibitor (HDIs) improves juvenile arthritis in a clinical trial [73]. HDIs are also effective in
treatment of rheumatoid arthritis, lupus and type 1 diabetes in experimental settings [53]. It remains to be tested whether environment through DNA methylation and histone acetylation triggers autoimmune responses in myasthenia gravis. Current understanding of myasthenia gravis has prompted the development of some potential therapies for the disorder (Table 1).

**Table 1: Future therapeutics for myasthenia gravis.**

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<td>APCs treated with IL-10, IFN-γ and TGF-β suppress T cell autoimmunity</td>
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<tr>
<td>Elimination of T cells</td>
<td>APCs expressing FasL</td>
<td>T cells</td>
<td>FasL elicits death signals to T cells</td>
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Note: CR1: Complement Receptor 1; HDACs: Histone Deacetylases; DNMT: DNA Methyltransferase; APCs: Antigen-Presenting Cells; FasL: Fas Ligand

1) **Complement inhibitors**: Binding of autoantibodies to autoantigens will activate complement pathways, leading to destruction of target cells (e.g., muscle). Complement inhibitors having been tested in experimental settings include soluble complement receptors and anti-complement antibodies. For example, soluble complement receptor 1 and anti-C6 antibodies are effective in animal models of myasthenia gravis [74,75]. The efficacy of these in treating myasthenia gravis patients awaits clinical trials.

2) **Epigenetic therapeutics**: The most studied epigenetic therapeutics so far is HDIs. The HDIs promote acetylation of histone proteins, leading to an increase in gene expression (e.g., pro-apoptotic and anti-inflammatory genes). Originally developed as chemotherapy for cancers [76], they have been tested to treat some autoimmune disorders including rheumatoid arthritis, lupus, type 1 diabetes and juvenile arthritis as described above. The second class of epigenetic therapeutics includes inhibitors of DNA methyltransferase (DNMT). DNMT inhibitors interfere with DNA methylation and they are currently being tested for their efficacy in treating autoimmune disorder in animals [77]. Similar to complement inhibitors, HDIs and DNMT inhibitors are non-specific to myasthenia gravis.

3) **Modified antigen presenting cells**: Dendritic cells, after exposed to IFN-γ, IL-10 or TGF-β *in vitro* and injected into rats with myasthenia gravis, ameliorate the muscle condition [78-80]. Treated antigen presenting cells seem to reduce production of anti-AChR antibodies and therefore increase the antigen-specific tolerance. This approach is more specific to myasthenia gravis than complement inhibitors and epigenetic therapeutics. It remains to be tested in clinical trials.

4) **T cell vaccination**: One approach to T cell vaccination is to utilize anti-T cell receptor antibodies that recognize the antigen-binding sites of T cell receptor. The antibodies interfere with binding between autoantibodies and autoantigens. The anti-T cell receptor antibodies can be produced *in vitro* and administered through injection or they could be induced *in vivo* using synthetic peptides [81,82]. This has been used in clinical trials for several autoimmune disorders including multiple sclerosis, rheumatoid arthritis and psoriasis [81]. Second approach to T cell vaccination is to use synthetic peptides that are variants of epitopes recognized by pathogenic T cells. The peptides can bind both the T cell receptors and the MHC II molecules but can’t stimulate the T cells. As a result, synthetic peptides compete with endogenous self-antigens for binding to T cells, whereby they turn off the autoimmune responses. T cell vaccination is a specific approach to myasthenia gravis but its efficacy *in vivo* awaits clinical trials.

5) **Elimination of B cells**: When the AChR protein conjugated with a toxin is administered to rats with myasthenia gravis, the conjugate elicits cellular toxicity, leading to elimination of B cells [83]. This approach is specific to myasthenia gravis. However, the side effect has to be carefully monitored during clinical trials since the toxin may be toxic to other cells as well.

6) **Elimination of T cells**: CD4+ T cells that recognize the autoantigen AChR can be eliminated by Fas-mediated apoptosis. This approach utilizes antigen presenting cells that
express three proteins: AChR, Fas ligand (FasL) and truncated FADD [84]. FasL triggers the death signal upon the target T cells while truncated FADD protects the antigen presenting cells from self-destruction. This approach is effective in cultured cells and specific to myasthenia gravis [84]. However, its efficacy needs to be tested in animals first.

**Conclusion**

Myasthenia gravis is perhaps the best characterized autoimmune disorder. Fine mapping of molecular components of signal pathways at neuromuscular junctions and simplicity of the symptom have all made this disorder a powerful model for understanding pathogenesis of autoimmune disorders. Unravelling the link between environment and epigenetics may provide a key to further understanding the causes of myasthenia gravis and a new rationale for designing specific therapeutics for the autoimmune disease.

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**Conflicts of Interest**

The authors declare that there is no conflict of interests.

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