The two faces of MuSK antibody pathogenicity and their cause and consequences in myasthenia gravis

Vergoossen, D.L.E.

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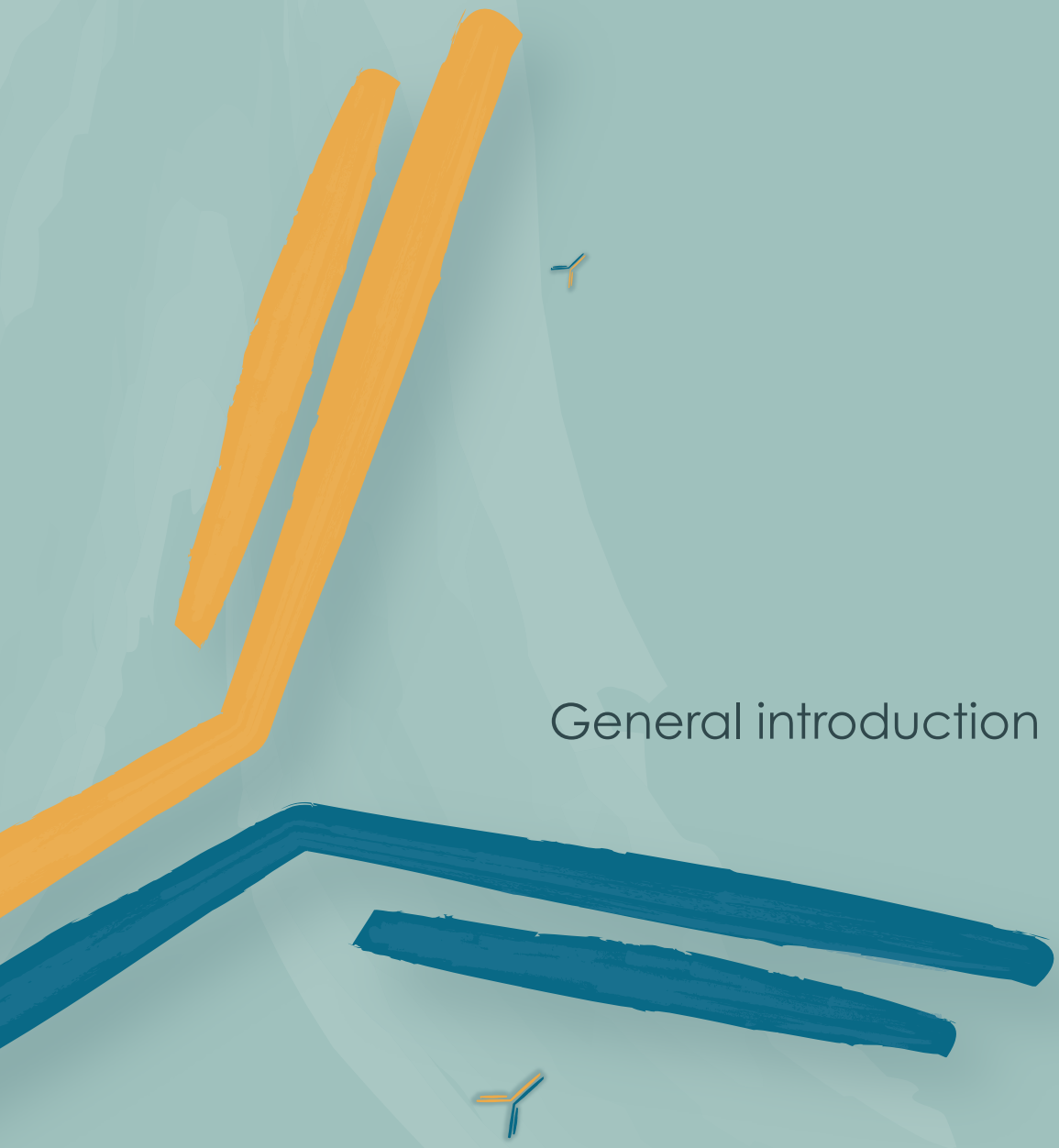

General introduction

MuSK antibodies, lessons learned from poly- and monoclonality
Dana L.E. Vergoossen, Roy Augustinus, Maartje G. Huijbers

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Abstract

Muscle-specific kinase (MuSK) plays a critical role in establishing and maintaining neuromuscular synapses. Antibodies derived from animals immunized with MuSK protein were important tools to help detect MuSK and its activity. The role of antibodies in MuSK-related research got an extra dimension when autoantibodies to MuSK were found to cause myasthenia gravis (MG) in 2001. Active immunization with MuSK or passive transfer of polyclonal purified IgG(4) fractions from patients reproduced myasthenic muscle weakness in a range of animal models. Polyclonal patient-purified autoantibodies were furthermore found to block agrin-Lrp4-MuSK signaling, explaining the synaptic disassembly, failure of neuromuscular transmission and ultimately muscle fatigue observed *in vivo*. MuSK autoantibodies are predominantly of the IgG4 subclass. Low levels of other subclass MuSK antibodies coexist, but their role in the pathogenesis is unclear. Patient-derived monoclonal antibodies revealed that MuSK antibody subclass and valency alters their functional effects and possibly their pathogenicity. Interestingly, recombinant functional bivalent MuSK antibodies might even have therapeutic potential for a variety of neuromuscular disorders, due to their agonistic nature on the MuSK signaling cascade. Thus, MuSK antibodies have proven to be helpful tools to study neuromuscular junction physiology, contributed to our understanding of the pathophysiology of MuSK MG and might be used to treat neuromuscular disorders. The source of MuSK antibodies and consequently their (mixed) polyclonal or monoclonal nature were important confounding factors in these experiments. Here we review the variety of MuSK antibodies described thus far, the insights they have given us and their potential for the future.

1. Introduction

Antibodies are one of the most efficient effector molecules of the immune system that protect us from potentially harmful pathogens. In addition, due to their specificity and the fact that they can be easily manipulated and produced, they have become important scientific tools and are widely applied as therapeutics. For an excellent review on (human) IgG structure and function [1] is recommended. However, when antibodies develop against a self-antigen, this may result in autoimmunity. One such autoimmune disease is muscle-specific kinase (MuSK) myasthenia gravis (MG). MuSK MG is a remarkable autoimmune disease, as it is hallmarked by predominant IgG4 autoantibodies [2]. MuSK MG thereby belongs to a new niche of autoimmune diseases characterized by predominant pathogenic IgG4 autoantibodies [3-5]. The reason for and importance of the dominant IgG4 response in these disorders is not fully understood. MuSK MG is an interesting model disease for unravelling these research questions.

During the last three decades antibodies have proven important tools to provide insight in the physiological role of MuSK at the neuromuscular junction (NMJ) and the pathomechanism of MuSK MG. These studies also illustrate that the source of MuSK (auto)antibodies in these experiments is critical for interpretation of the results. In this thesis, we provide a comprehensive overview of MuSK antibody research, the insights different antibodies have given us and their therapeutic potential.

2. MuSK antibodies as molecular biology tools to uncover the role of MuSK at neuromuscular synapses

MuSK and its essential role at the NMJ were discovered in the mid-nineties [6, 7]. To further study the role of MuSK at the NMJ, a range of MuSK-specific antibodies were generated (a summary of these antibodies and the main study conclusions are given in Table S1). The majority of antibodies were raised using different domains of rat MuSK in rabbits or goats [7-14]. Epitope specificity and antibody isotype were investigated for only a limited number of these antisera. In addition, polyclonal antibodies were generated against different domains of mouse, chicken, torpedo and human MuSK [10, 15-22]. Most antibodies result from immunization of laboratory animals in research groups with a particular interest in MuSK and NMJs; though, some commercial antibodies became available. These tools gave insight in MuSK localization, structure, signaling and interactors at the NMJ.

MuSK was found to be concentrated at the skeletal muscle NMJ, where it colocalizes with densely packed acetylcholine receptors (AChRs) [7, 16, 18, 23-25]. Though most studies have focused on the role of MuSK in the NMJ, expression of MuSK mRNA and protein has also been confirmed in multiple brain regions and other non-muscle tissues like retina, testis and hepatocellular carcinoma cell lines [22, 26-28]. The role of MuSK in these cells and organs has received little attention so far. One explanation for this is the lack of tools to visualize the MuSK protein, *in situ*. Although MuSK could be immunostained in astrocytes of rat retina [28], currently available antibodies (both of human and non-human origin) are unable to label MuSK in the central nervous system (personal observation). One explanation might be that alternatively spliced or alternatively post-translationally modified versions of MuSK predominate in these organs, which creates a variation in the expression of epitopes or their accessibility. Indeed, depending on the species, skeletal muscle MuSK has two or three N-linked glycosylation sites [29] and alternative splice variants have been described in the central nervous system [26]. To study the role of MuSK in other organs, new antibodies recognizing these tissue-specific variants will be essential.

MuSK is a single pass transmembrane receptor tyrosine kinase consisting of three extracellular N-terminal Ig-like domains, a Frizzled-like domain (Fz-domain) and an intracellular kinase domain [14, 30, 31]. Detection of these specific domains is possible with antibodies binding the N-terminal Ig-like domain [32, 33], the second Ig-like domain [34] or Fz-domain [35] (Table S1 and S5).

Absence of MuSK is incompatible with life, as MuSK is essential for prepatterning of AChRs, synapse formation and maintenance of adult NMJs [36, 37]. Mice lacking MuSK thus fail to form NMJs and die perinatally due to respiratory failure. To unravel the role of MuSK at the NMJ in more detail, immunoprecipitation using MuSK antibodies and consequently checking its phosphorylation status has been highly informative for deciphering MuSK signaling. MuSK was identified as one of the long sought components of the agrin-mediated AChR clustering signaling cascade [9, 15] (Figure 1A). However, agrin does not interact with MuSK directly. It requires low-density lipoprotein receptor-related protein 4 (Lrp4) to co-stimulate MuSK [38, 39]. Immunoprecipitation of truncated or mutated variants of MuSK and Lrp4 revealed that the fourth propeller domain of Lrp4 binds to the Ig1-like domain of MuSK [39]. In fact, two heterodimers of agrin and Lrp4 are required to bind and force dimerization of MuSK [40]. Antisera selective for the N-terminal domain of MuSK confirmed the

importance of MuSK dimerization as a critical step in this pathway, as bivalent IgG was able to stimulate MuSK phosphorylation and AChR clustering whereas monovalent Fab binding to MuSK did not [20, 32, 35].

With the help of antibodies binding specific MuSK peptide sequences containing phosphorylated tyrosine residues, it was furthermore found that agrin (and likely subsequent Lrp4 binding and MuSK dimerization) induced specific phosphorylation of Y553, Y750, Y754, Y755 in the juxtamembrane and activation loop of MuSK [10, 12]. MuSK is only then considered an active kinase and duly activates a variety of intracellular pathways resulting in for example synaptic gene expression and AChR clustering. Downstream of kinase 7 (Dok7), a cytoplasmic adapter, is furthermore required to maintain MuSK kinase activity and recruits further substrates like Crk and Crk-L [11, 18, 19, 21]. MuSK kinase activity and presence is further regulated by its internalization [41, 42], phosphatases like Shp2 [20] and regulation of its expression [43, 44]. Importantly, perturbation of MuSK signaling in mature synapses, either through selective inhibition using for example RNAi [45], or by (auto)antibodies blocking its function [46], results in synaptic disassembly and causes severe neuromuscular transmission deficits (see also sections 3 and 5 on MuSK antibodies derived from MuSK MG patients).

In addition to these key players in MuSK signaling, immunoprecipitation with MuSK antibodies revealed that MuSK has several extracellular interactors like wnts, Collagen Q (ColQ), biglycan and bone morphogenetic proteins (BMP)-4 [47-51] and intracellular interactors like 14-3-3 γ, Src homologous and collagen protein D (ShcD) and Dishevelled [13, 17, 52]. Knowledge on the biological importance of these interactions is unfortunately still limited and their relevance may differ between species [53]. Together MuSK antibodies have been instrumental tools to build the current model of synapse establishment and maintenance via the agrin-Lrp4-MuSK signaling cascade we have today (Figure 1A).

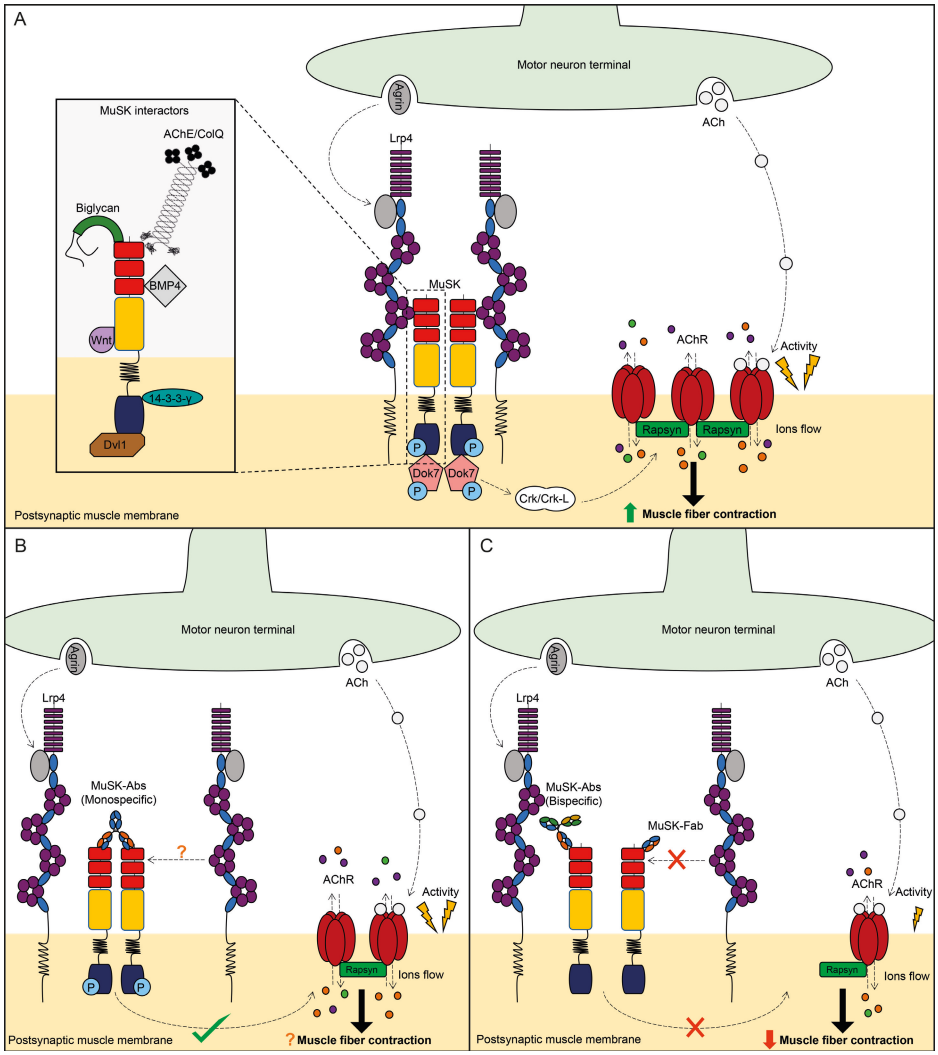


Figure 1. An overview of the MuSK signaling cascade. MuSK activation and MuSK-interacting proteins in healthy individuals (A). Agrin is released by the motor neuron, binds Lrp4 and together they stimulate MuSK dimerization and activation of its intracellular kinase domain. Activation of MuSK further requires Dok7 to remain active and stimulate downstream signaling towards AChR clustering. Binding of functional bivalent MuSK antibodies can bypass the need for agrin-Lrp4 in this pathway and directly stimulate MuSK dimerization and phosphorylation (B). MuSK is thereby fully activated, and AChR clustering is partially induced. Monovalent antibody binding by bispecific MuSK antibodies or Fab-fragments inhibits the binding of Lrp4 to MuSK, MuSK dimerization and AChR clustering (C). The loss of AChR clustering impairs neuromuscular transmission which results in myasthenic muscle weakness observed in experimental animal models of this disease and MuSK MG patients.

3. MuSK antibodies in MG patient serum or plasma

MG is an acquired neurological autoimmune disease hallmarked by fatigable skeletal muscle weakness. In the late seventies Lindstrom, Lennon and Seybold discovered that (auto)antibodies against AChR cause this disease [54]. In 2001, Hoch et al. characterized the second subgroup of MG patients that were hallmarked by MuSK autoantibodies [55]. Approximately, 4-8% of all MG patients have MuSK autoantibodies [56, 57]. The diagnosis of MuSK MG is currently based on the detection of MuSK antibody titers in blood using standardized radio-immunoprecipitation, cell-based assays or ELISA. For an extensive overview on (MuSK) MG, the disease subgroups and clinical aspects the recent reviews of Gilhus, Verschuuren and Evoli et al. are recommended [56, 57]. Although MuSK is expressed in all skeletal muscles, MuSK MG patients particularly suffer from predominant ocular, facial and bulbar muscle weakness [58-60]. The disease can progress into a generalized severe form and respiratory crises occur relatively often in this specific MG subtype. Most MuSK MG patients rely on life-long immunosuppressive therapies. To improve treatment success or even develop new therapeutic options, it is essential to understand the pathophysiology. Studies using patient serum have greatly advanced our understanding of the clinical relevance and mechanistic effects of MuSK autoantibodies in this disease (Table S2).

Patient serum titers correlate with disease severity, providing the first evidence for the pathogenicity of MuSK autoantibodies [61, 62]. In addition to the diagnostic value, MuSK antibody titers are good predictors of treatment responsiveness within an individual patient, but cannot be compared between patients [61, 63]. In other words, similar MuSK antibody titers can result in different disease severities in two individual patients. MuSK autoantibodies in patient serum were surprisingly shown to be predominantly of the IgG4 subclass; although, lower levels of other subclass MuSK antibodies can coexist [2, 62]. The main immunogenic region (MIR) of MuSK for these antibodies resides in the N-terminal Ig-like 1 domain; however, antibodies to all other extracellular domains have been detected [55, 63, 64]. The (pathogenic) effects of antibodies outside the MIR are not well understood. However, their presence suggests that epitope spreading has occurred during the development of the immune response against MuSK.

The functional consequence of serum antibodies binding to MuSK was first studied in *in vitro* settings using the C2C12 myotube cell line or TE671 muscle cells [55, 65]. Patient serum was shown to inhibit agrin-induced

AChR cluster numbers and AChR expression [55, 65]. Some studies had contradictory results, which may be the result of low MuSK antibody titers [66]. Importantly, many of these studies did not normalize for MuSK antibody-specific titers and as such are difficult to compare. In patient biopsies, little to no loss of AChRs density and structure, IgG deposition, or complement was observed [67, 68]. These studies, however, investigated the intercostal muscle and biceps brachii muscle, which are likely less affected by MuSK antibodies. A number of MuSK MG patients experience permanent muscle atrophy in bulbar muscles [58]. In line with this, MuSK MG patient serum increased the expression of atrophy-related genes like *Striated Muscle RING-Finger protein-1* (*MURF-1*) and *atrogin-1* [65, 69]. MuSK MG patient plasma also moderately inhibited synapse regeneration, when NMJs were challenged with notexin induced muscle damage [70].

Characterization of patient serum and plasma has resulted in some of the first important insights in the immunological and mechanistical features of this disease. Due to the clinical relevance of patient serum and easy accessibility, it furthermore created the opportunity to study large cohorts in different countries. However, the heterogeneity and relatively low level of autoantibody titers in serum, made more detailed studies towards the pathogenesis in a laboratory setting challenging. To unravel the pathomechanism of MuSK MG further, purified IgG fractions and experimental animal models have been instrumental.

4. MuSK antibodies derived from active immunization models

Once MuSK was identified as a new antigen in MG, it became possible to generate an animal model of MuSK MG through active immunization. Mice, rats and rabbits of different strains were injected with exogenous MuSK to elicit an antibody-mediated immune response (Table S3). Six to eight weeks after initiation of these vaccinations, an immune response could be detected and the animals developed a range of myasthenic symptoms [71-77]. Most experienced difficulties breathing, which is one of the key symptoms in MuSK MG [58, 60]. Not all vaccinations resulted in phenotypical MG and the disease severity between individual animals differed [71-75, 77, 78]. Disease severity in these animals correlated with the dose of MuSK immunogen and the antigen-specific antibody titer [71, 72, 76, 77].

Interestingly, active immunization of mice with MuSK results in predominant non-complement fixing IgG1 antibodies to MuSK [74, 77, 79, 80]. Although the functional features of mouse IgG1 do not completely recapitulate the features of human IgG4, it is a striking observation that MuSK seems to steer

this type of antibody response. Importantly, the MuSK antibody-subclass is not critical for the development of experimental autoimmune MuSK MG (EAMG) as IgG1-deficient mice can also mount an antibody response to MuSK and develop myasthenia [79]. As such active immunization with MuSK recapitulates the symptoms and antibody features observed in patients and provided evidence for the pathogenicity of MuSK autoantibodies.

Active immunization with MuSK further gave the opportunity to investigate the pathophysiology of MuSK MG *in vivo*. For example, compound muscle action potential (CMAP), a (clinical diagnostic) measure of the ability of a group of muscle fibers to generate an action potential, showed a decrement in these animals similar to what is seen in patients [71, 72, 75-78]. Several studies also investigated the effects of the anti-MuSK response on a single synaptic level with electrophysiological experiments. They demonstrated reduced miniature endplate potential or current (MEPP or MEPC) and end plate potentials or currents (EPP or EPC) [74, 75, 78]. The effect of MuSK antibodies on quantal content remained a matter of debate after these studies [74, 75].

On a molecular biology level, these studies also confirmed a loss of AChR clustering and fragmented NMJs in for example diaphragm, soleus, masseter and thoracic muscles [71, 74-76, 78]. Limb muscle were relatively spared compared to bulbar muscles, highlighting the sensitivity of bulbar muscles also in these models. Interestingly, MuSK expression seems to be lower in these muscles compared to limb muscles. This provides a possible explanation for the predominant bulbar muscle weakness in MuSK MG patients, as critical levels of MuSK will more quickly be reached in tissues with lower expression levels and thus will show muscle weakness earlier [71, 81].

Importantly, serum extracted from immunized mice and rabbits inhibited agrin-induced AChR clustering in C2C12 myotubes, similar to experiments using patient serum [55, 72, 73, 75, 82]. Interestingly, in the absence of agrin, rabbit-derived MuSK antibodies induced MuSK and AChR phosphorylation *in vitro,* while they strongly inhibit agrin-induced AChR clustering [73]. As these antibodies are polyclonal, it was suggested that some antibodies fully inhibit the cascade resulting in AChR clustering loss, while others moderately activate this cascade. As described previously, Hopf and Hoch demonstrated that bivalent antibody binding to MuSK was sufficient to induce downstream activation of MuSK and AChR clustering [32]. Bivalent IgG derived from these rabbits activated MuSK and Dok-7 phosphorylation and AChR clustering independent of agrin,

but inhibited agrin-induced AChR clustering [82]. In contrast, monovalent Fabs inhibited all these processes. Later studies confirmed that MuSK antibody valency influences their effect on the MuSK signaling cascade (see section 6 on patient-derived monoclonal MuSK antibodies) [33, 82, 83]. Retrospectively, this may explain the same observed moderate activation of AChR clustering seen with patient serum [55].

The active immunization studies uniquely contribute to our understanding of MuSK MG by showing that an autoimmune response against MuSK in different species causes muscle weakness, and can recapitulate the symptoms seen in MuSK MG patients. Furthermore, these animal models have given us great insight into the *in vivo* and *in vitro* pathophysiology of MuSK MG by demonstrating that MuSK antibodies, dependent on the antibody titers, alter the efficiency of neuromuscular transmission and reduce AChR density at NMJs. Lastly, active immunization has been a great source of MuSK antibodies and revealed that antibody valency may contribute to the manner by which a MuSK antibody affects downstream MuSK signaling. However, in interpreting these studies it is important to remember that rodent and rabbit IgG have different properties compared to human IgG; especially, when it comes to IgG4 [84]. Human IgG4 can undergo Fab-arm exchange, which has not been demonstrated under physiological conditions in rodents and rabbits [84]. Even though Mouse IgG1 is not a great complement fixer, it binds complement better than human IgG4, possibly overestimating the pathogenic effects of MuSK antibodies in mice [80]. Lastly, the method by which the antibody response is induced in these animals may not reflect the manner by which humans encounter antigen and mount a MuSK-specific response. *In vivo* models based on human IgG were explored to expand our understanding of natural occurring MuSK antibodies in patients.

5. MuSK MG patient-purified IgG

IgG can be purified from patient serum or plasmapheresis material via an ammonium sulfate–based precipitation method or by using an IgG-specific affinity resins [46, 85]. These methods also enable manipulation of the concentration of (antigen-specific) antibody titers in a range of experimental procedures. This is important as low levels of MuSK antibodies in patients do not always result in overt muscle weakness and higher concentrations of MuSK-specific antibodies are needed to reproduce disease in experimental models. Purified patient-derived IgG has thereby enabled the development of passive transfer models to study

the onset of disease, the pathophysiology and potential therapeutics (Table S4). Purified IgG was furthermore used to shed light on the molecular mechanism by which the antibodies cause myasthenia, the effects they have on other MuSK-interacting proteins and the functional characteristics of IgG4 MuSK antibodies.

MuSK is highly conserved in different species and MuSK MG patient-derived purified IgG(4) can bind and immunostain MuSK in whole mount mouse NMJs [46]. Purified IgG1-3 fractions from the same patients did not, suggesting that MuSK-specific antibodies in this fraction were either absent or too low to detect. One of the major questions in MuSK MG was whether IgG4 MuSK antibodies, due to the anti-inflammatory reputation of IgG4, could truly induce myasthenia [66-68, 86]. Several studies therefore passively transferred patient-derived purified IgG(4) into mice. This resulted in dose-dependent induction of myasthenic features, including muscle weakness, weight loss and CMAP amplitude decrement on repetitive nerve stimulation [46, 75, 85, 87]. Depending on the dose and patient, the symptoms occurred one to two weeks after initiation of the exposure to IgG(4). Similar experiments using the IgG1-3 fractions from the same patients did not result in (subclinical) myasthenia [46]. Moreover, passive transfer of patient-derived purified IgG(4) reduced AChR density, similar to what was earlier seen in *in vitro* myotube cultures. This likely contributes to the failure of neuromuscular transmission observed in the experimental mice [75, 85, 87-91]. In line with the observation in actively immunized mice, *ex vivo* electrophysiology experiments revealed reduced MEPP frequency and MEPP and EPP amplitude [46, 75, 87]. Surprisingly, compensatory upregulation of quantal content, which is observed in animal models for AChR MG, did not occur in these mice. Bulbar muscles seem particularly affected in these animal models; although, MuSK antibody binding could be detected in a range of limb, neck and bulbar muscles *ex vivo*.

Passive transfer of patient-derived purified IgG might also result in cognitive abnormalities in mice, although from this study it is unclear what percentage of patient antibodies reached MuSK in the central nervous system [92]. MuSK is expressed in the brain, with a possible role in hippocampal long-term potentiation [26]. Whether MuSK antibodies can influence brain functioning warrants further investigation.

These passive transfer models also facilitate preclinical testing of potential therapeutics. For example, *in vivo* reduction of passively transferred IgG by inhibiting its recycling through neonatal Fc receptors quickly ameliorated myasthenic symptoms, while neurotransmission and synaptic structure

seemed marginally altered [93]. A phase II clinical trial also reported a beneficial effect of this treatment in AChR MG patients [94]. The group of Phillips *et al.* elegantly investigated the effect of β-adrenoceptor agonist albuterol and 3,4-diaminopyridine and showed that these treatments provided significant improvement in muscle function, while synaptic functional and morphological features seemed marginally altered [95, 96]. Together these studies provide evidence that minimal improvement on single synaptic level can have profound effects on whole body muscle function. Acetylcholine esterase (AChE) inhibition often does not benefit MuSK MG patients or even worsens the disease [58, 97]. Pyridostigmine treatment in a passive transfer model using total IgG from MuSK MG patients indeed worsened the disease and was shown to reduce AChR cluster area even further [95]. When passive transfer of patient IgG is stopped when disease symptoms are at its worst, phenotypical remission in the mice can be observed after approximately 7 days (personal observation).

The mechanism by which MuSK antibodies induce MG are at least two-fold: increased internalization and subsequent depletion of membrane-bound MuSK and inhibition of agrin-Lrp4-MuSK signaling [83, 88, 91, 98]. Both effects ultimately result in reduced AChR cluster numbers and loss of the post-synaptic scaffold culminating in impaired neuromuscular transmission. IgG4 MuSK antibodies did not block MuSK dimerization directly [98]. In C2C12 myotubes, the effects of patient-purified IgG is ambiguous as both activation and inhibition of MuSK tyrosine phosphorylation was observed [88, 98]. These contradicting observations might be due to the ratio of bivalent and monovalent MuSK antibodies in polyclonal IgG (as discussed in section 4) which likely differs between patients, and is not analyzed in these studies. Increased MuSK internalization was reported with patient-purified IgG, while patient-purified IgG4 fraction did not affect MuSK internalization [83, 88, 98]. It is therefore tempting to speculate that the bivalent anti-MuSK IgG1-3s in the patient-purified IgG preparation are promoting MuSK internalization. However, the single study that has investigated the effects of MuSK antibodies specifically in the IgG1-3 fraction of a MuSK MG patient did not find increased internalization. Since these studies do not report MuSK-specific titers in their preparations, it is impossible to determine whether this discrepancy is due to anti-MuSK IgG1-3 titers or other (technical) factors. Further studies are necessary to conclusively confirm whether bivalent MuSK antibodies can induce MuSK internalization, which would suggest antibody valency and subclass are important for this mechanism.

It was evident that impairment of MuSK signaling is involved in the pathomechanism of MuSK MG; however, it was still unclear how autoantibodies affect interaction with other MuSK-interacting proteins. One of these proteins is AChE, which binds to MuSK through ColQ. MuSK antibodies block binding between ColQ and MuSK [90]. This is particularly interesting because this may contribute to sensitivity to AChE inhibition which is observed in both MuSK MG patients and mice, as mentioned above [58, 95, 97]. Whether interaction with other proteins like biglycan and BMP4 is impaired in MuSK MG patients is yet unknown.

Previous studies have shown that IgG4 can undergo Fab-arm exchange [99]. Fab-arm exchange is the process in which a half-molecule of one IgG4 exchanges stochastically with a half-molecule of another IgG4. Affinity purification of patient-derived MuSK antibodies and their light chain specificities indicated that up to 99% of the anti-MuSK IgG4s in blood, are bispecific and bind to MuSK in a monovalent fashion [100]. Forced Fab-arm exchange of patient-derived IgG with normal human IgG4 did not alter the pathogenicity of the antibodies further. These experiments suggest that MuSK IgG4 antibodies engage in Fab-arm exchange and that the vast majority is bispecific and functionally monovalent.

Studies characterizing MuSK MG patient-derived purified IgG and its subclasses have given us further insights in the pathomechanism of MuSK MG and provided new means to perform preclinical testing of a variety of (new) treatments. Due to the polyclonal nature of purified IgG from patients, high batch-to-batch, interindividual variability and limited availability, it is challenging to interrogate the effect of different antibody characteristics such as epitope, affinity and valency, and their role in MuSK MG pathogenesis. Patient-derived monoclonal antibodies form an exciting new tool to further unravel the effects of MuSK (auto)antibodies.

6. Patient-derived monoclonal MuSK antibodies

In contrast to polyclonal patient-derived purified IgG, patient-derived monoclonal antibodies are relatively easy tools to manufacture in a homogenous and reproducible manner. Furthermore, effector functions, the binding domains and thus their specificity can be easily manipulated. Monoclonal antibody sequences can be obtained from plasmablasts or by cloning the B cell receptor from memory B cells. It is important to realize that each method used to isolate such sequences introduces technical biases. Furthermore, plasma cells, that are responsible for the ongoing antibody response, usually reside in the bone marrow [101].

Therefore, one cannot expect the isolation of monoclonal antibodies using these techniques to give a complete and comprehensive overview of all antibody-mediated (auto)immune responses ongoing in an individual. It does allow us to study a small proportion of the circulating (antigen-specific) immune responses. Once antibody sequences have been isolated, the heavy and light chain sequences are cloned into an IgG backbone of choice and transfected in eukaryotic cell lines to produce recombinant monoclonal antibodies. In the past year these methods have advanced our knowledge of the genetic make-up of such MuSK monoclonal antibodies and their functional effects (Table S5).

Until now, 12 monoclonal MuSK antibody sequences from four patients have been isolated and characterized [33, 34, 102]. From the majority of patients, it was not possible to isolate MuSK antibody sequences. The fact that these numbers are relatively low likely reflects low numbers of circulating MuSK-specific B cells, and the ones that do circulate are difficult to capture. In spite of the dominant IgG4 antibody response in serum, relatively low numbers of IgG4 clones (N=3) were isolated. The other clones were of the IgG1, IgG3 or IgM isotypes. These antibodies made use of different V_h and V_l genes; although, the IgG1 and IgG3 clones from one patient were clonally related [33]. High levels of somatic hypermutation were reported (except for the IgM clone), suggesting antigen-driven affinity maturation. Sequence analysis of the Fc-tail of one of the IgG4 clones confirmed the presence of residues critical for Fab-arm exchange [33]. The monoclonal antibodies either bound the Ig-like 1 domain and the Ig-like 2 domain selectively. This matches with earlier observations, where epitope mapping of polyclonal serum identified the first Ig-like domain as the MIR and Ig-like 2 domain antibodies as the second most common epitope [2, 63]. All monoclonal antibodies described above bind whole mount mouse NMJs, providing new exciting tools to induce MG *in vivo* in the future.

Recombinant antibodies made based on these sequences were further explored for their effects on the MuSK signaling cascade. Most antibodies were produced in an IgG4 or IgG1 backbone. Recombinantly produced IgG4 monoclonal antibodies, when not exposed to other IgG4 molecules in a reducing environment, are functionally monospecific and bind in a bivalent manner to MuSK. To mimic functional monovalent binding of bispecific, Fab-arm exchanged IgG4 in patients, Fab-fragments were made by papain digestion [33]. While Fab-fragments inhibited both agrin-induced MuSK phosphorylation and AChR clustering, monospecific functionally bivalent MuSK IgG4 only partially inhibited

agrin-dependent AChR clustering [33, 34]. Contrarily, in the absence of agrin these monospecific functionally bivalent antibodies induced MuSK phosphorylation similar to agrin and partially induced AChR clustering [33]. This suggests that functionally monovalent Fab-fragments can recapitulate the inhibitory effect of Fab-arm exchanged patient IgG on both MuSK-phosphorylation and AChR clustering. This agonistic effect of functionally bivalent anti-MuSK IgG shows overlap with earlier described antibodies produced in active immunization models [73, 82]. This data suggests that anti-MuSK IgG4 becomes more pathogenic after Fab-arm exchange. This also suggests that class switching towards functionally monovalent IgG4 autoantibodies may be particularly detrimental in MuSK MG.

The study of monoclonal MuSK antibodies and their pathogenic effects is still in its infancy. The first observations are remarkable and have given us the current models for their pathophysiological effects (Figure 1B& 1C). It is essential to confirm this in animal models. The isolation of more MuSK monoclonal antibodies seems important to further elucidate the etiology and development of this immune response.

7. The therapeutic potential of MuSK antibodies

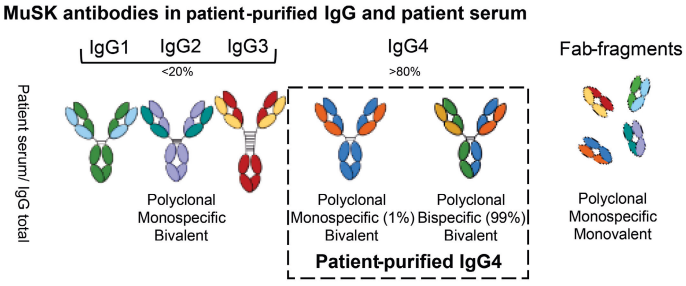
Many neuromuscular disorders are hallmarked by impaired NMJs. Due to the importance of MuSK signaling for establishing and maintaining synapses, it is tempting to speculate that stimulating MuSK might have therapeutic potential for these disorders [103]. Following this hypothesis, it was shown that MuSK overexpression preserved innervation and motor function for more than a month in a mouse model for amyotrophic lateral sclerosis (ALS) [104]. In addition, several monoclonal MuSK binding scFvs were identified using phage display, which induced MuSK phosphorylation and AChR cluster formation [35]. One of these MuSK binders was produced in a (murinized) IgG format and also tested in ALS mice [105, 106]. Both studies passively transferred antibody #13 in SOD1-G93A mice and demonstrated that treatment with antibody #13 improved innervation of the NMJ and slowed down muscle denervation, compared to mock treated mice (Table S6). Cantor *et al.* further demonstrated improved motor neuron survival and muscle function, resulting in a marginally extended lifespan [105]. The effects on muscle function and life-span were not observed by Sengupta-Ghosh *et al.*, which might reflect large differences in their experimental procedures [106]. These studies demonstrate that MuSK agonists have the ability to at least preserve structural integrity of neuromuscular synapses in ALS mice, more research is needed to confirm

improvement of muscle function. Evaluating the therapeutic potential of MuSK agonistic antibodies in other neuromuscular disorders could form an important new line of research.

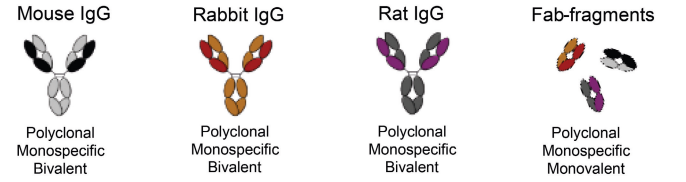
8. Conclusions

MuSK antibody research has greatly advanced our understanding of the physiological role of MuSK at the NMJ and the pathomechanism of MuSK MG. Recently, therapeutic potential of MuSK antibodies has enhanced the interest for these antibodies from a different point of view. It is important to realize that with each different antibody format or source different research questions can be answered and experimental bias is introduced. Figure 2 gives an overview of the structural and functional characteristics of each of the antibody sources in MuSK antibody research. For MuSK antibodies, the structure and valency seem particularly crucial and may explain contradictory experimental outcomes from the past. The isolation of more patient-derived MuSK antibody sequences and understanding their functional effects are anticipated to provide more insight in the development of MuSK MG and their application as therapeutics in the future.

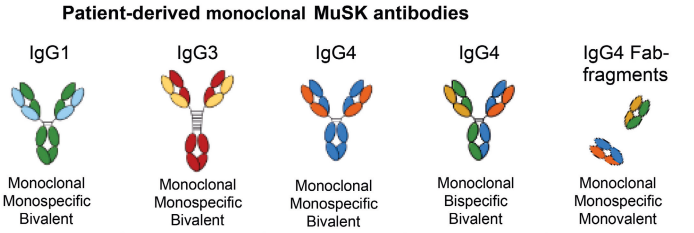
Legend for figure 2 (page 25). The structural characteristics of MuSK antibodies and their effects on MuSK signalling. Patient-derived purified IgG and serum are a heterogenous source of a polyclonal mixture of MuSK antibodies. IgG1-3 antibodies are expected to bind in a bivalent manner and activate complement while the majority of IgG4 MuSK antibodies will become bispecific and functionally monovalent for MuSK and will not activate complement. The distribution of MuSK antibodies over the different subclasses likely differs between individuals and was estimated to be >80% IgG4 in one study [83]. While the functional effects of anti-MuSK IgG1-3 vary, both IgG4 and monovalent Fab-fragments derived from polyclonal patient-purified IgG and patient serum consistently show inhibitory effects. Bivalent antibodies of mouse or rabbit origin fully activate MuSK, while AChR clustering is inhibited. These antibodies are expected to bind complement to varying degrees. Fab-fragments derived from active immunization-derived antibodies also cause inhibition of both MuSK phosphorylation and AChR clustering. Patient-derived recombinant antibodies, when functionally bivalent, activate MuSK phosphorylation and AChR clustering (partially), while monovalent Fab or bispecific MuSK antibodies inhibit all these processes. +/++/+++ =mild/moderate/strongly stimulating, -/--/--- = mild/moderate/strongly inhibiting, blank = not investigated.



MuSK internalization	+ +	+ +	- - -	
MuSK phosphorylation	- - -		- - -	
AChR clustering	- - -	- - -	- - -	- - -
Ability to activate complement	+ + +	+ + +	- - -	



MuSK internalization				
MuSK phosphorylation	+ + +	+ + +		- - -
AChR clustering	- -	- -	- - -	- - -
Ability to activate complement				



MuSK internalization					
MuSK phosphorylation	+ +		+ + +	- - -	- - -
AChR clustering			+ +	- - -	- - -
Ability to activate complement	+ + +	+ + +	- - -	- - -	

Figure 2

9. Scope of the thesis

The studies with MuSK antibodies from different sources and with different characteristics highlight the importance of understanding how MuSK MG patient antibodies and their specific characteristics cause MuSK MG. So far, it is not understood why MuSK antibodies are predominantly IgG4. The predominance of IgG4 MuSK antibodies in MuSK MG patients could theoretically be explained by a predisposition of MuSK MG patients to develop IgG4 responses. If that would be the case, IgG4 might also be the preferred response to other antigens and an overall increased serum IgG4 would be expected in these patients. Furthermore, abnormalities in the levels of other serum immunoglobulin subclasses or isotypes may help uncover the etiology of the disease. Therefore, in **Chapter 2**, the general immunoglobulin response of MuSK MG patients (IgG4 autoimmune disease) is investigated and compared to that of AChR MG patients (IgG1/3 autoimmune disease) and healthy donors.

IgG4 MuSK antibodies derived from patients can become functionally monovalent due to Fab-arm exchange. Whether this monovalent binding influences the effects they have on MuSK, the neuromuscular junction and their pathogenicity is investigated in **Chapters 3, 4 and 5** using recombinant monoclonal MuSK antibodies from patient-derived B-cell receptor sequences. **Chapter 3** describes the isolation of patient-derived B-cell receptor sequences and investigates how valency of MuSK antibodies influences the effect on agrin-induced MuSK signaling *in vitro*. In **Chapter 4**, we developed a technology to generate stable monovalent IgG4 MuSK antibodies and studied the *in vivo* pathogenicity of both monovalent and bivalent MuSK antibodies by passive transfer studies. In **Chapter 5**, we studied the consequences of monovalent and bivalent MuSK antibodies with different characteristics on MuSK functioning and the neuromuscular junction *in vitro* and *in vivo* to understand the mechanisms underlying the pathogenic effects of MuSK antibodies.

The consequences of MuSK antibodies in disease, but also as a therapeutic agent, may not be limited to the neuromuscular junction. For example, there are clinical observations that suggest that in some MuSK MG patients the central nervous system might also be involved. Therefore, in **Chapter 6**, we explored where in humans the genes involved in autoimmune and congenital myasthenia gravis are expressed outside muscles, with a specific focus on the central nervous system. These anatomical locations may be at risk for non-motor symptoms or (side-) effects of therapeutic approaches targeting these proteins.

Together, the studies in this thesis aim to elucidate the cause and consequence of (the pathogenicity of) IgG4 MuSK antibodies in MuSK MG.

10. Supplementary information

Supplementary tables can be found here:

11. References

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