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

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## Colofon

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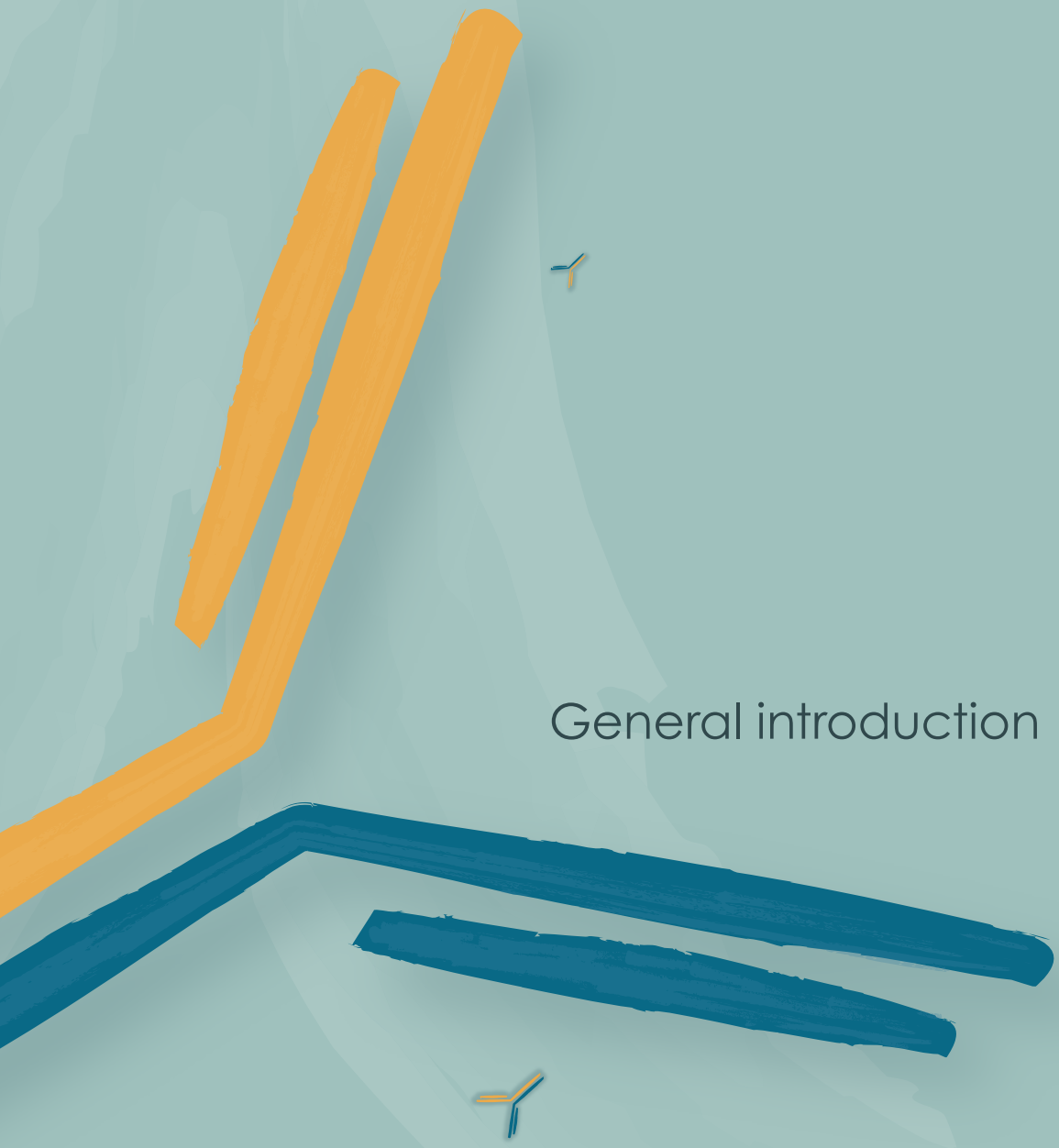

# General introduction

MuSK antibodies, lessons learned from poly- and monoclonality
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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).

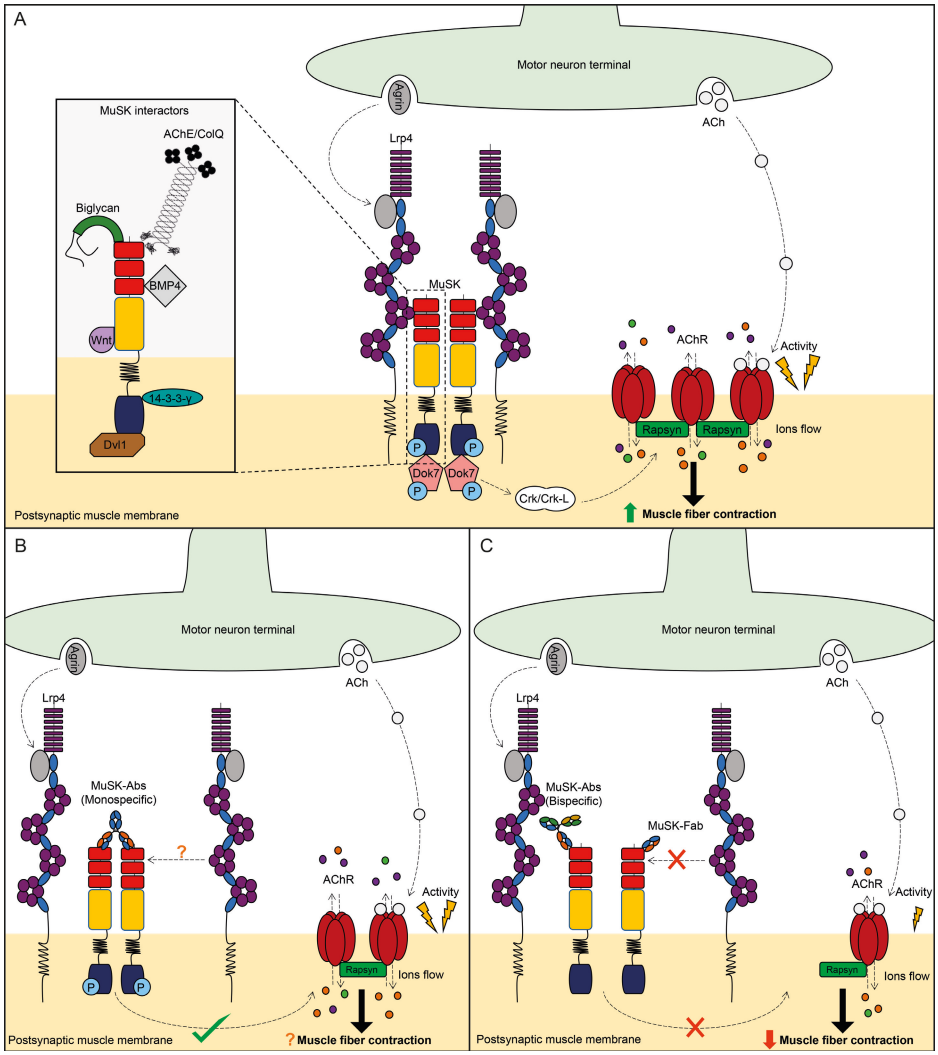



*Figure 1. An overview of the MuSK signaling cascade. MuSK activation and MuSK-interacting proteins in healthy individuals (A). Agrin is released by the motor neuron, binds Lrp4 and together they stimulate MuSK dimerization and activation of its intracellular kinase domain. Activation of MuSK further 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

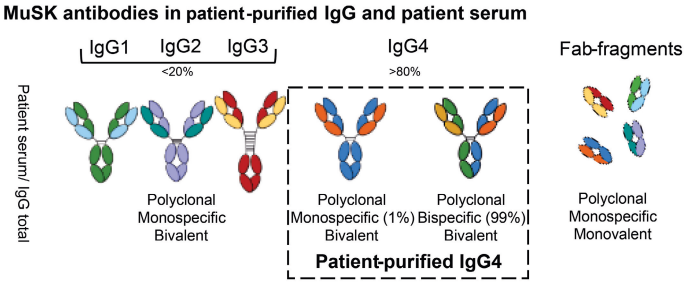
Many neuromuscular disorders are hallmarked by impaired NMJs. Due to the importance of MuSK signaling for establishing and maintaining synapses, it is tempting to speculate that stimulating MuSK might have therapeutic potential for these disorders [103]. Following this hypothesis, it was shown that MuSK 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.

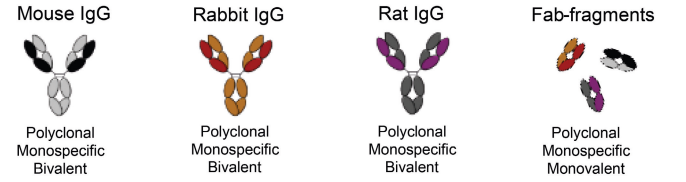
## 8. Conclusions

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.

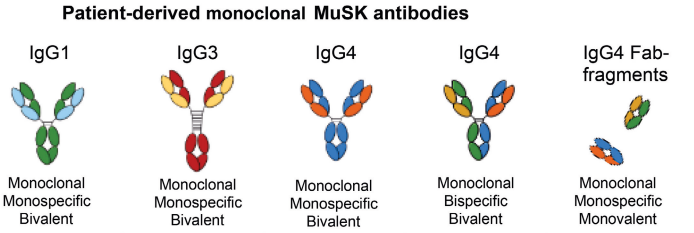
*Legend for figure 2 (page 25). The structural characteristics of MuSK antibodies and their effects on MuSK signalling. Patient-derived purified IgG and serum are a heterogenous source of a polyclonal mixture of MuSK antibodies. IgG1-3 antibodies are expected to bind in a bivalent manner and activate 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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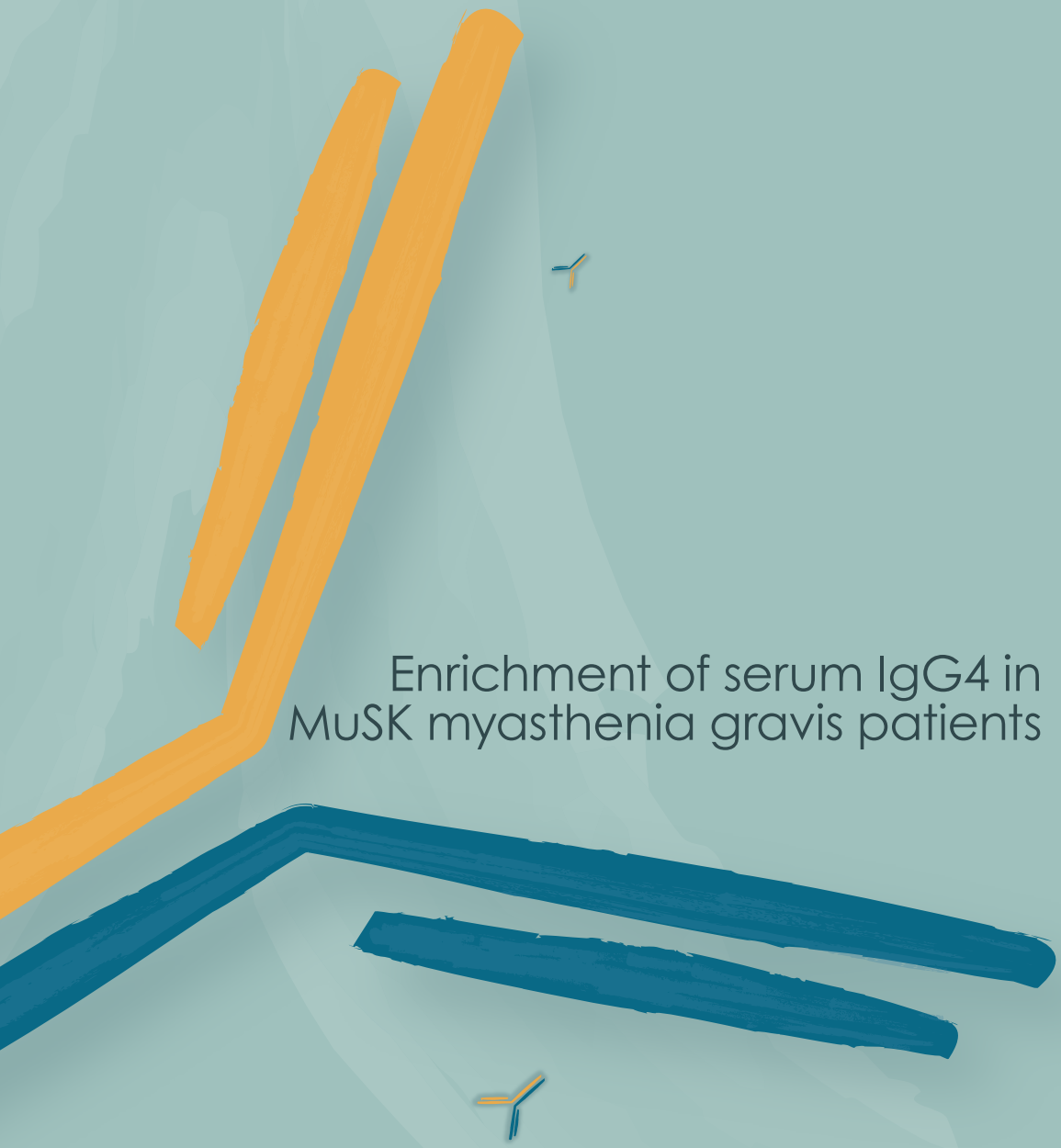

# Enrichment of serum IgG4 in MuSK myasthenia gravis patients

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## Abstract

Muscle-specific kinase (MuSK) myasthenia gravis (MG) is a neuromuscular autoimmune disease belonging to a growing group of IgG4 autoimmune diseases (IgG4-AIDs), in which the majority of pathogenic autoantibodies are of the IgG4 subclass. The more prevalent form of MG with acetylcholine receptor (AChR) antibodies is caused by IgG1-3 autoantibodies. A dominant role for IgG4 in autoimmune disease is intriguing due to its anti-inflammatory characteristics. It is unclear why MuSK autoantibodies are predominantly IgG4. We hypothesized that MuSK MG patients have a general predisposition to generate IgG4 responses, therefore resulting in high levels of circulating IgG4. To investigate this, we quantified serum Ig isotypes and IgG subclasses using nephelometric and turbidimetric assays in MuSK MG and AChR MG patients not under influence of immunosuppressive treatment. Absolute serum IgG1 was increased in both MuSK and AChR MG patients compared to healthy donors. In addition, only MuSK MG patients on average had significantly increased and enriched serum IgG4. Although more MuSK MG patients had elevated serum IgG4, for most the IgG4 serum levels fell within the normal range. Correlation analyses suggest MuSK-specific antibodies do not solely explain the variation in IgG4 levels. In conclusion, although serum IgG4 levels are slightly increased, the levels do not support ubiquitous IgG4 responses in MuSK MG patients as the underlying cause of dominant IgG4 MuSK antibodies.

## 1. Introduction

Human immunoglobulin G (IgG) is divided into four subclasses depending on small variations in the constant domain (Fc). These variations dictate their functionalities, such as their ability to activate complement, bind to Fc receptors on immune cells or undergo Fab-arm exchange [1]. In a healthy adult population, the following reference ranges have been determined as normal by the national Dutch blood bank: IgG1 (4.9-11.4 g/L), IgG2 (1.5-6.4 g/L), IgG3 (0.11-0.85 g/L) and IgG4 (0.03-2.0 g/L) [2]. IgG subclass levels can be abnormal due to a deficiency of IgG subclasses (hypogammaglobulinemia) or due to abnormal ratios of subclasses (dysgammaglobulinemia). Hypogammaglobulinemia is observed in diseases affecting class switching, such as CD40 ligand or activation-induced cytidine deaminase (cofactor) deficiencies, or in immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome [3, 4]. Dysgammaglobulinemia can be observed in for example selective IgG subclass deficiencies, monoclonal gammopathies or IgG4-related diseases [5-7].

Antibody-mediated autoimmune diseases are caused by antigen-specific autoantibodies, often predominantly of a specific IgG subclass. Muscle-specific kinase (MuSK) myasthenia gravis (MG) is part of a growing group of IgG4 autoimmune diseases (IgG4-AIDs), in which the majority of pathogenic autoantibodies are of the IgG4 subclass [8, 9]. Since IgG4 has anti-inflammatory characteristics, its role in autoimmune disease was surprising at first sight. In the past decade several studies have confirmed that IgG4 is indeed responsible for the pathology observed in these IgG4-AIDs. The predominant mechanism appears to be functional blocking of its antigen rather than antigenic modulation or complement activation common to IgG1-3 autoimmune diseases [8, 9]. However, why these diseases are dominated by IgG4 autoantibody responses is unknown. Altered levels of serum IgG subclasses have been found in several autoimmune diseases, such as primary Sjögren syndrome, systemic lupus erythematosus, rheumatoid arthritis, AChR MG and pemphigus [10-13]. Therefore, we hypothesized that patients with IgG4-AID may have a general predisposition to generate IgG4 responses. To assess possible dysregulation in the immunoglobulin response in MuSK MG, we measured IgG subclasses, Ig isotypes and antigen-specific titers in patients not under the influence of immunosuppressive treatments.

## 2. Materials and methods

### *2.1. Subjects*

MG patients were included from databases and biobanks at the Leiden University Medical Centre (LUMC, Leiden, the Netherlands), Policlinico Gemelli Università Cattolica del Sacro Cuore (Rome, Italy), Hospital Sant Pau UAB (Barcelona, Spain), Uppsala University (Uppsala, Sweden) and University of California Davis (Davis, CA, USA). The included serum samples from patients fulfilled the following criteria 1) positive for either AChR or MuSK antibodies, and 2) immunosuppression naïve or no immunosuppressive treatment for at least one year prior to the sample date. Patients who had received anti-CD20 treatment in their medical history were excluded from the study. Seventeen samples were excluded because the patients were receiving immunosuppression (IS) at time of sampling. These were used in a supplementary analysis examining IgG levels in IS vs non-IS treated patients. Samples and retrospective data were obtained from biobanks, in compliance with the institute's medical ethics committee guidelines. Demographics and clinical parameters were retrospectively collected from the medical history and gathered in an anonymized database. Sera were stored at -80 °C until analysis. Data from healthy adult donors were derived from the stem cell donor database of the Willem-Alexander Children's Hospital (LUMC, Leiden, the Netherlands).

### *2.2. Laboratory tests*

All measurements were performed by the clinical chemistry laboratory at the LUMC under standard operating procedures based on manufacturer's instruction. IgG subclasses, total IgG, total IgA and IgM were determined using nephelometric methods on BN-prospec (Siemens; IgG subclasses, total IgG, total IgA and IgM) or the turbidimetric assay using the Cobas8000 (Roche; total IgG, IgA and IgM). Total IgE was determined using the FEIA technique on ImmunoCAP250 (Thermo Fisher Diagnostics). AChR antibody titers were determined using radioimmunoprecipitation assay (RIA, RSR) and MuSK antibody titers with either RIA (RSR), or Enzyme-linked ImmunoSorbent Assay (ELISA, IBL, IgG4-specific).

### *2.3. Statistical analysis*

Statistical analyses were performed using SPSS statistics v25 (IBM). Demographics were analyzed with a Chi-square test (sex) or Kruskal-Wallis test (age). Absolute immunoglobulin levels were log-transformed before analysis. For group comparisons, ANCOVA with posthoc comparisons was

used and included age and sex as covariates. Elevated IgG4 proportions were tested with Fisher's Exact test.

To be able to combine MuSK antibody titers measured with the RIA and the ELISA, the values were ranked per variable for each assay separately. These sets of ranks were each converted to a scale from zero to one to normalize for the difference in the number of individuals analyzed between the two assays. The datasets for the MuSK RIA and MuSK ELISA were then combined and analyzed with a Spearman correlation.

Due to limited sample volume for some patient samples, we could not perform all predefined measurements. The group sizes available for individual measurements due to missing data are provided in the figure legends.



## 3. Results

### *3.1. Donor demographics*

Sera from five medical centers were included in this study. The MuSK MG group contained relatively more female patients, while AChR MG patients were generally older at time of sampling (Table S1). These demographic characteristics match the known epidemiological patterns for MuSK MG (female predominance) and AChR MG (bimodal age of disease onset, Table S1) [14]. Since the groups were significantly different in sex (Chi-square test, $p=0.024$) and age (Kruskal-Wallis, $p<0.001$) (Table S1), these were included as covariates in the statistical analyses. This is important because serum IgG4 levels can depend on both [15, 16]. Clinical information of the MG patient groups can also be found in Table S1.

### *3.2. Elevated and enriched serum IgG4 in MuSK MG patients*

IgG subclasses were measured in MuSK MG patients and compared with AChR MG patients (IgG1-AID) and healthy donors. MuSK MG patients had increased IgG1, similar to AChR MG patients (Figure 1A). In contrast, MuSK MG patients on average had higher serum IgG4 compared to both AChR MG patients and healthy donors, while no differences between groups were detected for IgG2 and IgG3 (Figure 1B-D). In line with previous findings, males had higher IgG4 levels than females ($p<0.01$) and this holds true for all groups (Figure S1) [15, 18]. Elevated IgG4 is defined as >1.35 g/L in IgG4-related disease [17]. In the MuSK MG group, 22% of patients had elevated serum IgG4 versus 3% in healthy donors and 10% in AChR MG patients, despite the female predominance in MuSK MG (Figure 1D, Fisher exact test, $p<0.01$).

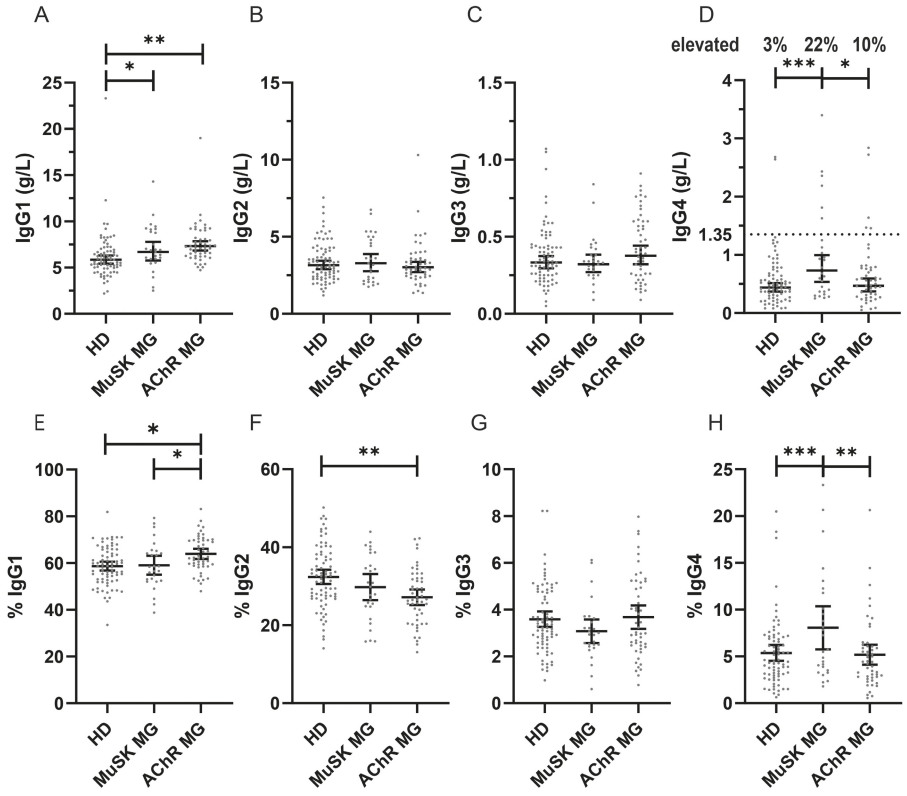


*Figure 1: Increased IgG4 in MuSK MG patients. Absolute values of IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D). Dotted line at 1.35g/L represents cutoff for elevated IgG4 in IgG4-RD [17]. IgG1 (E), IgG2 (F), IgG3 (G) and IgG4 (H) as a percentage of the sum of the IgG subclasses. Data are presented as geometric mean and geometric 95% confidence interval (CI) in A-D and as mean and 95% CI for E-H. * p<0.05, ** p<0.01, *** p<0.001. HD: n=77, MuSK MG: n=27, AChR MG: n=50. HD=healthy donors.*

To assess whether the subclass distribution differs between groups, we calculated the percentage of each subclass versus the sum of all IgG subclasses. In AChR MG patients, the relative proportion of IgG1 was higher, while IgG2 was relatively lower (Figure 1E-F). For MuSK MG patients, the relative proportion of IgG4 was significantly higher compared to the other subclasses (Figure 1H). The IgG subclass that is increased in each MG subtype is the subclass which dominates the antigen-specific autoantibodies.

Ig isotypes were measured to investigate whether an overall dysregulation of the immunoglobulin response is present. For a subset of patients, a separate measurement of total IgG revealed increased IgG for AChR

MG and a similar trend for MuSK MG (Figure 2A). The average serum IgM level in MuSK MG patients did not significantly differ from healthy donors and AChR MG (Figure 2B). A trend towards elevated serum IgA was present in both MG groups (Figure 2C). 43% of the MuSK MG patients had elevated IgE (>99 IU/mL) versus 31% of the AChR MG patients, this was not significantly different (Fisher's exact test, p=0.415). In the general adult population 32% have elevated IgE [19].

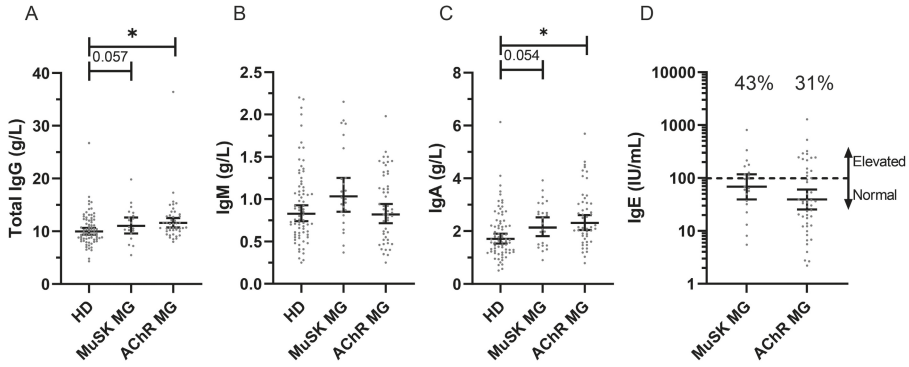


*Figure 2: Ig Isotypes. Absolute values of IgG (A), IgM (B), IgA (C) and IgE (D). Data is presented as geometric mean and geometric 95% CI. * p<0.05. HD: n=75, MuSK MG: n=24 (n=20 for IgG, n=21 for IgE), AChR MG: n=50 (n=44 for IgG, n=48 for IgE). HD=healthy donors.*

### *3.3. MuSK-specific titers correlate with serum IgG4*

To assess whether MuSK-specific antibodies contribute to the elevated serum IgG subclass levels, the relationship between MuSK antibody titers and absolute serum IgG1 or IgG4 was investigated, as these IgG subclasses showed elevated absolute levels in MuSK MG. A spearman correlation of ranks was performed to assess this relationship, because the data is non-parametric in nature. MuSK Ab titers significantly correlated with serum IgG4 (spearman's rho=0.511, p<0.05, n=22), but not with IgG1 (spearman's ρ=0.156, ns, n=22)(Figure 3). MuSK MG patients with higher serum IgG4 thus also more often have higher MuSK Ab titers.

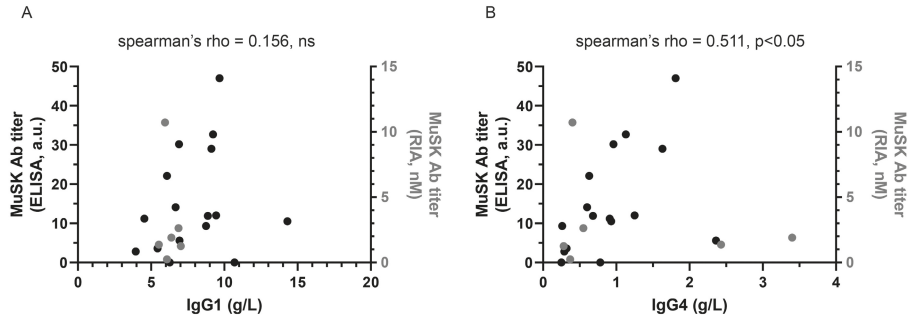


*Figure 3: MuSK antibody titers correlate with serum IgG4. Scatter plot of MuSK antibodies (Abs) versus IgG1 (A) or IgG4 (B). MuSK Abs detected by ELISA in black and by radioimmunoprecipitation assay (RIA) in grey.*

## 4. Discussion

IgG4 MuSK antibodies cause MuSK MG and correlate with disease severity both between and within patients [20-22]. In this study, we show that total serum IgG4 is increased in MuSK MG patients not under the influence of immunosuppressive treatment. MuSK MG patients with relatively high serum IgG4 also more often have relatively high MuSK titers, suggesting similar processes may underlie both. However, the observed individual variation suggests it is unlikely that the increased IgG4 levels are only due to MuSK-specific antibodies, indicating that MuSK MG patients experience an overall mild increase in IgG4 production. Absolute IgG1 levels were also elevated in both MuSK and AChR MG patients, resulting in an higher proportion of IgG1 vs all IgG subclasses in AChR MG patients only. Taken together, MuSK MG patients appear to have a (tendency to) mild increase in serum immunoglobulins, suggesting the antibody response in general may be more active. However, since circulating IgG4 levels fall within the normal range for most MuSK MG patients, it is unlikely that a predisposition to ubiquitously generate IgG4 responses causes the dominance of IgG4 antibodies targeting MuSK.

Total serum IgG4 is also selectively enriched in other IgG4-AIDs pemphigus vulgaris and pemphigus foliaceus [12]. In contrast, several recent studies did not find elevated serum IgG4 in various neurological IgG4-AIDs, including MuSK MG [18, 23, 24]. This may partly be explained by the low numbers of patient samples in these studies. Secondly, although samples were sometimes taken early in the disease course, a proportion of patients was receiving immunosuppression, which can reduce serum IgG(4) levels and thus influence how the immunoglobulin response relates to disease

activity (Figure S2, [25, 26]). To further support the observation that increased circulating IgG4 levels are a general phenomenon in IgG4-AIDs, sera of other IgG4-AID patient groups need to be investigated prior to starting immunosuppressive therapies.

IgG4-related diseases (IgG4-RD) are hallmarked by IgG4 producing B-cell infiltrates in organs, causing swelling and dysfunction [17, 27]. Over 50% of these patients have elevated serum IgG4 levels with some reaching an order of magnitude higher levels than normal [18, 28]. It has been hypothesized that IgG4-RD and IgG4-AID are similar disease entities [29]. However, our results and previous studies in pemphigus [12] found that serum IgG4 elevations are modest and less prevalent in IgG4-AIDs. Immune cell infiltrates have been found in pemphigus skin lesions, but it is unknown whether these are IgG4-producing B-cells [30]. Further, B-cell infiltrates were not observed at the neuromuscular junctions of intercostal muscles in MuSK MG patients [31-33]. Finally, IgG4-AIDs have a clear causative antigen, while this is not yet established for IgG4-RD [34]. Thus far, the overlap between these two disease classes is therefore limited.



The IgG4-response is associated with T helper 2 (Th2) cytokines interleukin (IL)-4, IL-13 and IL-10, regulatory T-cells and chronic antigen exposure [5]. Class-switching to IgE and IgG4 are linked by their dependency on IL-4 or IL-13, while IL-10 appears to direct a class switch to IgG4 over IgE [5]. The observed serum IgE levels in MuSK MG patients would be in line with a heightened Th2 response; although this was not statistically significant, likely in part because this analysis was underpowered. Surprisingly, *in vitro* activation of T-cells from MuSK MG patients suggests a higher Th1/17 versus Th2 response compared to healthy controls, although no differences could be found in plasma cytokine levels [35, 36]. Furthermore, less IL-10-producing regulatory B-cells were found *in vivo,* and *in vitro* CD40-stimulated B-cells produced less IL-10 [37, 38]. These results suggest that enhanced Th2 activation may not be the main driver of the IgG4 response in MuSK MG. However, the vast majority of the MuSK MG patients in these studies were on immunosuppressive medication, introducing a potential bias. An active immunization model of MuSK MG in mice did result in a predominant IgG1 (IgG4 equivalent in mouse) response accompanied by increases in IL-4 and IL-10, suggesting MuSK immunization can induce a Th2-like response [39]. In summary, it is yet unclear which immunological mechanisms cause the selective increase in IgG4 production or dominance in autoantibody subclass in IgG4-AIDs. In our experience it was very challenging to compile a set of samples fitting the immunosuppression naïve criteria, in spite of reaching out to 20

different MG expertise centers. The relatively low numbers studied here are a direct consequence of this and limit the study power. To further investigate the etiology of IgG4-AIDs it will be of importance to study immunosuppressive naïve serum and immune cells, as the exposure to such treatments may significantly limit our view on the immunological characteristics of these diseases.

## 5. Acknowledgements

We would like to thank all patients and healthy donors for donating serum and all clinicians for searching for serum from untreated MG patients. Thanks to Laurent Paardekooper for feedback on the manuscript.

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## Supplementary Material

*Table S1: Donor characteristics. The percentages of MG subtype , AChE inhibitors and thymectomy are calculated from the number of patients from whom the respective data are present.*

| | MuSK MG n=28 | | AChR MG n=51 | | Healthy donor n=77 | |
|---|---|---|---|---|---|---|
| **Sex, n (%)** | | | | | | |
| **Male** | 9 | (32) | 22 | (43) | 46 | (60) |
| **Female** | 19 | (68) | 29 | (57) | 31 | (40) |
| **Age, y** | 39 | (23.25-52) | 61 | (42-72) | 37 | (31-42) |
| **Missing, n (%)** | 2 | (7.1) | 0 | (0) | 0 | (0) |
| **Age disease onset , y** | 39.5 | (19.75-49.5) | 53 | (26-65) | | |
| ***Missing, n (%)*** | *2* | *(7.1)* | *0* | *(0)* | | |
| **MG subtype, n (%)** | | | | | | |
| **Ocular** | 0 | (0) | 8 | (22) | | |
| **Bulbar** | 4 | (19) | 3 | (8) | | |
| **Ocular Bulbar** | 0 | (0) | 1 | (3) | | |
| **Generalised** | 17 | (81) | 22 | (61) | | |
| **Limb-girdle** | 0 | (0) | 2 | (6) | | |
| ***Missing*** | *7* | *(25)* | *15* | *(29)* | | |
| **Immunosuppressive treatment, n (%)** | | | | | | |
| **Untreated** | 26 | (93) | 45 | (88) | | |
| **>1 year not treated** | 2 | (7) | 6 | (12) | | |
| **AChE inhibitors, n (%)** | 11 | (52) | 37 | (90) | | |
| ***Missing, n (%)*** | *7* | *(25)* | *10* | *(20)* | | |
| **Thymectomy, n (%)** | 0 | (0) | 13 | (35) | | |
| ***Missing, n (%)*** | *3* | *(11)* | *14* | *(38)* | | |

Age: median ($25^{th}$-$75^{th}$ percentile)

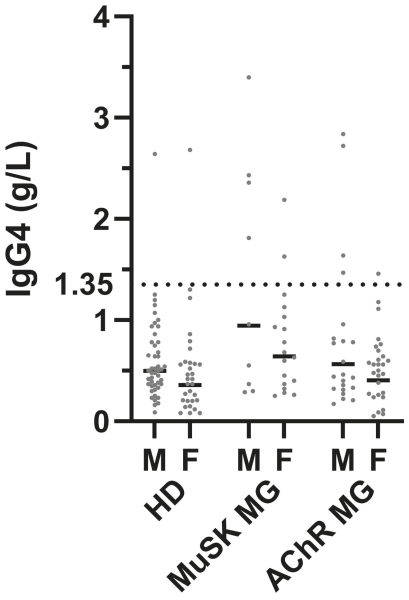


*Figure S1: Sex differences in IgG4 levels. Dotted line at 1.35g/L represents cutoff for elevated IgG4 in IgG4-RD [17]. Data are presented as geometric mean. HD: n=46 (M) and n=31 (F), MuSK MG: n=9 (M) and n=18 (F), AChR MG: n=22 (M) and n=28 (F). M=male; F=female; HD=healthy donors*

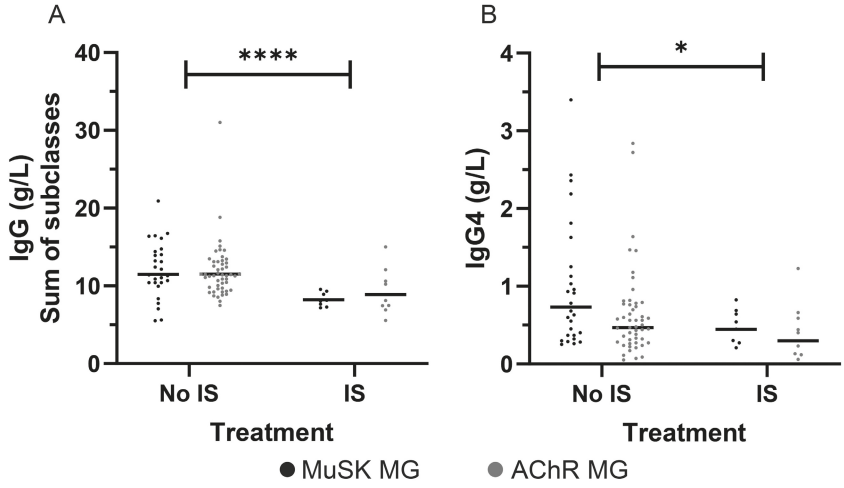


*Figure S2: Serum IgG (A) and IgG4 (B) in patients not receiving immunosuppression (No IS, cohort of primary analysis) or independent patients that were receiving immunosuppression at time of sampling (IS, excluded from primary analysis). Characteristics of patients on IS are presented in Table S2. Data are presented as geometric mean. * p<0.05, ** p<0.01, *** p<0.001. MuSK MG: n=27 (No IS) and n=8 (IS), AChR MG: n=50 (No IS) and n=9 (IS).*

*Table S2: characteristics of patients with IS used in Figure S2*

| | **MuSK MG + IS n=8** | **AChR MG + IS n=9** |
|---|---|---|
| **Sex, n (%)** | | |
| **Male** | 4 (50) | 1 (11) |
| **Female** | 4 (50) | 8 (89) |
| **Age, y** | 50 (45-59.75) | 53.5 (43.5-62.5) |
| **Missing, n (%)** | 0 (0) | 1 (11) |
| **Age disease onset , y** | 51 (43-59.75) | 38 (23-46.5) |
| ***Missing, n (%)*** | *4 (50)* | *2 (22)* |
| **MG subtype, n (%)** | | |
| **Ocular** | 0 (0) | 0 (0) |
| **Bulbar** | 0 (0) | 0 (0) |
| **Ocular Bulbar** | 0 (0) | 0 (0) |
| **Generalised** | 8 (100) | 9 (100) |
| **Limb-girdle** | 0 (0) | 0 (0) |
| ***Missing*** | *0 (0)* | *0 (0)* |
| **History immunosuppressive treatment, n (%)** | | |
| **Prednisone** | 7 (87.5) | 8 (89) |
| **Azathioprine** | 8 (100) | 8 (89) |
| **Ciclosporine** | 1 (12.5) | 3 (33) |
| **IVIg** | 1 (12.5) | 0 (0) |
| **Plasmapheresis** | 1 (12.5) | 1 (11) |
| **AChE inhibitors, n (%)** | 4 (50) | 2 (22) |
| ***Missing, n (%)*** | *0 (0)* | *0 (0)* |
| **Thymectomy, n (%)** | 2 (25) | 5 (56) |
| ***Missing, n (%)*** | *0 (0)* | *0 (0)* |

Age: median ($25^{th}$-$75^{th}$ percentile)


3

# MuSK myasthenia gravis monoclonal antibodies: valency dictates pathogenicity

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## Abstract

### Objective

To isolate and characterize MuSK monoclonal antibodies from MuSK MG patients on a genetic and functional level.

### Methods

We generated recombinant MuSK antibodies from patient-derived clonal MuSK-specific B cells and produced monovalent Fab fragments from them. Both the antibodies and Fab fragments were tested for their effects on neural agrin-induced MuSK phosphorylation and acetylcholine receptor (AChR) clustering in myotube cultures.

### Results

The isolated MuSK monoclonal antibody sequences included IgG1, IgG3 and IgG4 that had undergone high levels of affinity maturation, consistent with antigenic selection. We confirmed their specificity for the MuSK Ig-like1 domain and binding to neuromuscular junctions. Monovalent MuSK Fab, mimicking functionally monovalent MuSK MG patient Fab-arm exchanged serum IgG4, abolished agrin-induced MuSK phosphorylation and AChR clustering. Surprisingly, bivalent monospecific MuSK antibodies instead *activated* MuSK phosphorylation and partially induced AChR clustering, independent of agrin.

### Conclusions

Patient-derived MuSK antibodies can act either as MuSK agonist or MuSK antagonist, depending on the number of MuSK binding sites. Functional monovalency, induced by Fab-arm exchange in patient serum, makes MuSK IgG4 antibodies pathogenic.

## Introduction

Myasthenia gravis (MG) is a debilitating autoimmune disease where autoantibodies against neuromuscular junction (NMJ) proteins impair neuromuscular transmission and cause fatigable skeletal muscle weakness. About 5% of MG patients has autoantibodies against muscle-specific kinase (MuSK).[1] These autoantibodies are predominantly of the IgG4 subclass.[2] IgG4 is considered an "anti-inflammatory" antibody being unable to bind complement or many Fc receptors on immune cells.[3] IgG4 antibodies furthermore exchange Fab-arms with other IgG4 molecules which renders them functionally bispecific and monovalent, preventing antigen crosslinking and internalization.[4] Consequently, the pathogenicity of MuSK IgG4 autoantibodies was initially questioned.[5] However, retrospective longitudinal epitope mapping with polyclonal serum showed that disease severity correlates with IgG4 reactivity against the N-terminal Ig-like 1 domain of MuSK.[6] Furthermore, passive transfer of purified IgG4 from MuSK MG patients dose-dependently induced muscle weakness in mice.[7] Lastly, *in vitro* studies showed that MuSK IgG4 autoantibodies block MuSK-LDL receptor-related protein 4 (Lrp4) interaction thereby preventing acetylcholine receptor (AChR) clustering, which explains the impaired neuromuscular transmission in MG.[8-10] It is now well-established that MuSK IgG4 autoantibodies cause MG.

In some patients low titres of IgG1 and IgG3 MuSK autoantibodies coincide with high levels of IgG4 MuSK autoantibodies. Whether this IgG1 and IgG3 can cause MuSK MG remains enigmatic.[7,9,11]

To further understand the pathomechanism of MuSK MG and investigate whether the unique functional features of IgG4, like Fab-arm exchange, contribute to the pathogenesis we isolated and functionally characterized monoclonal MuSK antibodies from MuSK MG patients.

## Methods

### *Patient selection and study approval*

MuSK MG patients were recruited in our MG outpatient clinic at the Leiden University Medical Center and were selected based on the presence of a positive MuSK antibody test (RSR ltd). Both patients were symptomatic and on immunosuppressive treatment, while one patient had been previously treated with rituximab. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local medical ethics committee. Both patients signed informed consent.

*Isolation of monoclonal autoantibodies from MuSK MG patients*

MuSK-binding memory B cells were isolated using a fluorescence activated cell sorter (FACSaria, BD Biosciences) from cryopreserved peripheral blood mononuclear cells (PBMC) selecting for $CD19^+$, $CD20^+$, $CD27^+$ cells (using mouse anti-human monoclonals CD19-BV421 HIB19, CD20-AF700 2H7, CD27-APCHy7 M-T271 all from BD Biosciences; in 0.1 % BSA, 2 mM EDTA/Dulbecco's PBS). To remove dead cells and non-B cells a dump channel was included (labeled with 7-AAD from Thermo Fisher, CD3/FITC UCHT1 and CD14/FITC M5E2 from BD Biosciences, and CD56/FITC HCD56 from Biolegend). Antigen-specific cells were isolated using recombinant MuSK produced in E. Coli[6] labeled with R-PE (AS-72113, Anaspec) and MuSK produced in yeast tagged with DyLight 650 (a kind gift of Konstantinos Lazaridis and Socrates Tzartos, Thermofisher). Single sorted cells were cultured on irradiated CD40L cells (a kind gift from Kees van Kooten) in a 96 wells plate in complex RPMI medium.[12] After two weeks the medium was tested in duplicate for MuSK antibody production using the MuSK ELISA described previously.[6]

*RNA isolation, cDNA production and antibody sequence isolation*

Single wells containing MuSK antibody producing cells were lysed with 150 µl Qiazol and RNA was isolated using standard chloroform extraction and isopropanol/ethanol precipitation. RNA was rehydrated in 8µl $H_2O$ and stored at -80 °C until further use. cDNA was directly synthesized (without pre-amplification or purification) using Smartscribe reverse transcriptase (Takara Bio Europe) an Oligo-dT40VN primer (5'–AAGCAGTGGTATCAACGCAGAGTACT40VN-3') and a template switching oligo (5'-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3') for 10 rounds of amplification. Full length V(D)J were obtained by ARTISAN PCR using gene specific primers (general forward primer 5'-CTTAAGCAGTGGTATCAACGCAGAGTACATG-3', IgG HC 5'-GGAAGGTGTGCACGCCGCTGGTC-3', hkappaLC 5'-CTGATGGGTGACTTCGCMG-3', hlambdaLC 5'-CACACYAGTGTGGCCTTGTTGGCTTG-3').[13]

*Recombinant antibody production, purification and characterization*

Heavy and light chain sequences were ordered at Geneart (Thermofisher) in an IgG1 and IgG4 backbone pcDNA3.1 vector and transfected in suspension Freestyle HEK293-F cells (R790-07, Thermofisher) using Fectin (12347-019, Thermofisher) in FreeStyleTM 293 Expression Medium (12338, ThermoFisher). To increase transfection and production efficiency the cells were co-transfected with SV40 large T antigen, hp21 and hp27 (Kindly provided by Theo Rispens). After 6 days culture medium was collected,

cell debris was removed by centrifugation and IgG was purified using a HiTrap™ Protein A affinity column (17-0402-01, GE healthcare) on an Akta pure (GE Healthcare). Antibodies were dialyzed to PBS, filter-sterilized and stored at -20°C. Fab fragments were generated from these recombinant antibodies using papain according to manufacturer's instructions (20341, ThermoFisher).

To determine the binding characteristics of the recombinant antibodies we performed an epitope mapping ELISA.[6]

Recombinant antibodies were used to immunostain fixed mouse levator auris longus muscle at 1 µg/mL overnight at room temperature. Synaptic regions were labelled with 0,5 µg/mL AlexaFluor488 conjugated a-bungarotoxin (B13422, ThermoFisher) and bound recombinant antibodies were detected with 0,5 µg/mL AlexaFluor594 conjugated goat anti-human IgG (A110414, ThermoFisher) in PBS for one hour. Muscles were imaged on a Leica SP8 confocal laser-scanning microscope and analyzed using LasX software.



To determine the ability of the antibodies to inhibit MuSK phosphorylation and AChR clustering we exposed C2C12 myotubes cultures (Cell lines service) to them as described previously.[8] Differentiated myotubes were stimulated with 0.1 nM agrin (550-AG-100, R&D systems) in the presence or absence of 100 ng/mL recombinant antibodies or Fab fragments. For MuSK phosphorylation data, myotubes were lysed after 30 minutes of exposure and MuSK was precipitated using 5µL/sample rabbit anti MuSK polyclonal serum (ab94276, or ab94277 a kind gift of Steve Burden) during an overnight incubation at 4 °C. Bound antigen-antibody complexes were precipitated using protein A agarose beads (11134515001, Roche) which were extensively washed. Samples were subsequently ran on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. MuSK and phosphorylated MuSK was detected using goat anti-rat MuSK (AF562, R&D systems) and mouse anti-phosphotyrosine clone 4G10 (05-321, Millipore) as primary antibodies, and donkey anti-mouse-680RD (926-68072, Licor) and donkey anti-goat 800CW (926-32214, Licor). To confirm equal loading, whole cell lysates, that formed the immunoprecipitation input samples, were analyzed for tubulin levels using a mouse anti-a-tubulin antibody (T6199, Sigma) and donkey anti-mouse-800CW secondary antibody (926-32212, Licor). Bound antibodies were detected using the Odyssey CLx (Licor).

AChR clustering was studied after 16 hours of exposing myotubes to 100ng/mL recombinant antibodies or Fab fragments in absence or presence of 0,1 nM agrin. Subsequently the cells were washed three times with differentiation medium (DMEM, 31966 Gibco, 2% heat-inactivated horse serum 26050-088, Gibco, 1% pen/strep and 1 % L-glutamine) and incubated with 0,5µg/mL AlexaFluor488 conjugated α-bungarotoxin (B13422, ThermoFisher) in differentiation medium for 30 minutes at 37°C. After staining cells were fixed in 4% formalin solution for 5 minutes, washed with PBS and mounted using hardset mounting medium (H-1500, Vector laboratories). Twenty fields divided over two coverslips per condition were randomly selected, and imaged with Leica DM5500 microscope. AChR cluster count and size were analyzed using ImageJ (1.48v). MuSK phosphorylation studies were performed in duplicate and AChR clustering was performed in triplicate.

### *Statistics*

All data are expressed as mean ± standard error of the mean (SEM). Statistical significance of differences between treatment groups were tested with Student's t-tests, with corrections for multiple testing wherever appropriate. Differences with *P*-values <0.05 were considered statistically significant.

### *Data availability statement*

All data related to this manuscript will be made available upon request by a qualified investigator.

## Results

### *Patients with MuSK MG*

To obtain MuSK-specific B cells PBMC were isolated from two MuSK MG patients. The clinical characteristics of these patients are described in Table 1.

*Table 1: Clinical characteristics from study participants at the time of PBMC/blood donation.*

| Patient ID | Age (y) | Sex | MuSK titer (nmol/L) | Disease severity (QMG) | Thymus status | Treatment |
|---|---|---|---|---|---|---|
| 1 | 59 | M | 1.19 | 9/39 | Thymectomy 1987<br>No thymoma | prednisone, mycophenolate mofetil, pyridostigmine, plasmapheresis, two courses of rituximab 4 and 2 years before |
| 2 | 59 | V | 0.75 | Not available | No thymectomy<br>No thymoma | prednisone, azathioprine, plasmapheresis |

*Isolation and genetic characterization of patient-derived MuSK autoantibodies*

Antigen-specific single cell sorting yielded eight MuSK-binding B cells from two MuSK MG patients. The frequency of circulating MuSK clones was ~7 per 100 million PBMC for patient 1, and 2.5 per 100 million PBMC for patient 2. An overview of the isolated MuSK autoantibody characteristics is shown in Table 2. From six out of seven MuSK autoantibody producing clones from patient 1 we could derive the variable-region sequences of the heavy chain ($V_H$) and light chain ($V_L$). For one clone only the $V_H$ region could be sequenced.

Surprisingly, the majority (5/7) of the antibodies isolated were of the IgG1 isotype. We furthermore isolated one IgG4 and one IgG3 clone. All IgG1 clones and the IgG3 clone used the same $V_H$ and $V_L$ genes (*IGHV3-21 and IGKV1-39)*, suggesting a clonal relationship.

Fab-arm exchange is an important feature of IgG4 and might affect the functional characteristics of MuSK autoantibodies.[14] Previous work on polyclonal purified fractions suggested that MuSK MG IgG4 has the ability to undergo Fab-arm exchange.[14] We sequenced the Fc part of our MuSK monoclonal antibodies and confirmed that the IgG4 clone indeed possessed the serine at position 228 and the arginine 409 required to undergo Fab-arm exchange (data not shown).

N-linked glycosylation of the Fab can be important for antigen binding, e.g. in rheumatoid arthritis ACPA autoantibodies.[15] The NXS/T (where X can't be a proline) N-linked glycosylation motif was found in the variable

region of two of our monoclonal MuSK autoantibodies, suggesting that glycosylation of the Fab is not essential for all MuSK autoantibodies.

*Functional characteristics of recombinant MuSK monoclonal autoantibodies*

The Ig-like 1 domain of MuSK was previously recognized as the main immunogenic region of MuSK in polyclonal patient IgG4 fractions and serum.[6] For five of the patient-derived MuSK antibodies the epitope mapped to the first Ig-like domain of MuSK (Table 2). We have thus far identified no monoclonal autoantibodies against other domains. To establish the functional characteristics of the MuSK autoantibodies, recombinant antibodies were produced from an original IgG1 and IgG4 isolated clone. To assess the importance of autoantibody subclass in MuSK MG each of these variable-regions were subcloned in both an IgG1 and IgG4 backbone. We produced biotin antibodies as a negative control for all experiments.

To assess the ability of recombinant patient-derived IgG1 and IgG4 MuSK antibodies to bind to MuSK at the postsynaptic membrane of NMJs, we performed immunostaining on isolated mouse skeletal muscle. Both the IgG1 and IgG4 versions of the recombinant monoclonal MuSK antibodies clearly bound to NMJs (Fig 1, data shown for the IgG4 recombinant antibodies).

Successful neuromuscular transmission depends on properly clustered AChR, a process that is orchestrated through the agrin-Lrp4-MuSK signaling cascade.[16] Agrin is released by the motor nerve terminal and binds Lrp4, which subsequently binds MuSK and thereby stimulates MuSK homodimerization and trans-phosphorylation. Activation of MuSK phosphorylation stimulates a variety of intracellular signaling cascades of which one culminates in AChR clustering. Purified polyclonal patient IgG4 MuSK autoantibodies inhibit MuSK-Lrp4 interaction, subsequent MuSK dimerization and phosphorylation, and thereby prevent agrin-induced AChR clustering in C2C12 myotube cultures and cause myasthenia in mice.[7-9] Surprisingly, patient-derived recombinant MuSK monoclonal antibodies (both IgG1 and IgG4) activated rather than inhibited MuSK phosphorylation (Fig 2A). This effect was observed in both absence and presence of agrin. Activation of MuSK phosphorylation was concentration-dependent (Fig 2B) and differed slightly between the two clones. This shows that patient-derived bivalent monospecific MuSK antibodies binding the Ig-like 1 domain facilitate dimerization and activation of MuSK *in vitro*.

*Table 2: MuSK autoantibody characteristics. HC = heavy chain, VH = heavy chain variable region, LC = light chain, VL = light chain variable region*

| Clone | Pt ID | Isotype | HC VDJ genes | VH mutations V;J | LC VJ genes | VL mutations V;J | Glycosylation motif present | Epitope |
|---|---|---|---|---|---|---|---|---|
| 11-3D9 | 1 | IgG1κ | IGHV3-21*01F IGHD3-3*01F IGHJ4*02F | 16;6 | IGKV1-39*01F IGKJ2*01F | 16;4 | 1 in CDR1 of the HC | Ig-like 1 |
| 11-3F6 | 1 | IgG1κ | IGHV3-21*01F IGHD6-19*01F IGHJ4*02F | 19;6 | IGKV1-39*01F IGKJ2*02F | 30;1 | 1 in FR3 of the LC | Ig-like 1 |
| 11-8G4 | 1 | IgG1κ | IGHV3-21*01F IGHD6-19*01F IGHJ4*02F | 34;5 | N/A | N/A | No | N/A |
| 11-7C5 | 1 | | N/A | N/A | N/A | N/A | N/A | N/A |
| 13-3B5 | 1 | IgG4λ | IGHV1-2*02F IGHD3-10*01F IGHJ5*02F | 39;11 | IGLV3-25*03F IGLJ2*01F | 32;5 | No | Ig-like 1 |
| 13-3D10 | 1 | IgG1κ | IGHV3-21*01F IGHD3-16*01F IGHJ4*02F | 45;7 | IGKV1-39*01F IGKJ2*01F | 26;2 | No | Ig-like 1 |
| 13-4D3 | 1 | IgG3κ | IGHV3-21*02F IGHD3-16*01F IGHJ4*02F | 31;6 | IGKV1-39*01F IGKJ2*01F | 12;3 | No | Ig-like 1 |
| 16-8B3 | 2 | IgG1 | N/A | N/A | N/A | N/A | N/A | N/A |

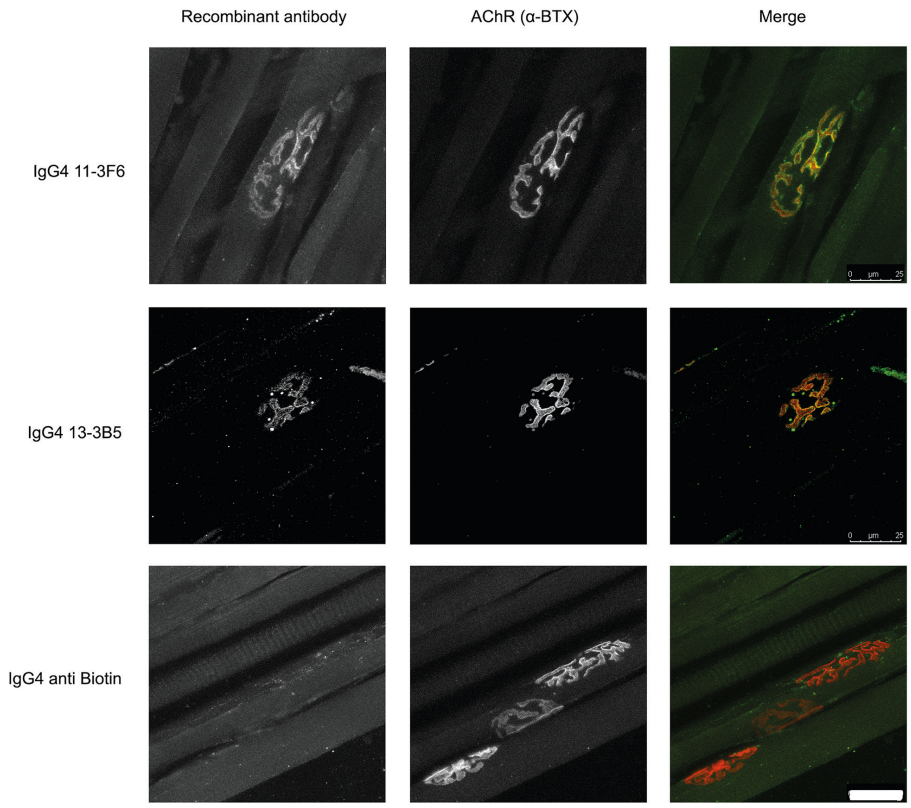


*Figure 1: Patient-derived recombinant MuSK antibodies bind mouse NMJs. Control biotin antibodies did not stain the NMJ. Scale bar is 25 um.*

Recombinant monoclonal IgG1 and IgG4 however both engage in bivalent monospecific antibody-antigen interactions. To investigate the functional effects of the bispecificity and functional monovalency of Fab-arm exchanged IgG4 MuSK antibodies in patients, we generated monovalent Fab fragments from these recombinant antibodies by papain digestion. *In vitro*, these Fab fragments inhibited agrin-dependent MuSK phosphorylation (Fig 2C) and AChR clustering similar to patient serum-derived MuSK IgG4 (Fig 2D). In spite of fully activating MuSK phosphorylation, bivalent monospecific MuSK antibodies partially inhibited agrin-dependent AChR clustering as compared to Fab fragments and a biotin control antibody. Importantly, AChR clustering could be partially induced using bivalent monospecific antibodies independent from agrin (Fig 2D). Thus, monovalent MuSK binding blocks MuSK signaling and the AChR clustering pathway, whereas bivalent

monospecific MuSK antibodies fully stimulate MuSK phosphorylation, and partially induce or restore AChR clustering in this tissue culture model.

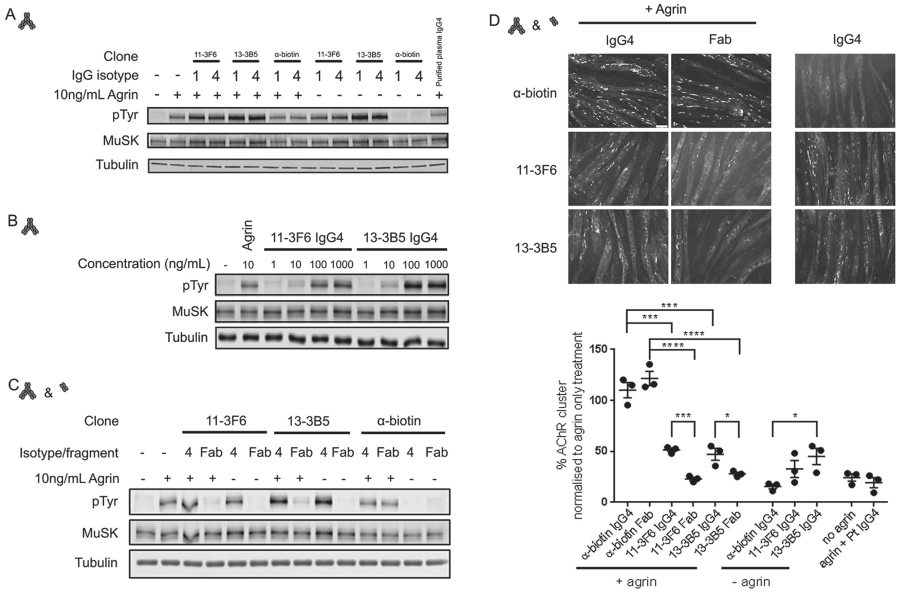


*Figure 2: Patient-derived recombinant MuSK antibodies can activate or inhibit MuSK phosphorylation and AChR clustering depending on the antibody valency. Bivalent monospecific recombinant MuSK antibodies (Clone# 11-3F6 and 13-3B5) activated MuSK phosphorylation in the presence and absence of agrin (A). Activation of MuSK phosphorylation was dose-dependent (B). Clone 13-3B5 was slightly more potent compared to 11-3F6. A biotin control antibody did not affect (agrin-dependent) MuSK phosphorylation. Monovalent Fab fragments generated from these recombinant MuSK monoclonals inhibited MuSK phosphorylation (C). Agrin-dependent AChR clustering was unaffected when exposed to a biotin control antibody or Fab fragments thereof (D). Bivalent monospecific recombinant IgG4 MuSK antibodies and Fab fragments inhibited the number of agrin-dependent AChR clustering significantly compared to the biotin control antibody (for IgG4: P<0.001, for Fab fragments: P<0.001, one-way analysis of variance (ANOVA) test, Dunnett corrected). Fab fragments reduced AChR clusters to the level of purified patient IgG4 and the "no agrin" condition. Bivalent monospecific antibodies significantly increased AChR clustering compared to Fab fragment-mediated inhibition that seems independent from agrin (11-3F6: P<0.001, 13-3B5: P=0.03, biotin antibody: P=0.30, unpaired t test, No agrin conditions 11-3F6: P=0.03, 13-3B5: P=0.20 one-way ANOVA Dunnett corrected). Data represent mean ± SEM. Scale bar represents 50 µm.*

## Discussion

In this study we provide new and surprising insight on the disease mechanism of MuSK MG by functional characterisation of patient-derived monoclonal MuSK antibodies. The major findings of this study are graphically depicted in figure 3. Previous studies with polyclonal purified IgG4 from MuSK MG patients showed that this IgG4 obstructs Lrp4-MuSK binding.[8, 9] This interaction is normally required for MuSK to become dimerized and phosphorylated. Polyclonal patient IgG4, by blocking Lrp4-MuSK interaction, prevents MuSK dimerization, phosphorylation and subsequent signalling towards AChR clustering leading to muscle weakness. Experiments by others furthermore confirmed that polyclonal MuSK IgG4 antibodies in patient serum have undergone Fab-arm exchange[14] and thus are functionally bispecific, binding to MuSK in a monovalent manner. We here show that monovalent Fab fragments, generated from patient-derived monoclonal MuSK antibodies, mimicked monovalent Fab-arm exchanged serum IgG4 and recapitulated the pathomechanism of MuSK MG. Surprisingly, bivalent patient-derived monoclonal MuSK antibodies had the complete opposite effect. These antibodies *activated*, rather than *inhibited* MuSK phosphorylation and partially induced AChR clustering. As MuSK phosphorylation and signalling requires MuSK dimerization, our data suggests that bivalent monospecific MuSK antibodies can force this dimerization, thereby stimulating MuSK phosphorylation and AChR clustering. This bypasses the need for agrin-bound Lrp4 to interact with MuSK. Because Fab-arm exchange renders circulating IgG4 functionally monovalent, serum MuSK IgG4 is unable to force dimerization and blocks MuSK signalling. Our data suggests that IgG4 MuSK autoantibodies become more pathogenic when, due to Fab-arm exchange, they have become functionally monovalent. MuSK autoantibody valency and, consequently, MuSK autoantibody subclass are thus key determinants in the pathogenic mechanism underlying MuSK MG. Inhibition of Fab-arm exchange might therefore have therapeutic potential in MuSK MG and perhaps also in other identified IgG4-mediated autoimmune disorders.[17, 18] Our experiments do not exclude the possibility that the low levels of IgG1-3 MuSK antibodies might damage NMJs through activation of complement or immune cell-mediated cytotoxicity.

The agonistic (i.e. bivalent) patient-derived MuSK monoclonal antibodies did not induce AChR clustering in the C2C12 myotube assay to the same degree as the natural agonist agrin. This is likely not a dose-dependent potency difference (data not shown). Alternatively, binding of bivalent

monospecific antibodies to the Ig-like 1 domain of MuSK might affect downstream aspects of the AChR clustering pathway differently from agrin-induced MuSK stimulation. These results show strong similarities to a study using IgG obtained from an active immunization model of MuSK MG in rabbits[19], to studies showing functionally bivalent antibodies binding the MuSK extracellular domain[20, 21], and to *in vitro* studies with polyclonal purified patient IgG1-3 fractions.[9] One explanation for the incomplete AChR clustering ability of these MuSK antibodies may be reduced Dok-7 protein levels, a protein acting downstream of MuSK essential for transducing the AChR clustering signal.[19] It is conceivable that binding of bivalent monospecific MuSK antibodies alters the interaction between MuSK and Dok-7 or might increase MuSK internalization and affect subsynaptic gene expression, ultimately lowering AChR cluster numbers.[11] It will furthermore be important to learn whether the level of AChR clustering that can be achieved with patient-derived bivalent monospecific MuSK antibodies is sufficient to maintain adequate neuromuscular transmission *in vivo*.



Recombinant MuSK agonistic antibodies have previously been described.[1, 21, 22] These antibodies bound to the Ig-like 1 domain or the Fz-domain of MuSK and also activated MuSK phosphorylation and AChR clustering *in vitro* and *in vivo*.[23] This suggests that MuSK signalling activation by bivalent antibodies might be epitope independent, and solely requires forced dimerization of two MuSK molecules. Interestingly, forced activation of MuSK signalling holds therapeutic promise in neuromuscular disorders characterized by NMJ deficits.[23]

MuSK autoantibodies can also inhibit MuSK-Collagen Q interaction.[24] It will be interesting to study the effect of these monoclonal antibodies on this interaction. Furthermore, although the Ig-like 1 domain of MuSK is considered the main immunogenic region (MIR), antibodies to other parts of the ecto-domain of MuSK have been found.[6, 25, 26] The here described methodology should enable isolation of MuSK monoclonal antibodies binding to different MuSK domains which will facilitate more detailed analysis of their (pathogenic) effects.

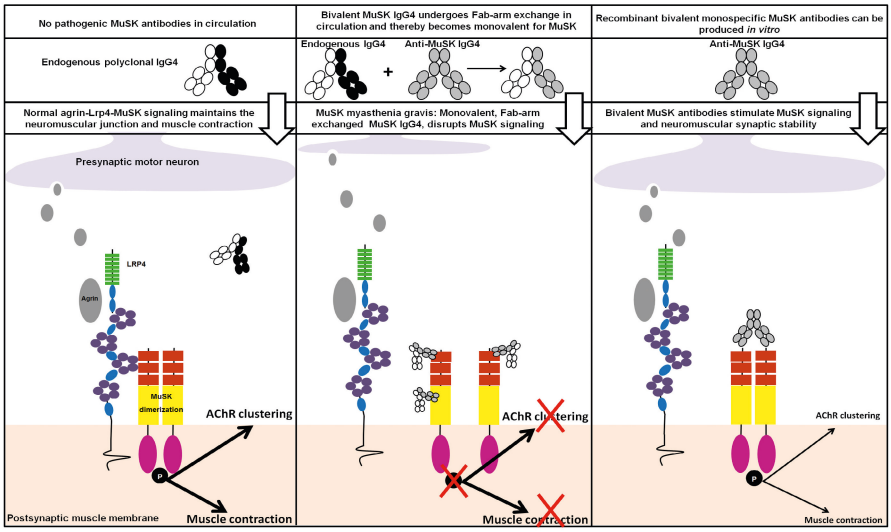


*Figure 3: Graphical depiction of the conclusions of this study.*

The frequency of MuSK-binding memory B cells was low in the patients studied. This may be explained by the immunosuppressive medication that the patients received. Both patients had, in spite of this medication, rather high levels of MuSK autoantibodies. Lighaam *et al.* showed that numbers of circulating IgG4 memory B cells tightly correlated with IgG4 titers[12]. This might however not reflect individual antigen-specific responses. As plasma cells mostly reside in the bone marrow the isolation of circulating memory B cells might not be a representative of the ongoing MuSK autoantibody production. We can also not exclude that the experimental procedures affected the number or isotype of the isolated MuSK-binding cells. Experiments testing the isolation and culture of IgG1 and IgG4 memory B cells using this method did show a slight (~10%) survival advantage for single IgG1 memory B cells (data not shown), however this is not sufficient to explain the lack of IgG4 MuSK-binding memory B cells isolated in these experiments.

The degree of somatic hypermutation, reflecting affinity maturation, was high in all MuSK antibody clones for both the $V_H$ and $V_L$, strongly suggesting antigen-driven selection. In two recent publications on MuSK monoclonals, B-cell populations, and repertoire analysis in MuSK MG patients, a different VDJ gene usage was reported.[27, 28]

At least 12 other autoimmune diseases affecting different organ systems are hallmarked by pathogenic IgG4 autoantibodies.[17, 18, 29] For several of them patient-derived monoclonal antibodies have been isolated and characterised. [30, 31] The VDJ gene usage differs between all of these diseases. It is not known why these diseases are caused by IgG4 autoantibodies. The role of antibody valency for these diseases has only partially been studied. In pemphigus vulgaris, where the antigen forms a heterodimer (in contrast to MuSK which forms a homodimer), pathogenicity seems more dependent on autoantibody titre and epitope.[18] It will be interesting to learn whether autoantibody valency and thus Fab-arm exchange is relevant in other IgG4-mediated autoimmune diseases.

In conclusion, the pathomechanism of MuSK MG is dependent on at least three factors: 1) autoantibody titre [6, 32], 2) recognized epitope [6, 8, 22], and, emerging from this and other[9, 19] studies 3) antibody valency/ autoantibody subclass. This study thereby presents a new pathomechanism in antibody-mediated autoimmunity, requiring Fab-arm exchange of IgG4 to render autoantibodies monovalent and pathogenic.



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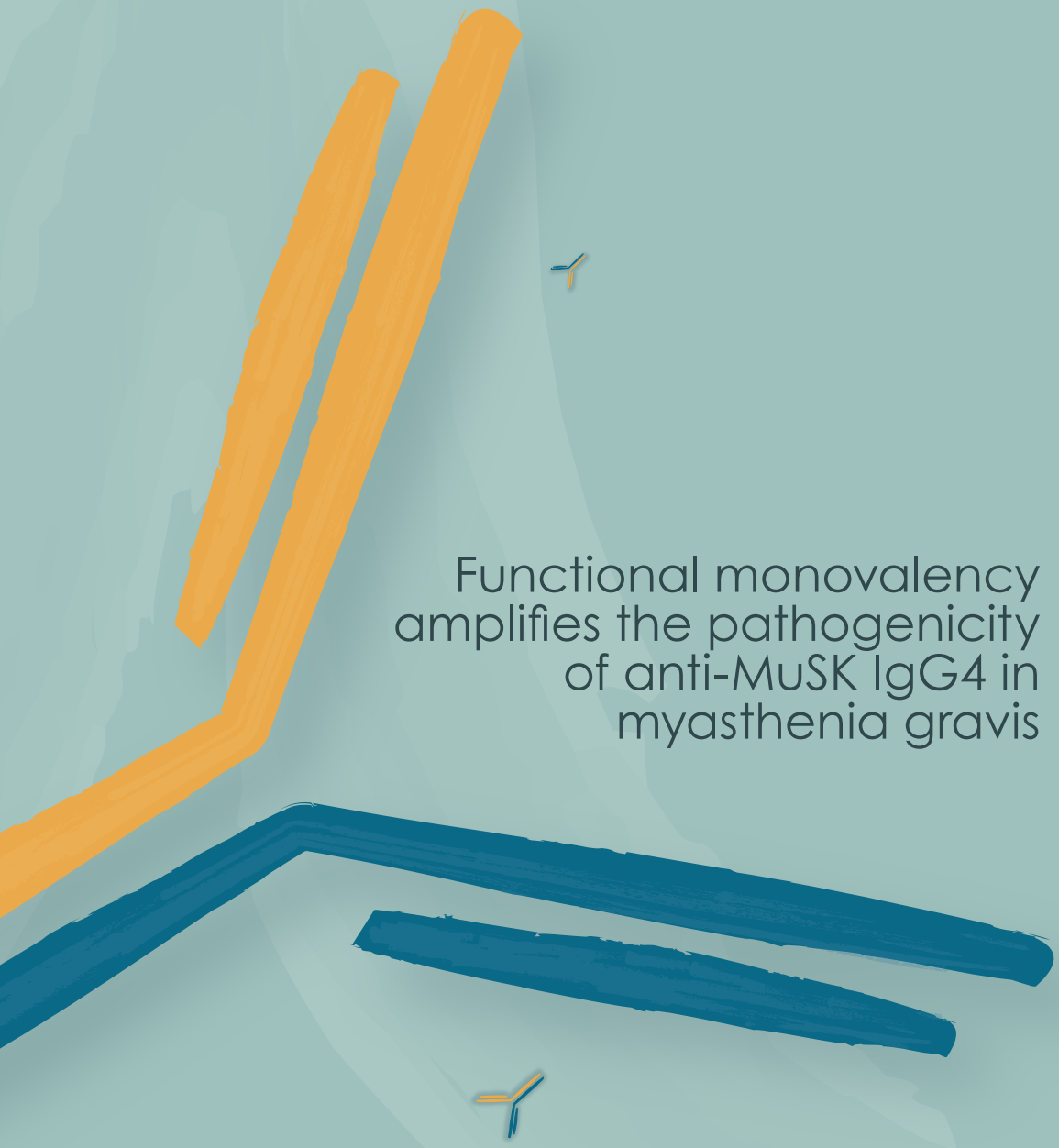

# Functional monovalency amplifies the pathogenicity of anti-MuSK IgG4 in myasthenia gravis

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## Abstract

Human immunoglobulin (Ig) G4 usually displays anti-inflammatory activity, and observations of IgG4 autoantibodies causing severe autoimmune disorders are therefore poorly understood. In blood, IgG4 naturally engages in a stochastic process termed Fab-arm exchange in which unrelated IgG4s exchange half-molecules continuously. The resulting IgG4 antibodies are composed of two different binding sites, thereby acquiring monovalent binding and inability to cross-link for each antigen recognized. Here, we demonstrate this process amplifies autoantibody pathogenicity in a classic IgG4-mediated autoimmune disease: muscle-specific kinase (MuSK) myasthenia gravis (MG). In mice, monovalent anti-MuSK IgG4s caused rapid and severe myasthenic muscle weakness, whereas the same antibodies in their parental bivalent form were less potent or did not induce a phenotype. Mechanistically this could be explained by opposing effects on MuSK signaling. Isotype switching to IgG4 in an autoimmune response thereby may be a critical step in the development of disease. Our study establishes functional monovalency as a pathogenic mechanism in IgG4-mediated autoimmune disease and potentially other disorders.

## Introduction

Recently, a growing class of antibody-mediated autoimmune diseases characterized by predominant pathogenic immunoglobulin (Ig) G4 responses was described [1-4]. IgG4 is a peculiar antibody with unique characteristics. It is for example unable to activate complement and has low affinity for Fcγ receptors on immune cells [5, 6]. It is therefore considered anti-inflammatory and the pathogenicity of IgG4 autoantibodies is sometimes questioned. IgG4 molecules furthermore have the unique ability to stochastically exchange half-molecules with other IgG4s in a dynamic process called Fab-arm exchange [7]. This is an efficient process resulting in the vast majority of IgG4 molecules in circulation being bispecific and functionally monovalent for each antigen recognized. Whether the unique functional characteristics of IgG4 (like Fab-arm exchange) influence their pathogenicity in IgG4 autoimmune diseases is not known.

Myasthenia gravis (MG) with antibodies against muscle-specific kinase (MuSK) is one of the first recognized IgG4-mediated autoimmune diseases. MuSK autoantibodies are predominantly of the IgG4 subclass; although anti-MuSK IgG1 and IgG3 may be present concurrently at lower titers [8]. Anti-MuSK IgG4s induce MG in a dose-dependent manner both in patients and in mice [9, 10]. MuSK, a receptor tyrosine kinase, has a crucial role in establishing and maintaining neuromuscular junctions (NMJs) by orchestrating postsynaptic acetylcholine receptor (AChR) clustering, which is critical for neurotransmission [11]. Most MuSK autoantibodies bind the extracellular N-terminal Ig-like 1 domain and thereby block the activation of MuSK by low-density lipoprotein receptor-related protein 4 (Lrp4) and agrin [8, 12-16]. This eventually leads to disassembly of densely packed AChRs in the NMJ, failure of neurotransmission and consequently muscle weakness [10, 17-20]. *In vitro* characterization of monoclonal antibodies derived from MuSK MG patients furthermore suggests that the valency of MuSK antibodies determines their effects on MuSK signaling [21-23]. Monovalent Fab fragments recapitulate the inhibitory effects of patient-purified IgG4 on MuSK signaling *in vitro* [12, 13, 21, 23]. Surprisingly, monospecific bivalent MuSK antibodies acted oppositely as (partial) agonists [21]. To investigate whether IgG4 predominance is critical for disease development in IgG4-mediated autoimmunity and study the role of Fab-arm exchange and autoantibody valency, we generated stable bispecific functionally monovalent MuSK antibodies and their monospecific bivalent equivalents and assessed their pathogenicity in NOD/SCID mice.

## Results

### *Generation of patient-derived stable bispecific monovalent IgG4 MuSK antibodies*

To investigate the role of MuSK antibody valency on their pathogenicity *in vivo*, pure and stable bispecific MuSK antibodies are needed. Remaining monospecific MuSK antibodies may have confounding effects, e.g. by competing for binding with MuSK and thereby masking effects of the bispecific MuSK antibodies. We therefore adapted the controlled Fab-arm exchange (cFAE) method to IgG4 [24, 25]. In addition to two previously identified patient-derived recombinant MuSK antibodies (13-3B5 and 11-3F6) [21], the b12 antibody was chosen to generate an innocuous arm in the bispecific MuSK antibody, because its antigen (the HIV-1 envelope protein gp120) does not exist in the model systems used [26]. To stabilize the antibodies under physiological conditions, the serine (S) at amino acid position 228 was converted into a proline (P) in the IgG4 heavy chain of the anti-MuSK antibodies (Fig. 1A). The b12 antibody was made suitable for efficient cFAE by altering S228P, F405L and R409K in its IgG4 heavy chain. For clarity, the parental monospecific IgG4 will be designated as bivalent, and the bispecific IgG4 as monovalent towards MuSK in the remainder of the manuscript.

The exchange efficiency and residual amount of the bivalent (monospecific) anti-MuSK antibodies were determined with capillary electrophoresis (CE) hyphenated with mass spectrometry (MS) [27]. The monovalent (bispecific) IgG4 was separated from its two bivalent parents with CE, permitting reliable determination of their relative amounts down to 0.5% (Fig. 1C, Fig. S1). The purity of the separated fractions was confirmed with MS (Fig. 1D). The efficiency of exchange was high for both antibodies, with <1% of bivalent MuSK antibody remaining. The b12 antibody was added in molar excess to drive exchange of the MuSK antibody to completion and on average ~30% excess b12 IgG4 is therefore apparent in the CE and MS analyses (Fig. 1B).

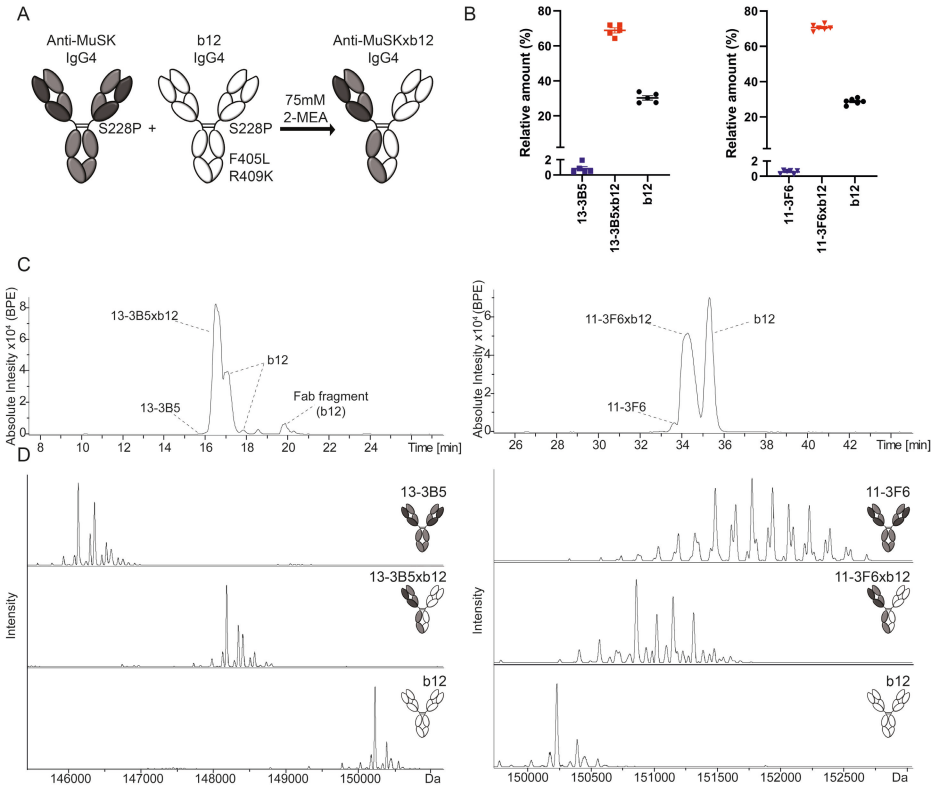


*Fig. 1. Generation of stable, MuSK MG patient-derived, bispecific, monovalent recombinant IgG4 MuSK antibodies. (A) Graphic depiction of the amino acid substitutions introduced for controlled Fab-arm exchange with IgG4. To make all antibodies resistant to Fab-arm exchange in vivo, serine 228 was converted to a proline, strengthening the interaction in the hinge region. To stabilize bispecific (monovalent) IgG4s a leucine (L) at position 405 and lysine (K) at position 409 were introduced in the b12 antibody to create complementary amino acids with the phenylalanine (F) and arginine (R) in the anti-MuSK IgG4s respectively. The interaction between the heterodimers is stronger than that between the homodimer, preventing in vivo and further in vitro exchange of the bispecific IgG4. (B) The exchange reaction with 75 mM 2-mercaptoethylamine (2-MEA) yielded high exchange efficiency with <1% contamination of the original monospecific (bivalent) anti-MuSK IgG4 for both clones (n=5). The b12 exchange partner was added in excess, yielding a remnant of ~30% after exchange. Representative examples of (C) base peak electropherograms (BPE) and (D) deconvoluted mass spectra of 11-3F6 and 13-3B5 respectively. The materials for 11-3F6xb12 exchange efficiency tests came from productions in both CHO and HEK293 cells. Representative images of 11-3F6 and 11-3F6xb12 deconvoluted mass spectra are from a production in CHO cells. Data represents mean ± SEM.*

*Monovalent MuSK antibodies are more pathogenic than bivalent MuSK antibodies in mice*

Passive transfer of MuSK antibodies to NOD/SCID mice is a well-established method to investigate the development of myasthenic muscle weakness without confounding immune reactions to human antibodies [10, 28]. Immunostaining of whole-mount levator auris longus muscle confirmed that both mono- and bivalent MuSK antibodies bound MuSK at the NMJ, while the control b12 antibody did not (Fig. S2A). The *in vivo* half-life ranged between 38-63 hours and varied by antibody (Fig. S3A and B). To ensure continuous *in vivo* exposure, an injection regimen of every 3 to 4 days was chosen for the passive transfer experiments. The minimum dose required to induce progressive phenotypical myasthenic symptoms was determined with monovalent 11-3F6xb12 IgG4. A dose of 2.5 mg/kg every 3 to 4 days resulted in progressive muscle weakness starting after about 5 days, leading to 20% body weight loss after 11 days (Fig. S3D-F). No clinical myasthenic muscle weakness or weight loss was observed at the 1.25 mg/kg dose. The serum antibody levels in these latter mice were approximately ten-fold lower compared to the 2.5 mg/kg regimen, indicating at these lower doses the pharmacokinetics do not change linearly (Fig. S3C). To compare the pathogenicity of monovalent vs. bivalent MuSK antibodies, we injected the bivalent or monovalent MuSK antibodies, or the b12 control at 2.5 mg/kg every 3 days (Fig. 2A).

Both monovalent anti-MuSK IgG4s induced rapid and severe myasthenic muscle weakness and body weight loss (Fig. 2, C-E and Fig. S4B-D for individual traces). The mice lost nearly all their grip strength and showed progressive fatigable muscle weakness in the inverted mesh test, starting within 1 week (Fig. 2, D and E). After a week, the mice also started to rapidly lose weight, likely as a consequence of the previously described bulbar muscle weakness, which makes it difficult to chew and swallow food (Fig. 2C) [18, 29, 30]. In sharp contrast, the bivalent 11-3F6 and 13-3B5 did not induce weight loss. Bivalent 11-3F6 furthermore did not induce any sign of clinical muscle weakness in grip strength or inverted mesh hanging time (Fig. 2, D and E). The bivalent 13-3B5 induced a mild, but statistically significant, loss of grip strength only at the final day of the experiment (Fig. 2D). Furthermore, three out of five mice in this group could not complete the three minutes hanging test on day 10 and/or 11, while all mice in the control group could (Fig. 2E and Fig. S4D).

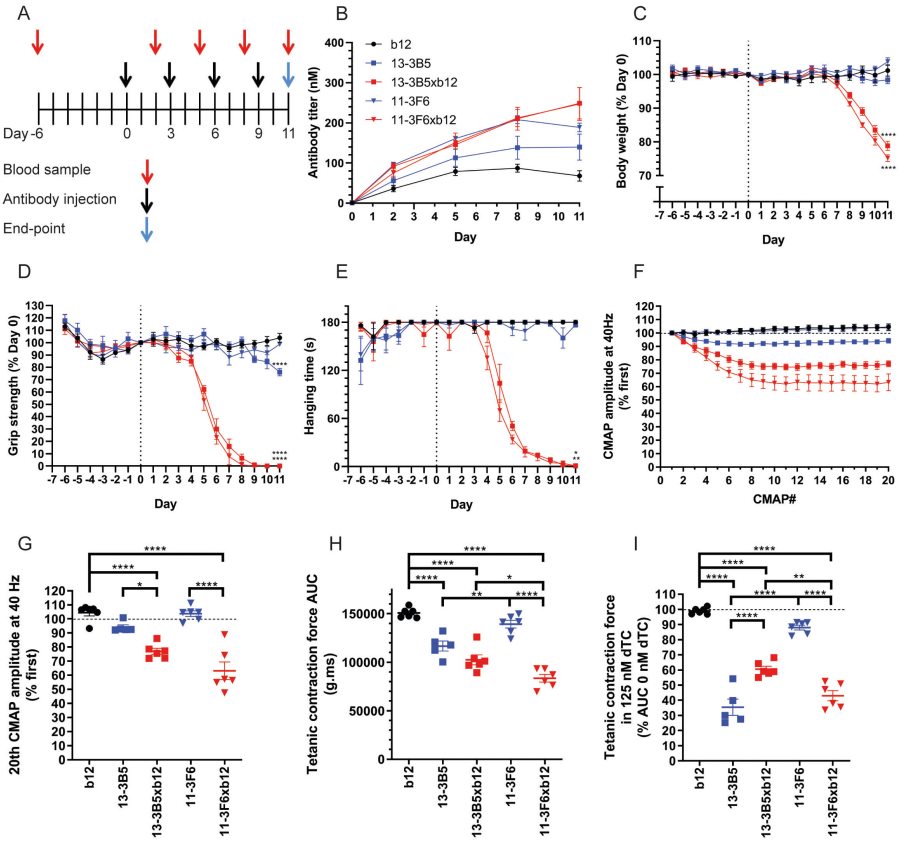


*Fig. 2. Monovalent MuSK antibodies induce rapid onset, progressive myasthenic symptoms in mice, whereas bivalent MuSK antibodies do not. (A) Experimental design of passive transfer with 2.5 mg/kg recombinant antibody (B) Serum antibody titers confirmed exposure. (C-E) Monovalent, but not bivalent, MuSK antibodies induced progressive weight loss and muscle weakness. Bivalent 13-3B5 induced a delayed, mild loss of grip strength. (F and G) Monovalent, but not bivalent, MuSK antibodies induced a significantly larger CMAP decrement upon repetitive stimulation at 40 Hz compared to the b12 control. (H) Ex vivo tetanic contraction force of the diaphragm was reduced by 13-3B5, 13-3B5xb12 and 11-3F6xb12 compared to b12. (I) The safety factor of neuromuscular transmission was assessed in the presence of 125 nM dTC. Exposure to all MuSK antibodies reduced the safety factor compared to the b12 control, although for 11-3F6 this was only a trend (p=0.079). 11-3F6 and 11-3F6xb12 n=6, 13-3B5 n=5, 13-3B5xb12 and b12 n=6 (hanging time n=5). Data represents mean ± SEM. One-way ANOVA with* ***Šidák-corrected comparisons*** *for all parameters (C, D, G, H and I) except hanging time, for which Kruskal-Wallis test was used (C). * <0.05, ** <0.01, *** <0.001, **** <0.0001 compared to b12 group, unless otherwise specified.*

To assess muscle function in more detail, repetitive nerve stimulation electromyography (EMG) on the left calf muscles was performed on the final day of the experiment. Decrement of the compound muscle action potential (CMAP) is a clinical electrophysiological hallmark of MG, and

is caused by loss of transmission in a progressive number of NMJs during the stimulations [31]. Both monovalent MuSK antibodies induced 20-30% decrement at 40 Hz simulation (approximately the physiological firing rate of motor neurons [32]), indicating severe muscle fatigability (Fig. 2, F and G). This decrement was significantly larger compared to the bivalent parent of both IgG4s. Notably, the bivalent antibodies did not differ from the b12 control group, indicating normal muscle functioning.

To monitor *in vivo* exposure to the antibodies, serum samples were taken before the first injection and 2 days after every following injection. Serum titers varied between individual mice (Fig. 2B and Fig. S4A), indicating individual differences in pharmacokinetics. Specifically, serum levels of the b12 antibody were lower compared to the different anti-MuSK clones, in spite of identical dosing. The levels of the MuSK antibodies were quite comparable, except at the final stage of the experiment, where titers of the bivalent IgG4s plateaued, while the monovalent anti-MuSK IgG4 serum levels continued to rise. Possible explanations could be the body weight and volume loss which mice injected with monovalent MuSK antibodies experienced at this stage, or kinetic differences between monovalent and bivalent MuSK antibodies due to receptor-mediated internalization and degradation.

To confirm that the IgG4s were stable *in vivo*, serum was collected at the final day of the experiment and analyzed with CE-MS. All five antibodies were successfully retrieved from the circulation and found to be intact at the end of the experiment (Fig. S5). The only modification observed was the *in vivo* removal of C-terminal lysine for 11-3F6 and 11-3F6xb12 (Fig. S5C and E). C-terminal lysine clipping of IgGs is a well-known natural process, which does not affect antigen-binding ability [33]. For both monovalent MuSK antibodies the proportion of excess b12 antibody seems to be reduced after injection, as seen in the base peak electropherogram (BPE) (Fig. S5D and E). The parental b12 control antibody therefore seems to be cleared slightly faster, but this is unlikely to have had an effect on the model as such. No other species or degradation products could be detected, indicating that the antibodies remained stable *in vivo* and ruling out confounding effects, like disassembly of bivalent MuSK antibodies.

In conclusion, all mice exposed to monovalent MuSK IgG4 rapidly developed severe myasthenic symptoms on all *in vivo* outcome measures. Mice exposed to the same dosing of bivalent MuSK IgG4 did not show overt phenotypical myasthenia. Thus, the functional monovalency of

these bispecific (Fab-arm exchanged) anti-MuSK IgG4s makes them much more pathogenic than their monospecific equivalents with functional bivalency for MuSK.

*Monovalent MuSK antibodies induce reduced ex vivo contraction force compared to bivalent MuSK antibodies*

To further characterize muscle function, the contraction force upon repetitive nerve stimulation was examined on the left hemidiaphragm *ex vivo*. Of note, functional effects on the diaphragm may be larger compared to other muscles, because it is directly exposed to the antibodies upon i.p. injection. Stimulation of the phrenic nerve at 40 Hz for 7 s resulted in tetanic contraction. Quantification of the area under the curve (AUC) revealed similar patterns between groups as seen with the CMAP decrement in the calf muscles. The monovalent MuSK antibodies reduced the contraction force most severely (Fig. 2H). Baseline contraction force upon exposure to bivalent 11-3F6 was indistinguishable from the b12 control group; while mice that received bivalent 13-3B5 showed a mild but statistically significant reduction of contraction force. Furthermore, the diaphragm contraction force was lower for the monovalent compared to their bivalent counterparts; although this was only statistically significant for 11-3F6xb12.



Subclinical signs of myasthenic muscle pathophysiology can be investigated by assessing the safety factor of neuromuscular transmission with the reversible AChR blocker d-tubocurarine (dTC). In the b12 control group, the tetanic contraction force of the diaphragm was not reduced by 125 nM dTC, indicating a healthy safety factor (Fig. 2I). Both monovalent anti-MuSK IgG4s substantially reduced the safety factor. In contrast, bivalent 11-3F6 did not show a statistically significant reduction of tetanic contraction, although a trend was seen (p=0.079). Diaphragms of mice exposed to bivalent 13-3B5 were affected by 125 nM dTC, indicating that although the clinical phenotype caused by 13-3B5 is mild, the NMJs have already substantial loss of AChR and are in a state of subclinical myasthenia (Fig. 2I). Taken together, monovalent MuSK IgG4 antibodies caused significant reduction in contraction force and safety factor of neurotransmission. Bivalent MuSK antibodies induced subclinical signs of myasthenia, but the extent was antibody-dependent.

*Monovalent and bivalent MuSK antibodies affect neuromuscular junction morphology*

Fragmented and reduced AChR area at NMJs is a well-described pathological feature, causing muscle weakness in MuSK MG animal

models [10, 17, 18, 20, 34-37]. To investigate the postsynaptic NMJ morphology after *in vivo* exposure to mono- and bivalent MuSK antibodies, AChRs were stained in whole-mount preparations of the right hemidiaphragm and epitrochleoanconeus (ETA) (Fig. 3 and Fig. S6A and D). Quantification of the total AChR signal per NMJ revealed that both monovalent anti-MuSK IgG4s and the bivalent 13-3B5 caused a strong reduction of AChRs, while mice exposed to bivalent 11-3F6 had more remaining AChRs compared to monovalent 11-3F6xb12 and bivalent 13-3B5 (Fig. 3A). The area of positive AChR signal was also significantly reduced in the muscles from mice treated with monovalent anti-MuSK IgG4s or bivalent 13-3B5 (Fig. 3B). The average intensity of post-threshold AChR staining was not statistically different between the conditions, likely due to animal-to-animal variance in these small groups (Fig. 3C). In sum, exposure to mono- and bivalent MuSK antibodies reduced the number of postsynaptic AChRs, with the effect of the bivalent MuSK antibodies being antibody-dependent.

Loss of appropriate pre- and postsynaptic alignment is also known to contribute to the NMJ dysfunction seen in MG animal models [2, 10, 17]. Therefore, this was assessed in the ETA muscle of a subset of animals (Fig. S6). The intensity and morphology of the presynaptic SV2 signal were not affected by exposure to these mono- and bivalent anti-MuSK IgG4s (Fig. S6B). Denervation assessed by the colocalization of presynaptic SV2 signal with postsynaptic AChR signal revealed a similar pattern as seen with the postsynaptic AChR signal (Fig. S6 A and C). Overall, this data suggests that postsynaptic pathology of the NMJ is the main cause of the neuromuscular dysfunction induced by these MuSK antibodies and that loss of AChR area results in less alignment with the pre-synapse.

*Antibody-dependent pathogenicity of parental bivalent MuSK antibodies*

To investigate whether both bivalent MuSK antibodies could be pathogenic, albeit at a higher dose or with prolonged exposure, we increased the dosing to 5 mg/kg and 10 mg/kg, and extended the duration of the passive transfer to 3 weeks (Fig. 4A). The mice exposed to bivalent 13-3B5 displayed overt signs of muscle weakness on grip strength and inverted mesh from day 9 to 12 onwards and progressively lost weight in week three (Fig. 4B-D and Fig S7B-D for individual traces). Grip strength slowly declined over a period of a week to a near complete loss on the final days of the experiment (Fig. 4C).

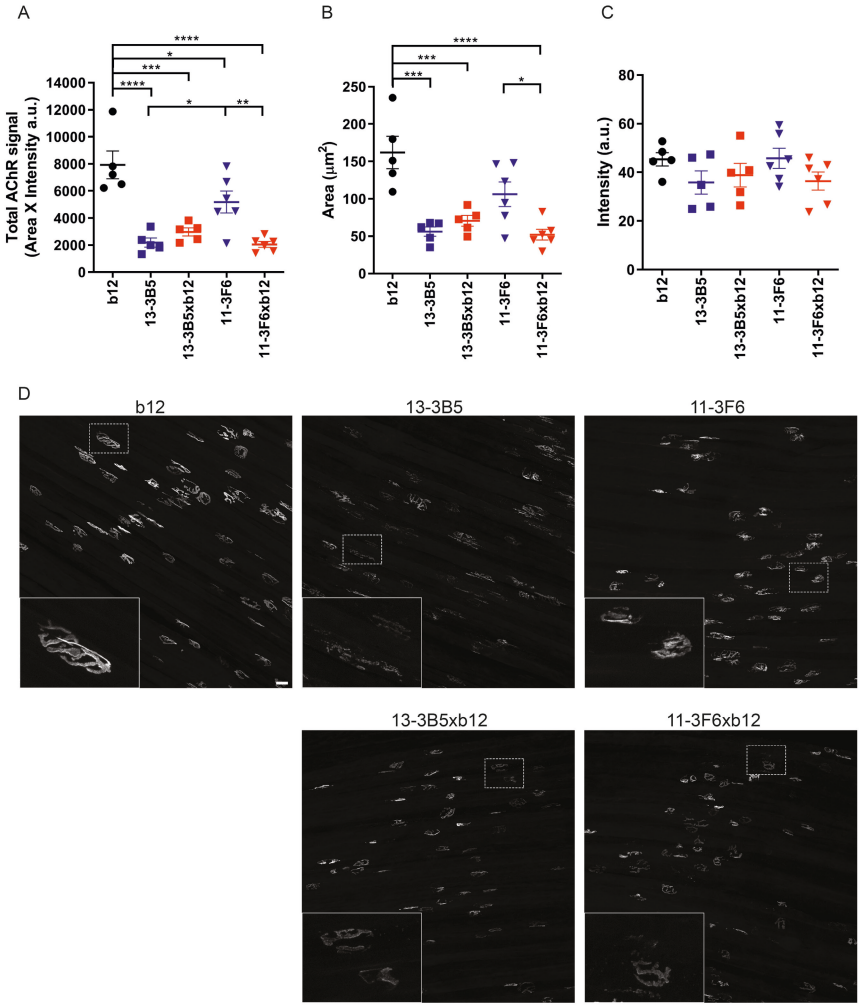


*Fig. 3. Monovalent and bivalent MuSK antibodies impair NMJ morphology to a different extent. To visualize the postsynaptic NMJ, AChRs were stained with AF488-BTX on diaphragm muscle preparations. Twenty randomly selected NMJs per diaphragm were analyzed and averaged. (A) Total AChR signal was calculated by multiplying the positive area with the average intensity per NMJ. Both bivalent and monovalent MuSK antibodies significantly reduced the total AChR signal compared to the b12-treated mice. Bivalent 13-3B5 and monovalent 11-3F6xb12 reduced the total AChR signal to a greater extend compared to monospecific 11-3F6. Bivalent 13-3B5 did not significantly differ from monovalent 13-3B5xb12. (B) To assess the size of the NMJs, a threshold was applied and the area of the positive signal was quantified. Exposure to monovalent MuSK antibodies or bivalent 13-3B5 resulted in less signal reaching the threshold and therefore smaller NMJs. Monovalent 11-3F6xb12 also caused significantly smaller NMJs compared to bivalent 11-3F6. (C) The average intensity of AChR staining surpassing the threshold did not significantly differ between groups. (D) representative maximum projections per condition with insets. Scalebar = 25 µm. 11-3F6 and 11-3F6xb12 n=6, 13-3B5, 13-3B5xb12 and b12 n=5. Data represents mean ± SEM. One-way ANOVA with Šidák-corrected comparisons for all parameter * <0.05, ** <0.01, *** <0.001, **** <0.0001.*

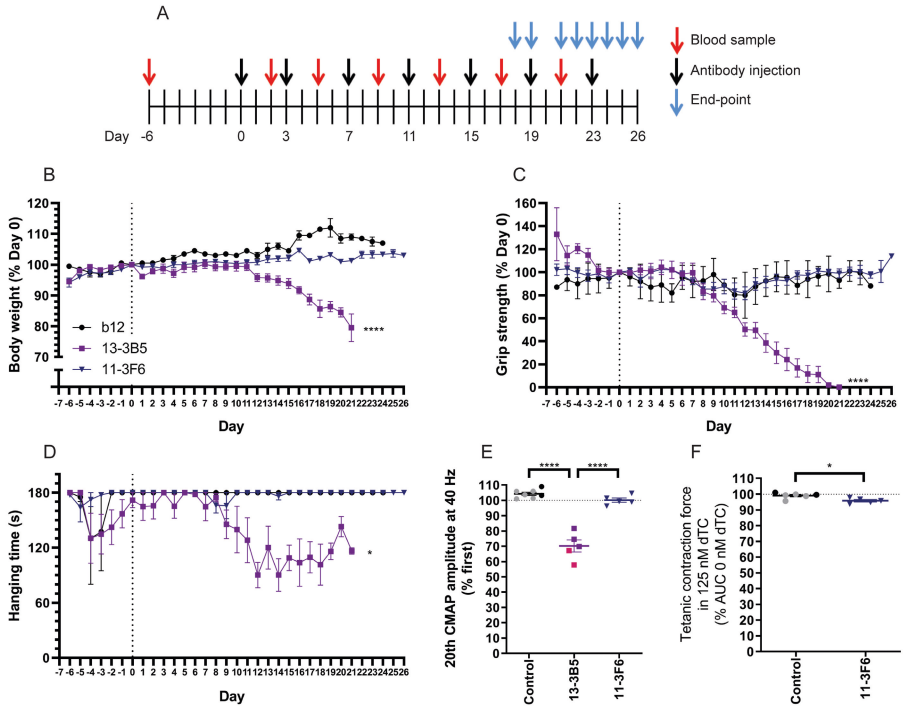


*Fig. 4. Antibody-dependent pathogenicity of bivalent MuSK antibodies. (A) Experimental design of passive transfer in NOD/SCID mice. Mice exposed to bivalent 13-3B5 started to (progressively) lose weight (B), grip strength (C) and hanging time on the inverted mesh (D) in the third and second week of the experiment respectively. Mice exposed to bivalent 11-3F6 did not show progressive loss on these parameters (B-D). (E) Mice exposed to bivalent 13-3B5 showed a significant ~30% CMAP decrement at the endpoint, while bivalent 11-3F6 did not induce a decrement. (F) Contraction force of the diaphragm was significantly, but very mildly (~3% reduction) affected by 125 nM dTC for mice exposed to bivalent 11-3F6. Data represents mean ± SEM. 13-3B5: 5 mg/kg (pink) n=2, 10 mg/kg (purple) n=4 (except for hanging time on inverted mesh and EMG n=3); 11-3F6: 10 mg/kg n=5; b12 (black): n=2, combined with untreated (grey) n=5 for CMAP or n=4 for tetanic contraction).*

Hanging time on the inverted mesh did not show a clear progressive decline in the majority of mice exposed to bivalent 13-3B5 (Fig. S7D). Lastly, exposure to bivalent 13-3B5 resulted in a significant CMAP decrement compared to both the control group and bivalent 11-3F6 (Fig. 4E). In sum, prolonged exposure to bivalent 13-3B5 IgG4 can cause progressive and severe myasthenic muscle weakness. Interestingly, the onset of symptoms is later and progresses slower compared to monovalent 13-3B5xb12.

Mice exposed to bivalent 11-3F6 did not show signs of myasthenic muscle weakness on any of the *in vivo* parameters at higher dose and after prolonged exposure (Fig. 4B-E). To detect possible subclinical pathology,

the safety factor of neurotransmission was assessed in the diaphragm. For reference, the relative tetanic contraction force of animals treated with the b12 antibody was supplemented with untreated healthy animals. The safety factor of mice exposed to 10 mg/kg bivalent 11-3F6 was slightly (~3%), but significantly reduced compared to control muscle (Fig. 4F). The effect size of the reduced safety factor seen in mice exposed to 2.5 mg/kg bivalent 11-3F6 for 11 days therefore does not seem to worsen over time and with higher dose (Fig. 2I). Antibody titers were comparable between mice injected with bivalent 13-3B5 or 11-3F6 (Fig. S7A). The differential effects between 13-3B5 and 11-3F6 therefore confirm bivalent MuSK antibodies can be pathogenic, but their pathogenic potential is antibody-dependent.

*Monovalent MuSK antibodies inhibit MuSK signaling in vitro*

C2C12 myotubes contain all the muscle-specific machinery to interrogate the agrin-Lrp4-MuSK-signaling cascade *in vitro*, except for neural agrin. Addition of agrin to these cultures activates this cascade, leading to MuSK phosphorylation and AChR clustering [38-40]. Monovalent MuSK antibodies were found to inhibit agrin-induced MuSK phosphorylation in a concentration-dependent manner (Fig. 5A and B). Bivalent anti-MuSK IgG4 in stark contrast (partially) induced MuSK phosphorylation and AChR clustering in both the absence or presence of agrin (Fig. 5C-F). These opposing effects extend previous observations using monovalent Fab fragments [21, 23]. The bivalent 13-3B5 and 11-3F6 antibodies induced MuSK phosphorylation to different extents, with 13-3B5 reaching supra-agrin levels, while maximum phosphorylation induced by 11-3F6 reached about 70% of the level of agrin. Bivalent anti-MuSK antibodies induced similar amounts of large AChR clusters (>15 $\mu m^2$), which are considered most mature and therefore relevant [41]. However, 13-3B5 also induced more smaller clusters compared to 11-3F6 (Fig. S2B). In summary, monovalent MuSK IgG4 abolished agrin-Lrp4-MuSK signaling *in vitro*, whereas bivalent anti-MuSK IgG4 partially activated this signaling, independent of agrin.

## Discussion

In this study, we demonstrate a new disease mechanism in autoimmunity related to the unique feature of IgG4 to undergo Fab-arm exchange. We provide evidence that autoantibody functional monovalency for MuSK amplifies the *in vivo* pathogenicity of IgG4 MuSK antibodies. Mechanistically, this may be explained by the respective antagonistic vs. agonistic effects of the Fab-arm exchanged monovalent and parental bivalent IgG4 antibodies on the MuSK signalling cascade. The sequence of events driving IgG4 autoantibody pathogenicity and main conclusions

of this study are summarized in Fig. 6. Thus, class switching to IgG4 not only may be a characteristic of IgG4-mediated autoimmunity, but also be a crucial step in symptom manifestation.

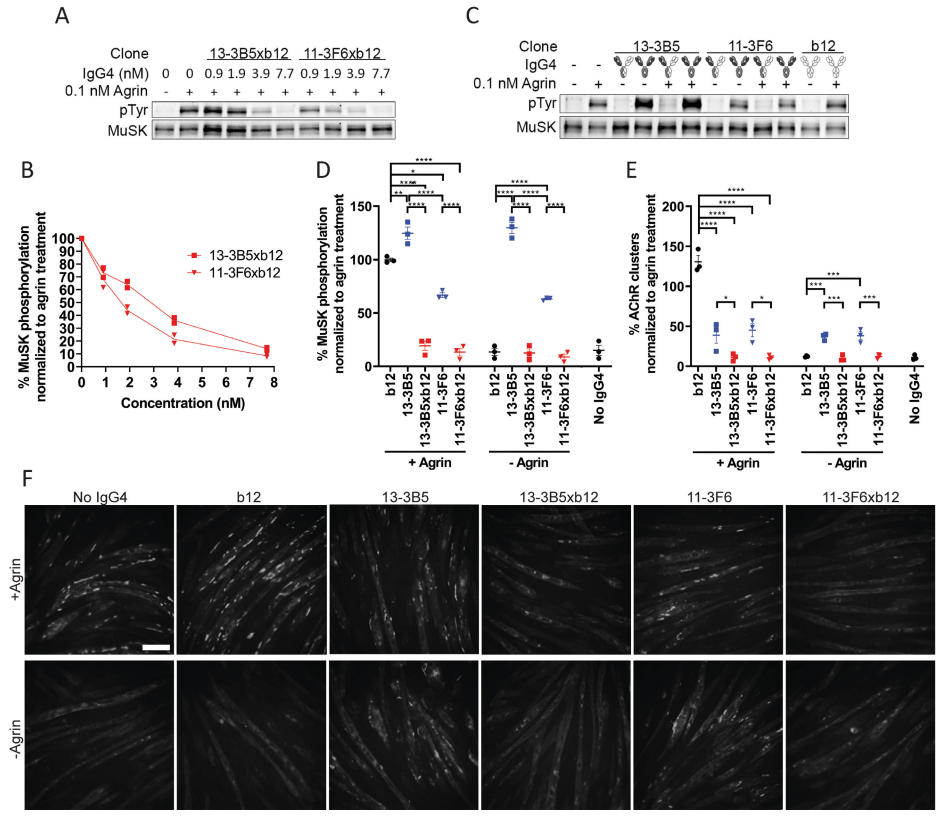


*Fig. 5. Monovalent IgG4 MuSK antibodies abolish agrin-induced signaling of the agrin-Lrp4-MuSK cascade. (A and B) Monovalent MuSK antibodies inhibited agrin-induced MuSK phosphorylation in a concentration-dependent manner in C2C12 myotubes (n=2). Maximum inhibition was reached at 7.7 nM. (C and D) Bivalent MuSK antibodies activated MuSK phosphorylation independent of agrin, while monovalent MuSK antibodies fully inhibited agrin-induced MuSK phosphorylation. The b12 control did not affect MuSK phosphorylation. To correct for loading differences the phosphotyrosine (pTyr) signal was divided by the amount of MuSK that was immunoprecipitated and normalized to the agrin only condition per replicate (n=3). (E and F) Monovalent MuSK antibodies completely inhibited agrin-induced AChR clustering (visualized by AF488-BTX staining). Bivalent MuSK antibodies partially induced AChR clustering independent of agrin, but partially inhibited agrin-induced clustering. Large (>15 µm$^2$) AChR clusters were counted and normalized to the agrin only condition per replicate (n=3). Data represents mean ± SEM. One-way ANOVA with Šidák-corrected comparisons (D and E) * <0.05, ** <0.01, *** <0.001, **** <0.0001. Scalebar=100 µm.*

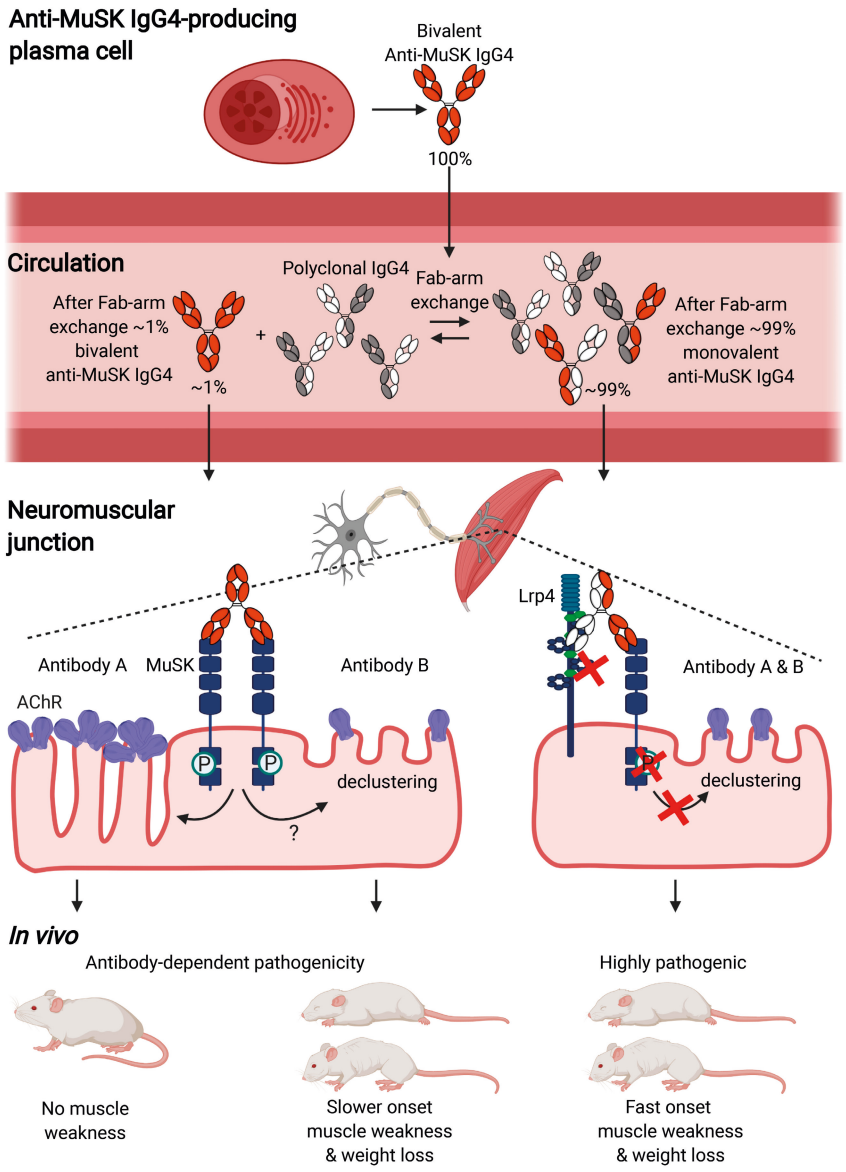


*Fig. 6. Functional monovalency amplifies the pathogenicity of anti-MuSK IgG4. Anti-MuSK IgG4 is produced as monospecific bivalent antibody. Upon reaching the circulation, Fab-arm exchange renders ~99% of IgG4 MuSK antibodies bispecific and functionally monovalent. Monovalent MuSK antibodies reach NMJs and upon binding to MuSK, block its function, induce AChR declustering resulting in fast onset progressive myasthenia. Monospecific functionally bivalent MuSK antibodies dimerize and activate MuSK signaling independent of agrin. Depending on the clone this direct effect on MuSK either results in more slowly progressing myasthenia due to depletion of AChRs, by a yet to be discovered mechanism, or induces no clinical phenotype with largely intact NMJs. P = phosphorylation. This figure was created with BioRender.com*

IgG4 is the only human antibody subclass able to undergo Fab-arm exchange under physiological conditions [42]. It furthermore has limited ability to stimulate inflammation. The relevance of (Fab-arm exchanged) IgG4 is thought to be inhibition of ongoing detrimental immune responses against exogenous antigens and allergens [5]. For example, in novice bee-keepers prolonged exposure to bee venom induces isotype/subclass switching to IgG4 which dampens allergic responses, rendering beekeepers resistant to bee stings [43]. Moreover, chronic inflammation due to worm infections is halted by class switching to IgG4 [44]. Why certain (auto)immune responses undergo a class switch to predominant IgG4 responses is not known. In MuSK MG, autoantibodies of the IgG1, 2 or 3 subclasses are monospecific and may activate complement. However, given their significantly lower levels in serum and potential agonistic effects, their contribution to the clinical manifestation in patients is uncertain [8]. It is possible that in IgG4-mediated autoimmune diseases a mild IgG1-IgG3 immune response against the antigen is ongoing, similar to bee keepers. This prolonged exposure at some point may induce an autoantibody class switch to high titers of functionally monovalent IgG4 and only then symptoms manifest. In the development of MuSK MG, high affinity binding of MuSK antibodies may furthermore be extra critical, as functional monovalent (germlined) monoclonal MuSK antibodies lost significant binding capacity and *in vitro* pathogenicity [23]. Affinity maturation thus seems an additional requirement for potentiating pathogenicity of monovalent MuSK antibodies. It will be exciting to learn what governs the development of IgG4 (auto)immunity.

Monovalent anti-MuSK IgG4s inhibited agrin-Lrp4-MuSK signaling *in vitro* and induced progressive myasthenic weakness and NMJ morphological abnormalities in NOD/SCID mice similar to polyclonal patient-purified IgG4 and monovalent Fab fragments [10, 12, 13, 21, 23, 45]. This suggests that Fab-arm exchanged anti-MuSK IgG4 in polyclonal patient IgG is the main pathogenic factor causing muscle weakness in patients and animal models. Importantly, this study using patient-derived monoclonal monovalent antibodies to model MuSK MG *in vivo*. These models are exciting new tools to perform preclinical therapeutic tests.

Patient-derived bivalent MuSK antibodies can potentially be pathogenic *in vivo,* but this differed considerably between the two antibodies studied here. The bivalent parent antibody 13-3B5 was able to cause overt myasthenic symptoms, but this required more time to start (2 to 3 weeks) and progressed more slowly, as compared to monovalent 13-3B5xb12. This time-course of myasthenic symptom development and progression resembles

what has been reported in MuSK active immunization models [19, 35, 36, 46, 47]. The MuSK antibodies induced during active immunization are functionally bivalent and monospecific, as rodent IgG is unable to exchange Fab-arms under physiological conditions [5]. Furthermore, MuSK immunization of mice induces a dominant mouse IgG1 response [46]. Mouse IgG1 activates complement weakly [48], suggesting that the effects of direct binding are the main cause of myasthenic symptoms upon active immunization. Therefore, the mechanism of MuSK MG in active immunization models is expected to resemble that of pathogenic monospecific, functionally bivalent MuSK antibodies like 13-3B5. Taking together, the opposing effects on MuSK signaling *in vitro* and the differences in timeline and development of muscle weakness and safety factor of neurotransmission in diaphragm NMJs *in vivo*, it would be interesting to investigate whether bivalent 13-3B5 is pathogenic through a different mechanism compared to the monovalent anti-MuSK IgG4s.

The bivalent 11-3F6 parent antibody did not induce any signs of clinical myasthenic muscle weakness, not even when doubling the exposure time and increasing the dose. Subtly different epitopes may differentially affect the conformation and activation of MuSK, and thereby the subsequent downstream signaling and pathogenicity of bivalent MuSK antibodies. Whether non-pathogenic MuSK antibodies such as 11-3F6 or #13 [49], can mitigate detrimental effects of pathogenic mono- and bivalent MuSK antibodies is not known.



It is important to note that a polyclonal pool of MuSK antibodies exists in patients. The question arises how representative the investigated clones are for the polyclonal response in different MuSK MG patients, warranting further research. However, because the clones bind the main immunogenic region of MuSK at two different epitopes within this region [8, 15, 16], they are likely representative for a substantial part of the polyclonal response range. Some autoantibodies may be functionally bivalent, for example when MuSK antibodies are of the IgG1, IgG2 or IgG3 subclasses or non-exchanged IgG4. These antibodies may be non-pathogenic (like 11-3F6) and may compete for binding with inhibitory monovalent anti-MuSK IgG4. Alternatively, some of these may be pathogenic (like 13-3B5) and together with monovalent anti-MuSK IgG4 induce disease. The net result on NMJ function and disease severity will depend on the complex combination of these antagonistic and agonistic effects. It is further important to realize that the (lack of) pathogenicity of the bivalent human MuSK antibodies in this study can be only mediated by a direct effect on MuSK, as the model system used does not allow for assessment

of for example complement activation [50]. This means it is still possible that if the subclass of the bivalent antibody allows for complement activation, it might be pathogenic in other model systems or in humans.

A critical step in this study was the generation of stable bispecific (monovalent) IgG4 antibodies. The cFAE method presented here broadens the toolbox available for generating therapeutic bispecific antibodies. By altering three residues in the Fc tail of IgG4 and adding excess of an irrelevant donor antibody (b12), <1% monospecific antibody contamination could be achieved. Furthermore, these bispecific IgG4s were stable after intraperitoneal injection in NOD/SCID mice. The field of bispecific antibody therapeutics has taken an exponential flight [51]. The therapeutic promise of bispecific antibodies lies in their ability to bring together two antigens that could not be brought in close proximity with monospecific bivalent antibodies. Because of their naturally flexible structure and anti-inflammatory nature, stable bispecific IgG4 have shown promise in preclinical tests for T cell redirection in the treatment of cancer and hemophilia A [52-54]. Our method adds to other currently available (IgG4) antibody technology platforms and can be used for development of antibody therapies in the future.

Taken together, IgG4 Fab-arm exchange is not an innocent bystander activity, but instead potentiates the pathogenicity of MuSK autoantibodies. This may be relevant for the growing number of IgG4-mediated autoimmune diseases and other disease settings where IgG4 plays a pathogenic role, such as where IgG4 blocks endogenous anti-tumor responses in melanoma patients [2, 4, 55, 56].

## Materials and Methods

### *Generation of bivalent and monovalent recombinant antibodies*

Anti-MuSK clones 11-3F6 and 13-3B5 were previously isolated from a MuSK MG patient and produced with a human IgG4 Fc [21]. The S228P amino acid change in the anti-MuSK antibodies was achieved by converting AGC>CCC through side-directed mutagenesis based on the QuikChange II system (Agilent). To make the b12 antibody suitable as an exchange partner for cFAE, the sequence was modified to achieve S228P, F405L and R409K amino acid changes in an pcDNA3.1 IgG4 backbone. Heavy and light chain sequences of the b12 antibody were ordered at GeneArt (Thermo Fisher). All sequences were verified using Sanger sequencing.

Recombinant monoclonal antibodies were produced in suspension FreeStyle HEK293-F cells as described previously [21] or using transient Chinese hamster ovary (CHO) cell-based expression (Evitria) (SI Materials and Methods). To generate monovalent MuSK antibodies, each of the anti-MuSK clones was combined with 1.3-1.4x molar excess of the b12 antibody and 75 mM 2-Mercaptoethylamine-HCl (Sigma-Aldrich) for five hours at 31°C [24]. Preparations were dialyzed back to PBS using dialyzer cassettes, filter sterilized and stored at 4°C until use. Exchange efficiency and monovalent antibody purity were assessed using CE-MS (SI Materials and Methods).

Concentrations of all recombinant antibodies were determined with nanodrop (ND-1000, v.3.8.1) using specific extinction coefficients predicted with the ProtPI tool based on the amino acid sequences (Table S1). Furthermore, antigen-binding capacity was assessed using MuSK or gp120 enzyme-linked immunosorbent assay (ELISA) (SI Materials and Methods). Antibody integrity was confirmed using PageBlue Coomassie stain, according to the manufacturer instructions (Thermo Fisher).

*Mouse passive transfer studies*

NOD/SCID mice were used to avoid a mouse immune response to the injected human recombinant IgG4. Mice were bred in the Leiden University Medical Center (LUMC) or purchased from Charles River. They were housed in sterile, individually-ventilated cages and provided with sterile food and drinking water *ad libitum*. Female mice were aged 8 to 10 weeks at the start of the experiment, unless otherwise specified.

The experimenters were blinded for the injected antibodies throughout all experiments and analyses (SI Materials and Methods). To compare pathogenicity of monovalent and bivalent MuSK antibodies, mice were i.p. injected with 2.5 mg/kg recombinant antibody every 3 days. Mice were allocated to a treatment group by a lab member not involved in the experiment. Serum samples were taken on day -6 and 2 days after every injection. Body weight, *in vivo* muscle strength and endurance were assessed daily. On day 11, mice were subjected to the endpoint analyses described below.

To further interrogate possible pathogenic effects of bivalent MuSK antibodies at higher doses over longer time, mice were injected with 5 mg/kg or 10 mg/kg on day 0, 3, 7, 11, 15, 19 and 23. Serum samples were taken prior to the first injection and two days after every injection. Mice that received 13-3B5 were sacrificed between day 18 and 21 as they lost

weight. Mice that received 11-3F6 were sacrificed sequentially between day 21 and 26, due to time restrictions of the end-point analyses. For one mouse that received 11-3F6, the first injection did not lead to systemic exposure as it could not be detected in the serum. Therefore, day 0 was moved to the day of the second injection for this animal. Mice were subjected to repetitive nerve stimulation EMG on the endpoint day. For the mice without signs of muscle weakness on the *in vivo* outcome measures, the diaphragm was prepared for measurement of *ex vivo* contraction force, described below. Untreated non-littermate NOD/SCID mice (males and females aged 2 to 4 months) were combined with the two animals that received the b12 control used as a healthy reference for EMG and contraction measurements.

Over all experiments, one mouse injected with 2.5 mg/kg 13-3B5 had to be excluded for all parameters, because the serum titers revealed lower antibody levels on two time-points in the experiment, likely due to misplacement of i.p. injections. For a further three mice (2.5 mg/kg b12, 2.5 mg/kg 13-3B5xb12 and 10 mg/kg 13-3B5), the measurements on the inverted mesh had to be excluded, because the animals did not complete 180 s hanging during the training period.

*Endpoint analyses*

After the daily measurements on the endpoint day, mice were subjected to repetitive nerve stimulation EMG of the calf muscles under anesthesia, described in detail previously [10]. One of the mice treated with 10 mg/kg bivalent 13-3B5 died from the anesthesia before EMG could be conducted. Substantial CMAP decrement can be measured at 40 Hz stimulation in passive transfer MuSK MG models [10]. Upon completion of the EMG, blood was collected by cutting the tail without recovery from anesthesia. Immediately thereafter, mice were killed by $CO_2$ inhalation. During dissection more blood was collected via vena cava puncture. The serum from the tail and vena cava was pooled and stored at -20°C until further analysis. The right hemidiaphragm and ETA were dissected for NMJ morphological analyses described below.

To measure *ex vivo* contraction force, the left phrenic nerve-hemidiaphragm was prepared as described previously [28]. Briefly, the hemidiaphragm was equilibrated in Ringer's medium. The phrenic nerve was supramaximally stimulated at 40 Hz for 7 seconds every 5 minutes until the preparation gave stable contraction. The safety factor of neuromuscular transmission was assessed by incubating the preparation

with 125 nM dTC (Sigma-Aldrich) and stimulating at 40 Hz for 7 seconds every 5 minutes until the preparation gave stable contraction.

*Neuromuscular junction morphology*

The most dorsal strip of the right hemidiaphragm and the whole ETA were pinned up in Sylgard lined dishes and fixed in 1% PFA in PBS for 30 minutes. All incubations were done at RT, unless otherwise specified. To enable parallel processing of all muscles in an experiment, they were stored floating in 1% PFA at 4°C for 3 to 7 days. Before staining, muscles were blinded to the phenotypes observed in the experiment until all morphology analyses were completed. The tissues were extensively washed with PBS, remaining 1% PFA was neutralized with 0.1 M glycine in PBS (1 hour) and muscles were blocked with 2% bovine serum albumin (BSA, Sigma), 1% Triton-X (Sigma) in PBS (2 hour). The muscles were subsequently incubated with 0.2 µg/mL mouse anti-SV2 (5ea, developmental studies hybridoma bank) in block ON at 4°C. After 6x 10 minutes washes with PBS, tissue was incubated with 2 µg/mL BTX-488 to visualize AChRs and 2 µg/mL Alexa Fluor 594-conjugated donkey anti-mouse IgG (A21203, Thermo Fisher) in 2% BSA in PBS for 2 hours. After 6x 10 minutes washes in PBS, muscles were mounted in Prolong Gold mounting medium (Thermo Fisher) and stored at 4°C until imaging. Due to technical issues with the staining two ETA muscles (both in the b12 group) and two diaphragm preparations (1x in b12 group and 1x in 13-3B5xb12 group) had to be excluded from further analysis. Consequently, for the ETA muscles the b12 control group was left with only two independent data points. Therefore, statistical analysis was not done on this dataset.

One high resolution Z-stack of a representative part of the muscle was taken using a 20x objective on a SP8 confocal laser-scanning microscope with Las X software (Leica). Z-stacks were converted into maximum projections and further analyzed using ImageJ 1.52n. For the diaphragm images, twenty *en face* NMJs were randomly selected in the 488-BTX-channel and analyzed for intensity and area using a manual threshold. For the analysis of the ETA, thirty enface NMJs were selected, to better capture the variation seen in these preparations. A global threshold was manually determined for the diaphragm and ETA separately. The total AChR signal (intensity X area in the 488-BTX channel) of all analyzed NMJs per image were averaged and used as an n=1 for visualization and further analysis. NMJ colocalization analysis was conducted on the ETA muscles (SI Materials and Methods).

*MuSK phosphorylation and AChR clustering*

C2C12 myoblasts were obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany) , tested for mycoplasma contamination and maintained for maximum 7 passages after thawing. MuSK phosphorylation and AChR clustering was assessed as described previously [21]. Briefly, for MuSK phosphorylation differentiated C2C12 myotubes were treated for 30 minutes. The concentration of monovalent MuSK antibodies was titered to achieve complete inhibition of agrin-induced MuSK phosphorylation. C2C12 myotubes were treated with this concentration (7.7 nM) of recombinant mono- or bivalent antibodies in the absence or presence of 0.1 nM neural agrin (R&D systems). MuSK was immunoprecipitated from whole lysate and detected on western blot. For AChR clustering, C2C12 myotubes were treated for 16 hours with 7.7 nM recombinant antibodies in 96-well plates. After treatment, cells were stained with 2 µg/mL BTX-488 (B13422, Thermo Fisher) and 2 µg/mL Hoechst 33342 (H1399, Thermo Fisher) for 30 minutes at 37°C before fixation with 4% paraformaldehyde (PFA). Twenty fields divided over 5 wells per condition were randomly selected in the brightfield channel on a Leica AF6000 microscope. AChR cluster count and size were analyzed using ImageJ 1.52n. A manual threshold was set for each independent replicate. Large (>15 $\mu m^2$) and all >3 $\mu m^2$ clusters were analyzed. Both assays were performed in triplicate.

*Statistics*

Data are expressed as mean ± SEM. Comparisons between three or five groups were analyzed using one-way ANOVA with Šidák-corrected comparisons for parametric data. Hanging time (non-parametric data) was analyzed with the Kruskal-Wallis test with Šidák-corrected comparisons. The following comparisons were pre-defined: b12 vs 13-3B5, b12 vs 13-3B5xb12, b12 vs 11-3F6, b12 vs 11-3F6xb12, 13-3B5 vs 13-3B5xb12, 11-3F6 vs 11-3F6xb12, 13-3B5 vs 11-3F6 and 13-3B5xb12 vs 11-3F6xb12. Comparisons between two groups were analyzed using two-tailed unpaired t-test. Measurements from the end-point day was used for statistical analysis by GraphPad Prism (version 8.1.1). Differences were considered significant at $p<0.05$.

*Study approval*

All animal studies were executed with approval of the Dutch national and local animal experiments committees, according the Dutch law and Leiden University guidelines.

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## Supplementary Information

### SI Materials and Methods

*IgG purification*

IgG was purified with a HiTrap MabSelect SuRe protein A affinity column (GE Healthcare) on an AKTA Pure (GE Healthcare). Antibodies were dialyzed to PBS in dialysis cassettes (Thermo Fisher) or desalted to PBS using an HiPrep 26/10 column (GE Healthcare) on an AKTA Pure, filter sterilized and stored at -20°C until use. Recombinant antibodies 13-3B5 and b12 used in all experiments were produced in HEK cells. For 11-3F6, a batch produced in HEK cells was used in Fig. 1 and 5 and Fig. S1, 2 and 5. A batch of 11-3F6 produced in CHO cells was used in Fig. 1, 2, 3 and 4, and Fig. S4, 5, 6 and 7.

*Capillary electrophoresis mass spectrometry*

Sheathless CE-MS was employed to assess exchange efficiency and purity of monovalent antibodies. Analyses were carried out on a CESI 8000 instrument (Sciex) coupled to an Impact Qtof mass spectrometer (Bruker Daltonics) equipped with a nanoelectrospray source. Porous-tip capillaries (91 cm x 30 µm ID) were obtained from Sciex. Capillaries were coated using polyethylenimine (Gelest) following the protocol described by Sciex [57]. The background electrolyte (BGE) consisted of 10% acetic acid or 30% acetic acid and 10% MeOH for 13-3B5(xb12) and 11-3F6(xb12), respectively. Before each run, the capillary was flushed for 4 min at 100 psi with the BGE. Separation was performed by applying -20 kV at 20°C. Samples were buffer exchanged to the relevant BGE using 30 kDa MWCO filters (Vivaspin, 3 cycles of 10000xg at 4 °C) and hydrodynamically injected for 15 s using 2.5 psi. The mass spectrometer was operated in positive ionization mode using a capillary voltage of 1200 V, a drying gas temperature of 120°C and a drying gas flow rate of 1.2 L/min. An ISCID energy of 100 eV was employed to obtain proper declustering of the antibodies. Quadrupole ion and collision cell energy were 5.0 and 20.0 eV, respectively. MS control and data acquisition and analysis were performed using QTOF control and data analysis software (Bruker Daltonics). Molecular mass determinations were performed using the Maximum Entropy deconvolution algorithm of the data analysis software. A baseline subtraction of 0.7 points was applied to the deconvoluted mass spectra.

Relative amounts of the antibodies were determined by integrating the area under the peaks observed in the BPE. For 13-3B5, the areas were

directly employed to determine the relative amounts of the antibodies. For 11-3F6, different ionization efficiency was observed compared to b12 as consequence of the Fab glycans. Therefore, the relative amounts were determined by adding known amounts of the antibodies and interpolating in the corresponding calibration lines.

*MuSK and gp120 ELISA*

MuSK or gp120 ELISA was used to assess antigen reactivity or quantify the serum titers of the recombinant antibodies in the NOD.CB17-Prkdcscid/J (NOD/SCID) mice. To measure mono- or bivalent variants of 11-3F6 and 13-3B5, 3 µg/mL of the complete extracellular region of MuSK, produced as described previously [16], was coated on MaxiSorp plates (Thermo Fisher). For the b12 antibody, MaxiSorp plates were coated with 1 µg/mL HIV-gp120 protein (Sigma-Aldrich). Samples were diluted in an eight-point two-fold dilution series. Serum samples started at 125x dilution in block. The original batch of antibody that was injected served as a standard in duplicate; the first dilution started at 0.5-1 nM. Mouse anti-human IgG4 (Nordic clone N315, Nordic MUbio) and rabbit anti-mouse-AP (D0314, Dako) were used as secondary antibodies and conjugate respectively. Plates were developed with pNPP (VWR) and the reaction was stopped using sodium hydroxide. All samples were tested in duplicate and quantified in SoftMax pro (version 7.0.3, Molecular Devices).

*Immunostaining of mouse NMJs using monovalent antibodies*

To determine binding capacity of recombinant mono- and bivalent MuSK antibodies to mouse MuSK, levator auris longus muscles of NOD/SCID mice were immunostained with 2 µg/mL recombinant antibody overnight at 4°C. The preparations were co-stained for synaptic regions with 2 µg/mL AlexaFluor594-conjugated α-bungarotoxin (BTX) (B13423, Thermo Fisher) and imaged as described previously [21].

*Blinding of in vivo experiments*

A researcher not involved in executing or analyzing the experiment made an inventory of body weight and assigned each mouse to a treatment group ensuring a roughly equal distribution of body weight. This researcher prepared the injection solution at the right concentration for each mouse solely labeled with the mouse code and provided this to the executing researcher. Deblinding was done after all data was collected.

*Half-life and in vivo dose-finding*

To determine antibody half-life, mice received a single 5 mg/kg intraperitoneal (i.p.) injection of one of the recombinant antibodies. Antibody titer was monitored in the serum obtained from blood drawn via tail vein cut. Because of animal ethical regulations, daily blood withdrawal was not allowed. Therefore, blood was drawn from mice per treatment group in an alternating fashion. Mouse 1 had blood samples (~50 µL) drawn 8, 72 and 144 hours after the injection using a tail cut. Mouse 2 had blood samples drawn 3 days before and 24, 96 and 168 hours after the injection. Samples were allowed to clot at room temperature, centrifuged at 10,000 RPM for 3 min and serum was stored at -20°C until analysis. Antibody titers were measured using the MuSK and gp120 ELISA (SI Materials and Methods). A one-phase exponential decay function with the plateau constrained at zero was fitted in GraphPad Prism (version 8.1.1) per mice to calculate the half-life. Both male and female NOD/SCID mice were used in this experiment.

To determine the minimum dose at which monovalent MuSK antibodies could be fully pathogenic, 11-3F6xb12 was i.p. injected on day 0, 3, 7, 11, 15 and 19 at doses of 1.25, 2.5 or 5 mg/kg (of the body weight at the beginning of the experiment). In addition to body weight, *in vivo* muscle strength and endurance were assessed on a daily basis using a grip strength meter and inverted mesh hang test as described previously [10]. To familiarize the mice with handling and the tests, the daily *in vivo* measurements were started 4-6 days prior to the first injection. Blood samples were taken on day -2, 5, 13 and on the final day of the experiment. Mice were killed if they lost >20% of their body weight compared to the first day of injection or when they reached the end of the experiment.

*NMJ colocalization analysis*

In the preparations of the ETA muscles, all *en face* NMJs were identified in the 488-BTX-channel. An NMJ was excluded if aspecific background in the SV2 channel overlapped with an NMJ. From the remaining NMJs, thirty were randomly selected for colocalization analysis with the EzColocalization plugin for ImageJ [58]. Manually-determined thresholds for each channel were assigned. As a measure of innervation, the fraction of the postsynaptic AChR signal (BTX) that overlaps with the presynaptic signal (SV2), weighted for signal intensity, was quantified for each NMJ with the Mander's colocalization coefficient M1 [59]. In addition, the total presynaptic signal (area x intensity in the SV2 channel) was assessed. All analyzed NMJs per image were averaged and used as an n=1 for visualization.

*Protein A purification of mouse serum*

To confirm the integrity of the recombinant antibodies was maintained throughout the *in vivo* experiment, they were purified back from the mouse serum with protein A. To ensure sufficient yield for CE-MS analysis, the mouse sera were pooled per condition. Protein A agarose (Roche) beads were equilibrated with PBS. Pooled serum diluted 1:1 with PBS was incubated with the protein A beads, rotating for 1 hour. Bound protein was eluted with 0.1 M Glycine HCl pH 2.5. Fractions were neutralized with 1:6 1 M Tris-HCl pH 8. Protein content was measured with nanodrop in IgG mode. Protein containing fractions were pooled and immediately processed further for CE-MS.

## SI Figures

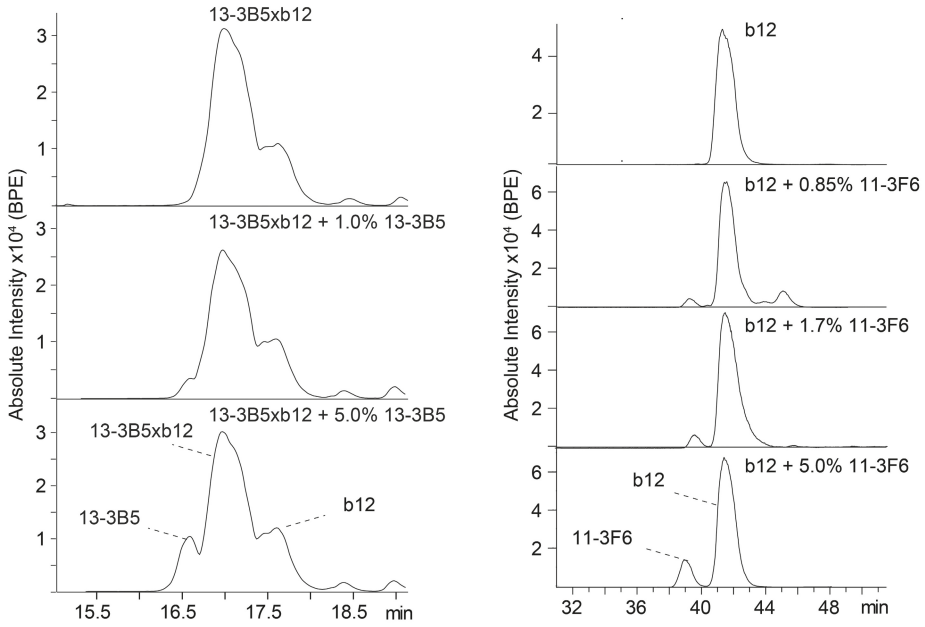



*Fig. S1. Detection limit of CE-MS for bivalent 13-3B5 and 11-3F6 is below 0.5%. To assess the detection limit, antibodies were added in small amounts and analyzed by CE-MS. From the titration curve it is extrapolated that CE-MS permits determination of the bivalent antibodies in relative abundances below 0.5%.*

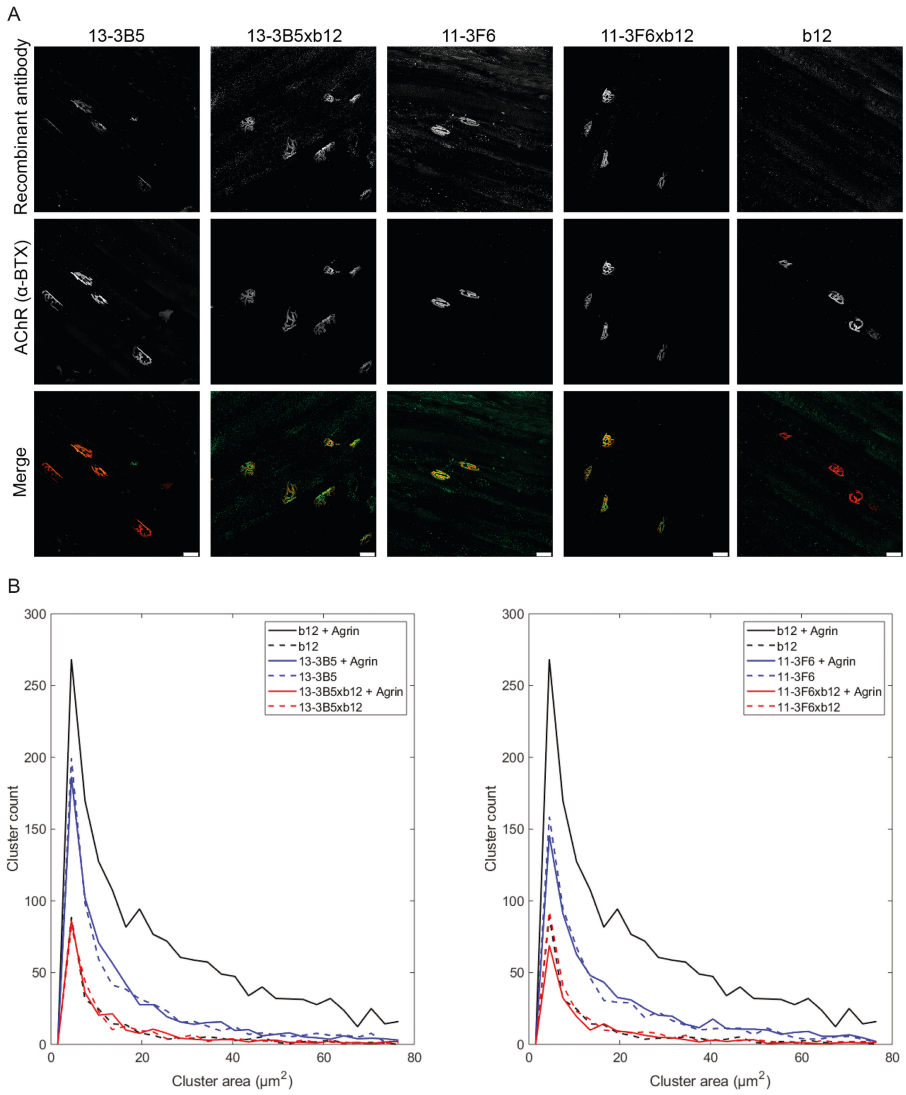



*Fig. S2. In vitro functional characterization of monovalent and bivalent IgG4 MuSK antibodies. (A) Bivalent and monovalent MuSK antibodies bind NMJs of NOD/SCID mice. The control b12 antibody did not show binding. α-BTX = alpha-bungarotoxin. Scale bar 25 μm. (B) Distribution of AChR clusters based on cluster size (cutoff 3 μm²) revealed that bivalent 13-3B5 induced more small clusters compared to bivalent 11-3F6 in C2C12 differentiated myotubes. The effect of the bivalent and monovalent anti-MuSK clones was independent of agrin. Distributions represent mean of three independent experiments.*

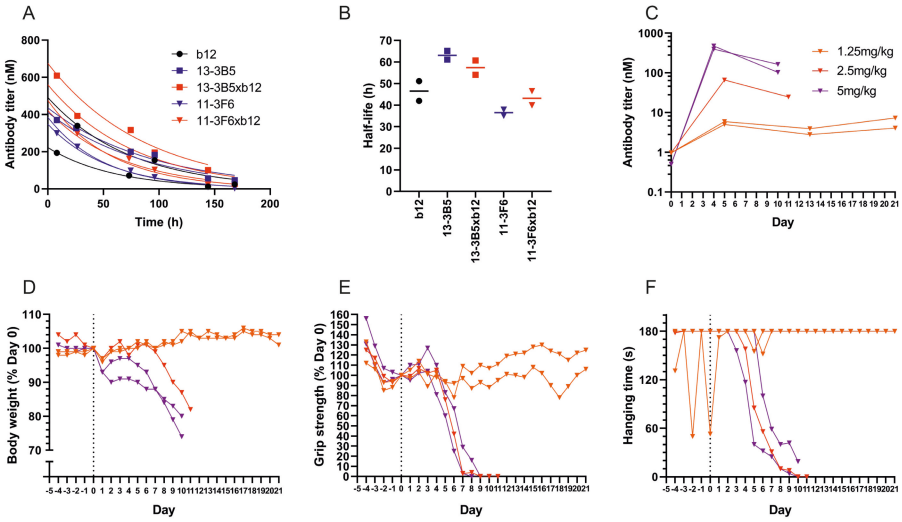



*Fig. S3. Half-life and in vivo dose-finding. (A and B) Serum antibody titer was assessed at different times after a single i.p. dose of 5 mg/kg recombinant antibody with antigen-specific ELISA (n=2). The half-life was calculated by fitting a one-phase exponential decay function with the plateau constrained at zero. Half-life ranged between 38-63 hours, depending on the antibody. (C) MuSK antibody titers of NOD/SCID mice injected with different doses of 11-3F6xb12 every 3-4 days. (D) Body weight, (E) grip strength and (F) inverted mesh hanging time revealed that 2.5 mg/kg every 3-4 days was the minimal dose required to cause progressive phenotypical myasthenia (n=1-2). Individual data are presented.*

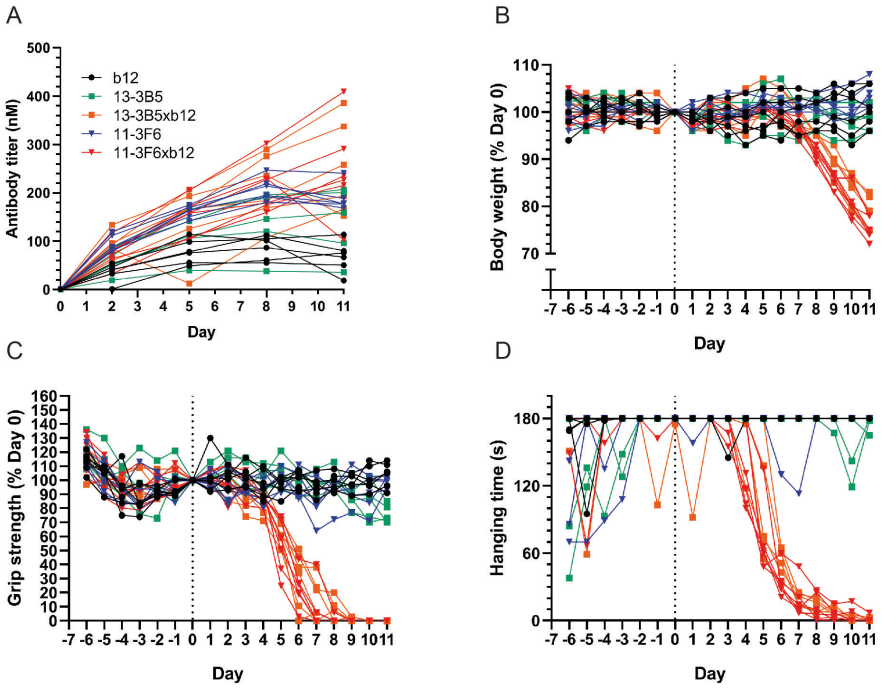



*Fig. S4. Individual mouse data of in vivo parameters. Data trajectories of Figure 3 visualized per mouse for (A) antibody titer, (B) body weight, (C) grip strength and (D) inverted mesh hanging time. 11-3F6 and 11-3F6xb12 n=6, 13-3B5 n=5, 13-3B5xb12 and b12 n=6 (hanging time n=5).*

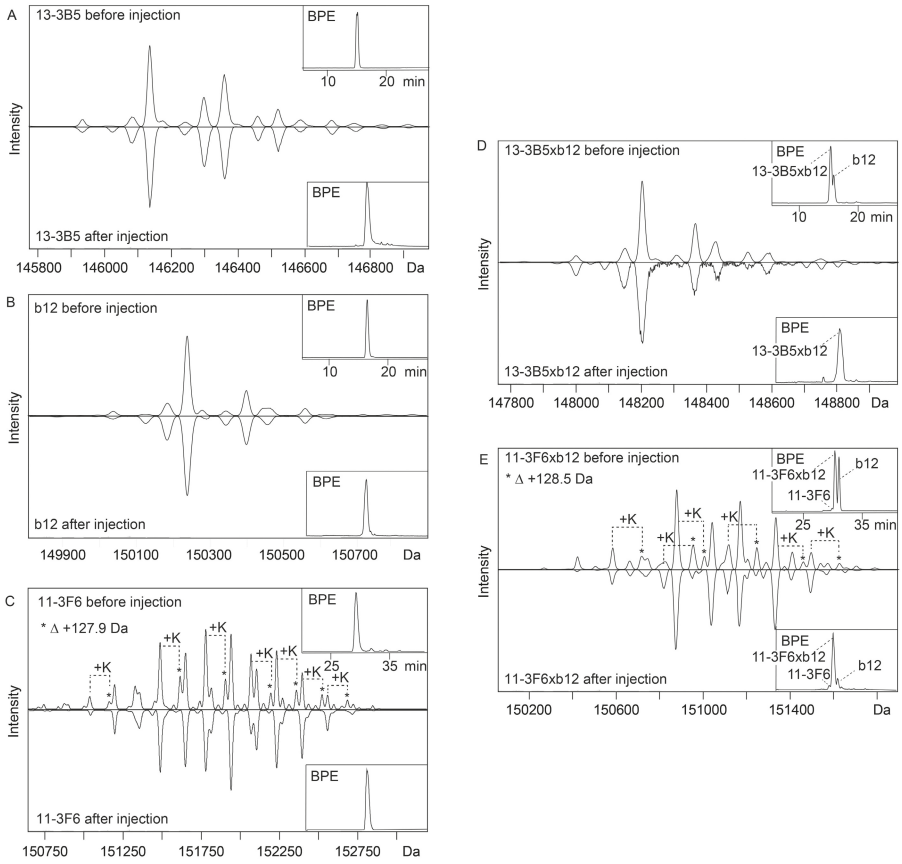



*Fig. S5. Recombinant bivalent and monovalent antibodies are stable in vivo. Deconvoluted mass spectra and base electropherograms (BPE) of bivalent and monovalent antibodies before (upper trace) and after (lower trace) injection in mice showed stability of the antibodies in vivo. (A) Bivalent 13-3B5, (B) bivalent b12, (C) bivalent 11-3F6, (D) monovalent 13-3B5xb12 and (E) monovalent 11-3F6xb12. 11-3F6 (C, upper trace) and 11-3F6xb12 (E, upper trace) contained variants with an additional lysine (K) at the C-terminus of the Fc tail, indicated by (*). C-terminal lysines on IgG Fc are known to be clipped in the circulation [33] and could consequently not be observed in the mass spectra of 11-3F6 (C, lower trace) and 11-3F6xb12 (E, lower trace) purified from mouse serum.*

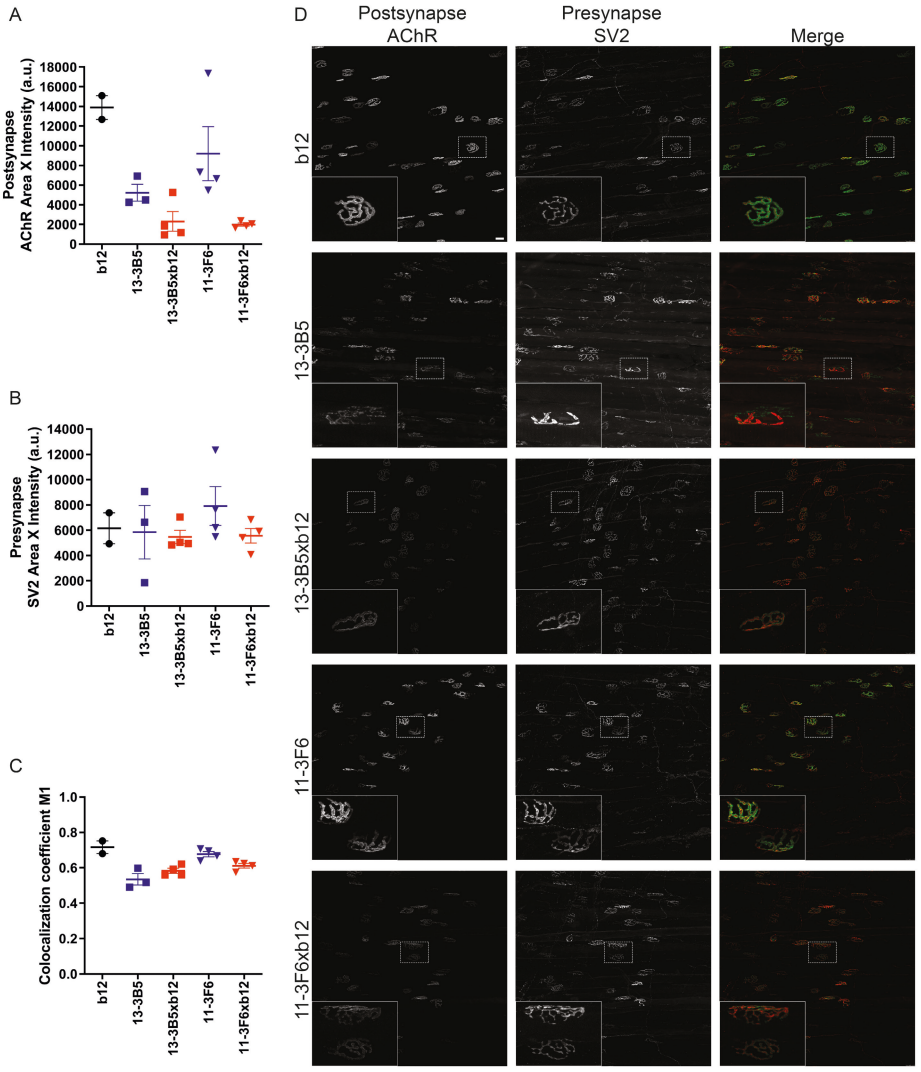



*Fig. S6. Altered NMJ morphology caused by bivalent and monovalent MuSK antibodies seems to be limited to the postsynapse. AChR staining (AF488-BTX) marks the postsynaptic NMJ area and synaptic vesicle protein 2 (SV2) staining marks the presynaptic NMJ area. Thirty randomly selected NMJs per epitrochleoanconeus (ETA) muscle were analyzed and averaged. (A) Exposure to bivalent or monovalent MuSK antibodies resulted in, on average, less postsynaptic signal. (B) Presynaptic morphology does not seem to be affected by exposure to monovalent or bivalent MuSK antibodies. (C) NMJ innervation by the motor neuron was assessed by the colocalization coefficient M1, which is an intensity weighted co-occurrence coefficient of presynaptic signal (SV2) with postsynaptic signal (AChR) compared to the total postsynaptic area. Monovalent MuSK antibodies and bivalent 13-3B5 seem to slightly reduce the overlap between the pre- and postsynapse. (D) Representative maximum projections with insets per condition. In the merged picture green = AChR, red = SV2. Scalebar = 25 µm. 11-3F6, 11-3F6xb12 and 13-3B5xb12 n=4, 13-3B5 n=3 and b12 n=2. Data represents mean ± SEM.*

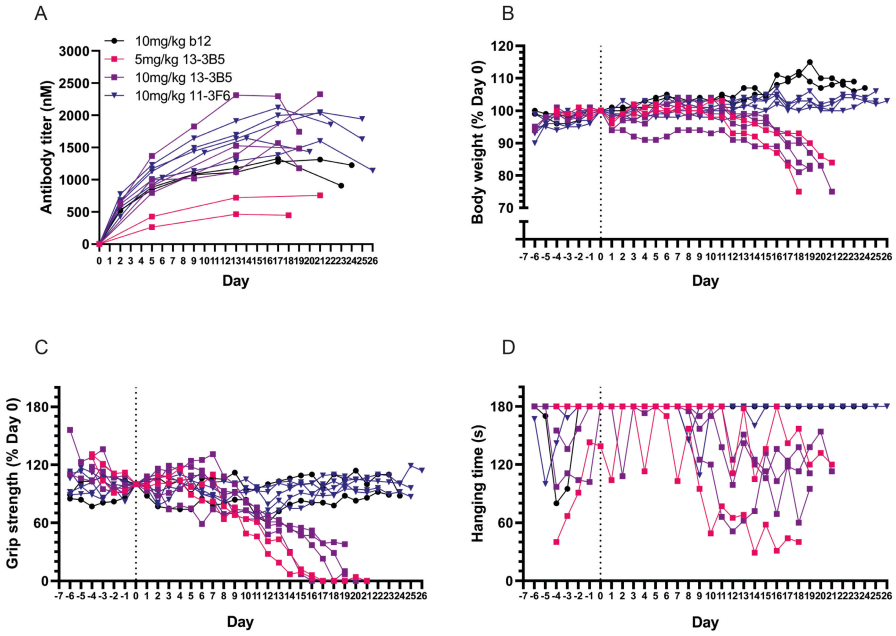
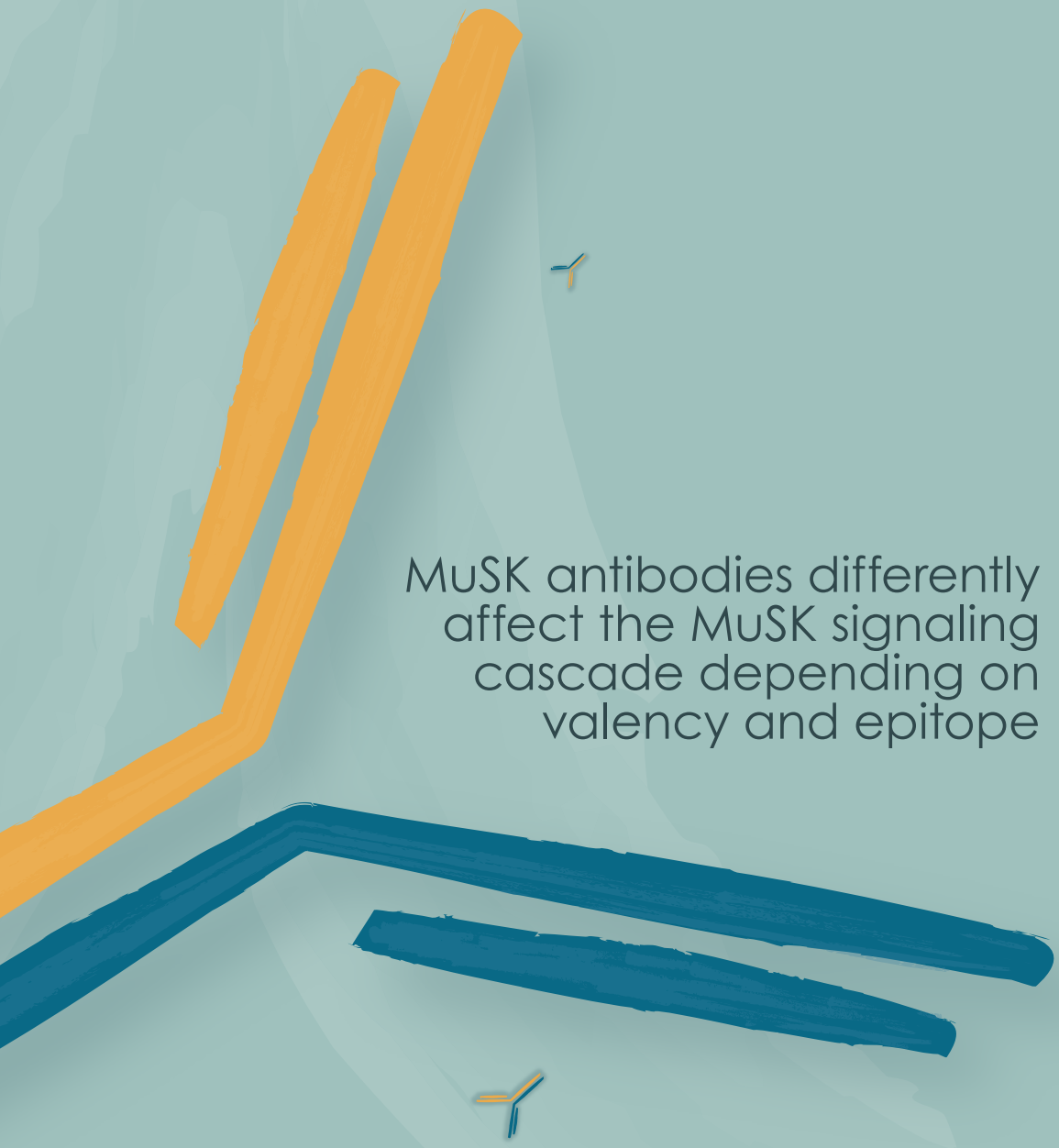



*Fig. S7. Individual mouse data of in vivo parameters. Data trajectories of Figure 5 visualized per mouse for (A) antibody titer, (B) body weight, (C) grip strength and (D) inverted mesh hanging time. 10mg/kg b12 n=2, 5mg/kg 13-3B5 n=2, 10mg/kg 13-3B5 n=4 (hanging time n=3), 10mg/kg 11-3F6 n=5.*

*Table S1: Predicted molecular characteristics of recombinant antibodies,based on amino acid sequence (glycosylation was not considered).*

| Recombinant antibody | Mass (kDa) | Molar extinction coefficient (x$10^3$ $M^{-1}cm^{-1}$) | Absorption coefficient |
|---|---|---|---|
| **b12** | 147.67 | 218.42 | 1.48 |
| **13-3B5** | 143.57 | 215.44 | 1.50 |
| **13-3B5xb12** | 145.62 | 216.93 | 1.49 |
| **11-3F6** | 144.76 | 196.42 | 1.36 |
| **11-3F6xb12** | 146.22 | 207.42 | 1.42 |

# 5



# MuSK antibodies differently affect the MuSK signaling cascade depending on valency and epitope

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*Manuscript in preparation*

## Abstract

Muscle-specific kinase (MuSK) plays a central role in forming and maintaining healthy neuromuscular junctions (NMJ). Antibodies against MuSK impair MuSK functioning and thereby cause myasthenia gravis (MG). MuSK autoantibodies are predominantly IgG4, which bind in a monovalent fashion to MuSK due to Fab-arm exchange. Monovalent and bivalent MuSK antibodies have opposite effects on MuSK activation. How valency and other antibody characteristics affect MuSK's interaction with partner molecules and intracellular pathways downstream of MuSK is largely unknown. To further understand the pathogenic mechanisms underlying MuSK MG, we have investigated how MuSK antibody binding affects MuSK functioning with a panel of human (patient-derived) monoclonal MuSK antibodies. We found valency-dependent effects on inhibition of agrin-induced MuSK activation, Dok7 binding to MuSK and NMJ gene expression. Monovalent binding to the frizzled domain of MuSK did not inhibit agrin-induced MuSK activation, in contrast to binding to the Ig-like 1 domain. In addition, the kinetics of Dok7 degradation induced by bivalent MuSK antibodies appear to depend on epitope binding between and within structural domains of MuSK. None of the clones tested (both bivalent and monovalent) increased MuSK internalization. The net pathogenic effect of polyclonal MuSK antibodies in individual MuSK MG patients thus likely depends on autoantibody titer, and the unique composition of MuSK autoantibodies varying in epitope-binding and valency.

## Introduction

The neuromuscular junction (NMJ) is a specialized synapse where motor neurons and skeletal muscles communicate. Most proteins in the NMJ are indispensable as neuromuscular communication is essential for basic muscular functions such as breathing [1]. Muscle-specific kinase (MuSK) is a transmembrane tyrosine kinase which forms a central signaling hub essential for forming and actively maintaining the NMJ [1]. MuSK has three extracellular immunoglobulin-like (Ig-like) domains followed by a frizzled-like domain (Fz-domain), a transmembrane domain and an intracellular kinase domain [2]. The interaction between its Ig-like domain 1 and lipoprotein receptor-related protein 4 (Lrp4) is crucial for activation and autophosphorylation of the MuSK kinase domain through dimerization of two MuSK molecules [3, 4]. The interaction between Lrp4 and MuSK is greatly enhanced once neuronal agrin released from the motor nerve terminal has bound Lrp4 [4]. Upon dimerization and autophosphorylation of MuSK, downstream of kinase 7 (Dok7) is recruited to the intracellular phosphotyrosine-binding site, stabilizing and enhancing phosphorylation of both MuSK and Dok7 [5, 6]. This initiates further downstream signaling leading to cytoskeletal reorganization and clustering of acetylcholine receptors (AChRs) by scaffold protein rapsyn, and contributes to transcriptomic regulation of NMJ-specific genes [1, 7]. In addition, the MuSK Ig-like 1 domain is involved in tethering the Collagen Q (ColQ) - acetylcholine esterase (AChE) complex to the NMJ [8]. AChE breaks down acetylcholine, the main neurotransmitter responsible for neurotransmission at the NMJ and thereby regulates neuromuscular communication. Because MuSK has multiple functions in the NMJ, perturbing or modifying its functioning can have many consequences.

Antibodies against MuSK cause the neuromuscular autoimmune disorder myasthenia gravis (MuSK MG) characterized by fatigable skeletal muscle weakness [9]. MuSK autoantibodies are predominantly of the IgG4 subclass and thus become in great majority bispecific and functionally monovalent through the stochastic process of Fab-arm exchange [10-12]. Besides IgG4 MuSK antibodies, some patients also have low levels of co-occurring functionally bivalent and monospecific IgG1, IgG2 or IgG3 MuSK antibodies [10, 13, 14]. The term "bivalent" will be used for functionally bivalent and monospecific antibodies and the term "monovalent" for functionally monovalent and bispecific antibodies. Bivalent MuSK antibodies can (partially) activate MuSK (agonists), while monovalent MuSK antibodies inhibit agrin-induced MuSK activation (antagonists) [15-17]. These opposing effects are related to the natural dimerization of MuSK

that occurs in a healthy synapse to keep the MuSK kinase constitutively active and the NMJ intact. Monovalent MuSK antibodies block Lrp4-MuSK interaction thereby preventing dimerization and activation of the kinase and AChR clustering leading ultimately to synaptic disintegration and skeletal muscle fatigue [13, 18]. In contrast, bivalent MuSK antibodies are thought to force dimerization of MuSK, therefore bypassing the need of agrin and Lrp4 in activating MuSK [15, 19]. Experiments with recombinant monoclonal antibodies based on anti-MuSK B-cell receptor (BCR) sequences isolated from MuSK MG patients show that monovalent MuSK antibodies are more pathogenic than their bivalent equivalents and cause rapid onset fatigable muscle weakness in mice [16]. However, the pathogenicity of bivalent MuSK antibodies varies between clones. While *in vitro* their AChR clustering capabilities are relatively equal, some bivalent MuSK antibodies can cause myasthenic muscle weakness with slower disease progression (compared to monovalent MuSK antibodies) in mice, while others are non-pathogenic, even after long-term exposure [15, 16, 20]. We therefore hypothesize that the mechanism by which MuSK antibodies impair MuSK signaling may differ between clones and depend on antibody valency.

Here we investigated the effect of six MuSK antibody clones in monovalent and bivalent format on Dok7, MuSK internalization and NMJ gene expression.

## Results

### *MuSK antibodies differentially affect MuSK and Dok7 depending on valency and epitope.*

To investigate how MuSK antibodies with different characteristics affect MuSK-mediated signaling, we generated a panel of six human recombinant MuSK antibodies (Figure 1A). The sequences of five MuSK clones were isolated from MuSK MG patient BCR sequences and one MuSK clone was identified after phage display selection and screening [15, 21, 22]. All clones were produced in a bivalent manner. Monovalent equivalents were generated by IgG4 controlled Fab-arm exchange with the b12 control antibody and are described as [clone]xb12 [16]. The residual amount of bivalent MuSK antibody after the controlled Fab-arm exchange method was two percent or less, validating the relative purity of the monovalent MuSK antibodies (Figure S1A) [16, 23]. Due to Fab-glycosylation, the 11-3D9xb12 mixture was too complex to calculate relative amounts of each variant. However, the deconvoluted mass spectra in this mixture confirmed the generation of the bispecific,

monovalent variant for this clone (Figure S1B). Five out of six clones bind the Ig-like 1 domain of MuSK, while one clone (mAb13) binds the Fz domain [15, 24].

C2C12 myotube cultures are a well-established cell model to interrogate MuSK signaling *in vitro* as they endogenously express the muscle-specific signaling components of this pathway. All five monovalent MuSK antibodies binding the Ig-like 1 domain of MuSK fully inhibited agrin-induced MuSK phosphorylation, while their bivalent equivalents do not (Figure 1B and C). Monovalent mAb13xb12 binding the Fz domain of MuSK did not inhibit MuSK phosphorylation, even at higher concentrations (Figure 1B, 1C and S2A). Bivalent mAb13 was able to induce MuSK phosphorylation in the absence of agrin, while monovalent mAb13xb12 was not (Figure S2B). For agrin in combination with bivalent 13-3B5 or mAb13, MuSK phosphorylation exceeded the levels of agrin with the b12 control antibody, while for bivalent 13-3D10 the MuSK phosphorylation levels were slightly lower (Figure 1B and C). This suggests that the bivalent MuSK antibody is facilitating MuSK phosphorylation over agrin. Taken together, the inhibition of MuSK by monovalent MuSK antibodies seems to depend on which the structural domain of MuSK is targeted, while the agonistic capacity of bivalent MuSK antibodies does not.



Dok7 binding to MuSK is critical to stabilize and enhance MuSK phosphorylation and propagate intracellular signaling [5, 6]. If Dok7 cannot properly bind to phosphorylated MuSK, this can contribute to antibody pathogenicity. To assess how much Dok7 interacts with MuSK upon MuSK antibody-binding, endogenously expressed MuSK was immunoprecipitated and assessed for co-immunoprecipitation of Dok7. Inhibition of MuSK phosphorylation by monovalent MuSK antibodies resulted in less Dok7 bound to MuSK compared to their bivalent equivalents for the MuSK Ig-like 1 binding 13-3B5, 11-3F6, 11-3D9 and 13-4D3 clones (Figure 1D). No large differences were found in the recruitment of Dok7 to MuSK between for the 13-3D10 and mAb13 clones and between individual bivalent or monovalent anti-MuSK clones. To investigate if equal amounts of Dok7 was available for binding to MuSK in all conditions, we investigated total Dok7 levels in whole cell lysate (WCL). Bivalent 13-3B5 and 11-3F6 significantly reduced Dok7 protein levels compared to agrin with their monovalent equivalents (Figure 1E). In addition, agrin with 13-3B5 has significantly lower Dok7 levels compared to agrin with control antibody b12. Tendencies for lower Dok7 levels compared to agrin were also seen for some of the other, especially bivalent, MuSK clones.

A

| MuSK clone | MuSK domain | Functional Valency | *In vivo* muscle weakness |
|---|---|---|---|
| 13-3B5 | Ig-like1 | Bi | Slow[1] |
| | | Mono | Rapid[1] |
| 11-3F6 | Ig-like1 | Bi | No[1] |
| | | Mono | Rapid[1] |
| 13-3D10 | Ig-like1 | Bi | No[2] |
| | | Mono | n.a. |
| 11-3D9 | Ig-like1 | Bi | No[2] |
| | | Mono | n.a. |
| 13-4D3 | Ig-like1 | Bi | No[2] |
| | | Mono | n.a. |
| mAb13 | Fz | Bi | No[2] |
| | | Mono | n.a. |

[1]Vergoossen et al. 2021 PNAS
[2]Lim et al. in prep.

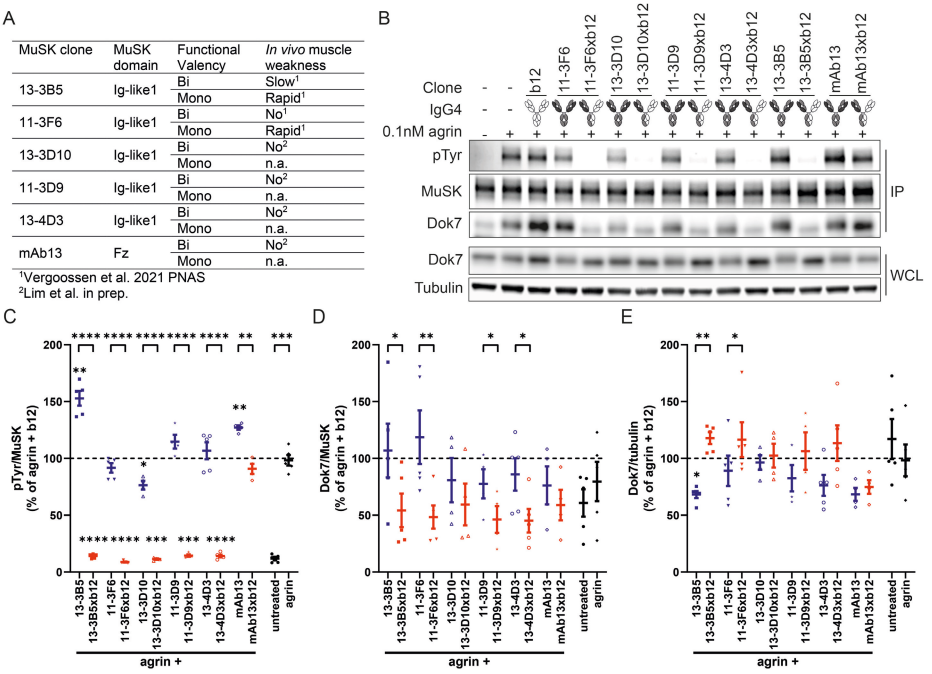


*Figure 1. MuSK antibodies differentially affect MuSK and Dok7 depending on valency and epitope. A. Panel of anti-MuSK clones and variants with antibody characteristics. B. Representative blots of MuSK phosphorylation signal and MuSK-Dok7 co-immunoprecipitation. Quantification of phosphorylated MuSK (C), Dok7 interacting with MuSK (D) and Dok7 levels in whole cell lysate (E) after 30 min exposure. Data represents mean and SEM. n=5 for 13-3B5(xb12), 11-3F6(xb12), 13-4D3(xb12) and agrin and n=4 for 11-3D9(xb12), 13-3D10(xb12) and mAb13(xb12). Paired t-test on log-transformed data with Benjamini-Hochberg false discovery rate correction. IP = immunoprecipitation, WCL = whole cell lysate.*

To further investigate the kinetics of how MuSK activation by bivalent MuSK antibodies or agrin influences Dok7, we measured Dok7 levels in whole cell lysate over time. Bivalent mAb13 reduced Dok7 most similarly to agrin, starting at 2 hours and continuing to 6 hours (Figure 2). In contrast, bivalent MuSK clones binding the Ig-like 1 domain already significantly reduced Dok7 levels after 30 minutes, but do not appear to further decrease Dok7 levels at later time points (Figure 2). Consistently, Dok7 levels upon 30-minute exposure to bivalent 13-3B5, 13-3D10 and 13-4D3 were significantly lower than mAb13 (Figure 2C). Bivalent 13-3B5 reduced Dok7 levels more than all other Ig-like 1 domain binding clones at 2 and 6 hours and compared to bivalent 11-3F6 at 30 minutes (Figure 2C-E). Bivalent mAb13 induced significantly lower Dok7 levels compared to the 13-3D10 and 13-4D3 clones at 6 hours (Figure 2E). Agrin induced significantly lower Dok7 levels compared to bivalent 13-3D10 at 6 hours,

but no other significant differences between agrin and the bivalent MuSK mAbs could be detected after correcting for multiple comparisons (Figure 2E). Since mAb13 binds the Fz domain and the 13-3B5 clone binds a non-overlapping epitope compared to the other Ig-like 1 domain binding clones, Dok7 levels appear to be affected differently by bivalent MuSK antibodies depending on antibody epitope [20].

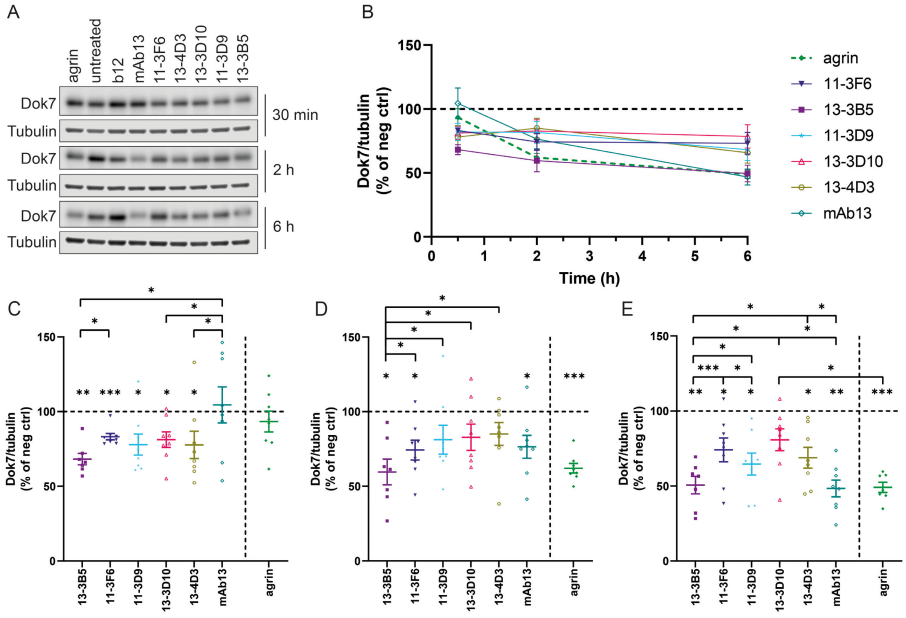


*Figure 2. Bivalent MuSK antibodies affect Dok7 levels differently over time depending on epitope and clone. A. Representative blots of Dok7 levels at 30 min, 2 h and 6 h. Quantification of Dok7 levels over time (B), after 30 min (C), 2 h (D) and 6h (E). Data represents mean and SEM and is normalized against b12 for MuSK mAbs, and against untreated for agrin. n=8 (except for 13-3B5 n=7) for 30 min and 2 h, n=8 (except for untreated, agrin and 13-3B5 n=7) for 6h. Paired t-tests on log-transformed data with Benjamini-Hochberg false discovery rate correction.*

### *MuSK antibodies do not deplete MuSK from the membrane of C2C12 myotubes*

To get more detailed understanding on what determines the pathogenicity of MuSK antibodies, we studied the 13-3B5 and 11-3F6 clones further. They differ in pathogenic capacity in bivalent form while binding the same domain of MuSK, preventing confounding effects of different target domains on MuSK [16]. Accelerated internalization is a common consequence of antigen-crosslinking by bivalent antibody binding. In addition, multiple receptor tyrosine kinases are known to rapidly internalize upon activation [25]. If MuSK is depleted from the membrane by antibody binding, this can contribute the pathogenicity

of MuSK antibodies. To investigate whether the amount of endogenously expressed MuSK on the membrane surface is affected by exposure to MuSK antibodies, we biotinylated and pulled down membrane surface proteins of C2C12 myotubes and probed this fraction for MuSK immunoreactivity. Neither monovalent nor bivalent MuSK antibodies binding the Ig-like 1 domain reduced the amount of surface MuSK after 30 minutes of exposure (Figure 3). Agrin, at the minimal dose for maximal activation (0.11nM) or a supramaximal dose (5nM), also did not reduce the amount of surface MuSK. Exposure to epidermal growth factor (EGF) for 15 minutes did significantly reduce the amount of surface EGF receptor in C2C12s, validating the method. Longer exposure to agrin or bivalent 13-3B5 also did not reduce endogenous MuSK on the surface membrane (Figure S3). Thus, the amount of MuSK on the membrane seems stable during active signaling and is not altered by exposure to these antibodies in C2C12 myotubes.

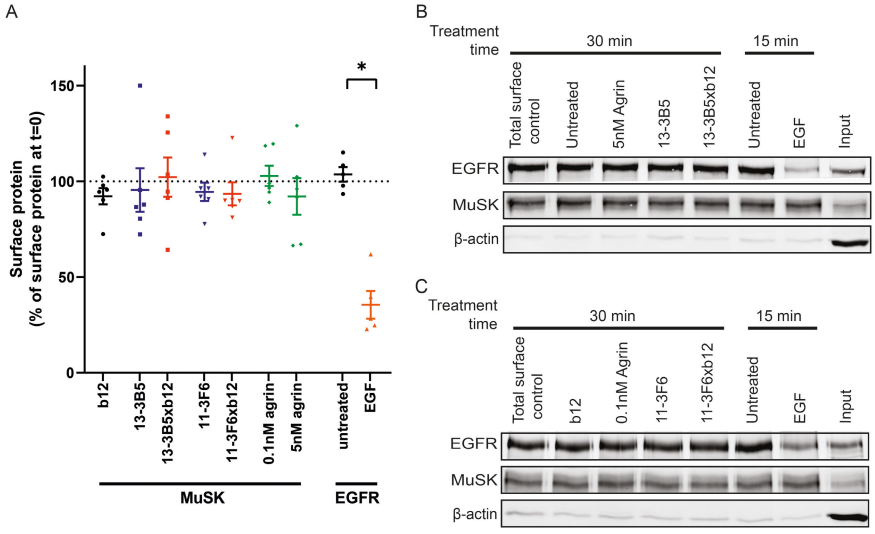


*Figure 3: MuSK surface depletion does not occur following exposure to monovalent or bivalent MuSK antibodies. A. Quantification of surface MuSK following 30 min exposure to monovalent or bivalent MuSK antibodies or agrin compared to t=0 in C2C12 myotubes (n=6). Significant surface depletion of the EGFR following EGF exposure could be detected with this method (n=5). Representative blots of surface MuSK and EGFR upon 13-3B5(xb12) (B) or 11-3F6(xb12) (C). Intracellular protein β-actin is not pulled down with this method. Data depicts mean ± SEM. Paired t-test on log-transformed data with Holm-Bonferroni correction.*

*Neuromuscular junction gene expression is differentially affected by monovalent and bivalent MuSK antibodies.*

Expression of synaptic genes for the specialized structure and function of the NMJ is tightly regulated and specific to subsynaptic nuclei in the synaptic region of muscles [7]. Disruptions in their expression may contribute to the pathomechanism of disease-causing MuSK antibodies. To investigate if altered NMJ gene expression may explain the pathogenic differences observed between MuSK antibodies, we measured RNA expression of NMJ genes with a direct link to MuSK signaling in masseter muscle of NOD/SCID mice exposed for 11 days or 3 weeks to bivalent or monovalent MuSK antibodies [16]. Briefly, monovalent MuSK antibodies to the Ig-like 1 domain caused severe myasthenic muscle weakness, lethal after 11 days (Figure 4A). In contrast, bivalent 11-3F6 did not cause overt muscle weakness after 3 weeks, while exposure to bivalent 13-3B5 resulted in subclinical myasthenic muscle weakness after 11 days progressing to lethal muscle weakness after 3 weeks of exposure [16].

Monovalent and bivalent MuSK antibodies caused different patterns of NMJ gene expression in the masseter muscle. In general, monovalent MuSK antibodies showed a (tendency to) decreased expression of a subset NMJ genes, while bivalent MuSK antibodies (a tendency to) increased expression of not exactly the same NMJ genes (Figure 4B). MuSK antibodies differentially affected the expression of *Lrp4*, *Chrne*, *Chrng*, *Colq* and *Ache* depending on valency. Most notably, monovalent MuSK antibodies strongly downregulated *Colq*, *Ache* and the epsilon subunit of the AChR (*Chrne*), while bivalent MuSK antibodies even after prolonged exposure did not (Figure 4G, J and K). In contrast, both bivalent MuSK antibodies strongly increased the expression of the gamma subunit of the AChR (*Chrng*), while this was not caused by exposure to monovalent MuSK antibodies at end-stage disease (Figure 4H). The alpha 1 subunit of the AChR (*Chrna1*) was only increased by bivalent 13-3B5 after 3 weeks of exposure (Figure 4F). Lastly, *Musk* is the only gene with a trend (p=0.07) to increased expression for all MuSK antibodies (both monovalent and bivalent) that caused a myasthenic phenotype (Figure 4D). Taken together, these data further support monovalent and bivalent MuSK antibodies cause myasthenic muscle weakness through different mechanisms.

MuSK-mediated signaling is involved in regulating NMJ-specific gene expression in subsynaptic nuclei [7]. To investigate if MuSK antibody binding directly affects (NMJ) gene expression in muscle, we measured the total RNA transcriptome of C2C12 myotubes exposed to agrin, bivalent 13-3B5, or agrin in combination with monovalent 13-3B5xb12 or 11-3F6xb12 for 16h, as at this time the full AChR clustering cascade is on.

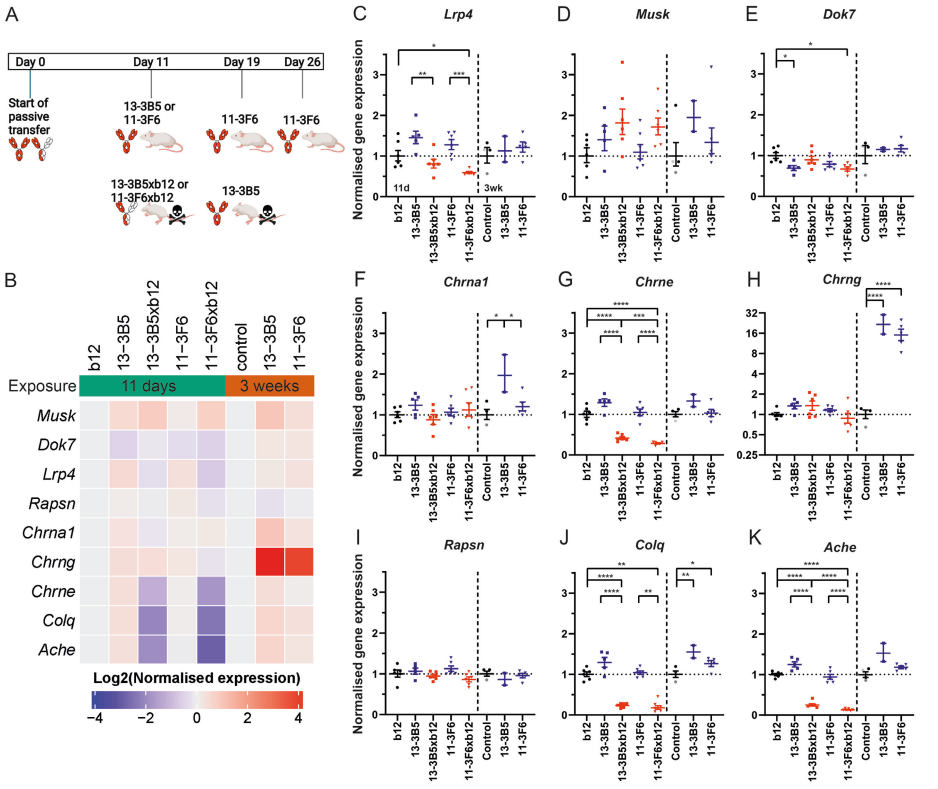


*Figure 4: Monovalent and bivalent MuSK antibodies have differential effects on NMJ gene expression in mouse muscle. A. Graphic summary of results and time frame of passive transfer experiments [16] B. Log2 normalised expression of NMJ genes in masseter muscle of NOD/SCID mice exposed to MuSK antibodies. Normalised gene expression of Lrp4 (C), Musk (D), Dok7 (E), Chrna1 (F), Chrne (G), Chrng (H), Rapsn (I), Colq (J), Ache (K) in masseter muscle of exposure to MuSK antibodies for 11 days (left of dotted line) or 3 weeks (right of dotted line). Data depicts geometric mean ± geometric SEM. 11 days: 2.5mg/kg b12, 13-3B5xb12, 11-3F6, 11-3F6xb12: n=6; 2.5mg/kg 13-3B5: n=5. 3 weeks: control is 10mg/kg b12 (black): n=2 combined with untreated or PBS-treated (grey): n=2; 10mg/kg 13-3B5: n=2; 10mg/kg 11-3F6: n=5. One-way ANOVA with Šidák-corrected comparisons for 11 day exposure. One-way ANOVA with Fisher's LSD test for 3 week exposure. Welch ANOVA when assumption of equal variance was not met. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0,0001*

No differentially expressed genes were found upon MuSK activation or MuSK-antibody binding (data not shown). To confirm we did not miss the gene expression window induced by MuSK signaling, we exposed C2C12s to agrin or bivalent MuSK antibodies for different durations between 30 minutes and 24 hours and measured the expression of *Chrne* and *Musk*, because their promotors have experimentally been shown to be directly regulated by MuSK signaling [26, 27]. MuSK activation by agrin or bivalent MuSK antibodies did not affect *Chrne* or *Musk* expression within

24 hours (Figure S4A and B). Neuregulin activates the other pathway regulating NMJ gene expression in muscles and did rapidly increase the expression of early growth response 3 (*Egr3*) and showed a tendency to increase *Chrne* expression after 24 hours (Figure S4A and C) [7]. This suggests gene expression changes relevant for the NMJ can be detected in C2C12 myotubes however, those mediated through MuSK may require more time. Exposure to MuSK activating compounds for more than 24 hours resulted in cell death, hindering the investigation of longer-term or indirect effects of MuSK signaling on gene expression. Taken together, the observed changes in NMJ gene expression in the masseter muscle are unlikely to be mediated by an acute effect of MuSK-antibody binding on MuSK signaling.

## Discussion

To further understand the mechanisms underlying MuSK MG, we tested how a panel of monovalent and bivalent (patient-derived) monoclonal MuSK antibodies affected MuSK-mediated signaling. Our data support the conclusion that the valency and epitope of MuSK antibodies have a significant effect on MuSK activation, Dok7 and NMJ gene expression.

MuSK antibodies binding the Ig-like 1 domain have valency-dependent effects on MuSK activation and Dok7. Monovalent MuSK antibodies inhibited agrin-induced MuSK phosphorylation and Dok7 binding to MuSK, while their bivalent equivalents induced these processes, confirming and extending on previous findings on MuSK phosphorylation [15-17]. These data support that the forced dimerization of MuSK by bivalent MuSK antibodies allows for Dok7 binding to the intracellular domain of MuSK. The expression of several NMJ genes was also differentially affected depending on MuSK antibody valency. Monovalent, but not bivalent, MuSK antibodies decreased the gene expression of *Colq*, *Ache* and *Chrne* and to a lesser extent *Lrp4*. In contrast, bivalent, but not monovalent, MuSK antibodies greatly increased *Chrng* expression, similar to what was seen upon active immunization with MuSK [28]. Murine antibody subclasses are not able to undergo Fab-arm exchange, therefore all antibodies in active immunization animal models are bivalent. Congruent with this, *Colq*, *Ache* and *Chrne* expression was not reduced upon MuSK active immunization [28]. Thus, the cellular consequences of monovalent and bivalent MuSK antibodies differ considerably also beyond the MuSK-induced AChR clustering pathway. Importantly, this emphasizes that active immunization and passive transfer studies of MuSK MG are models for considerably different aspects of the disease mechanisms in humans.

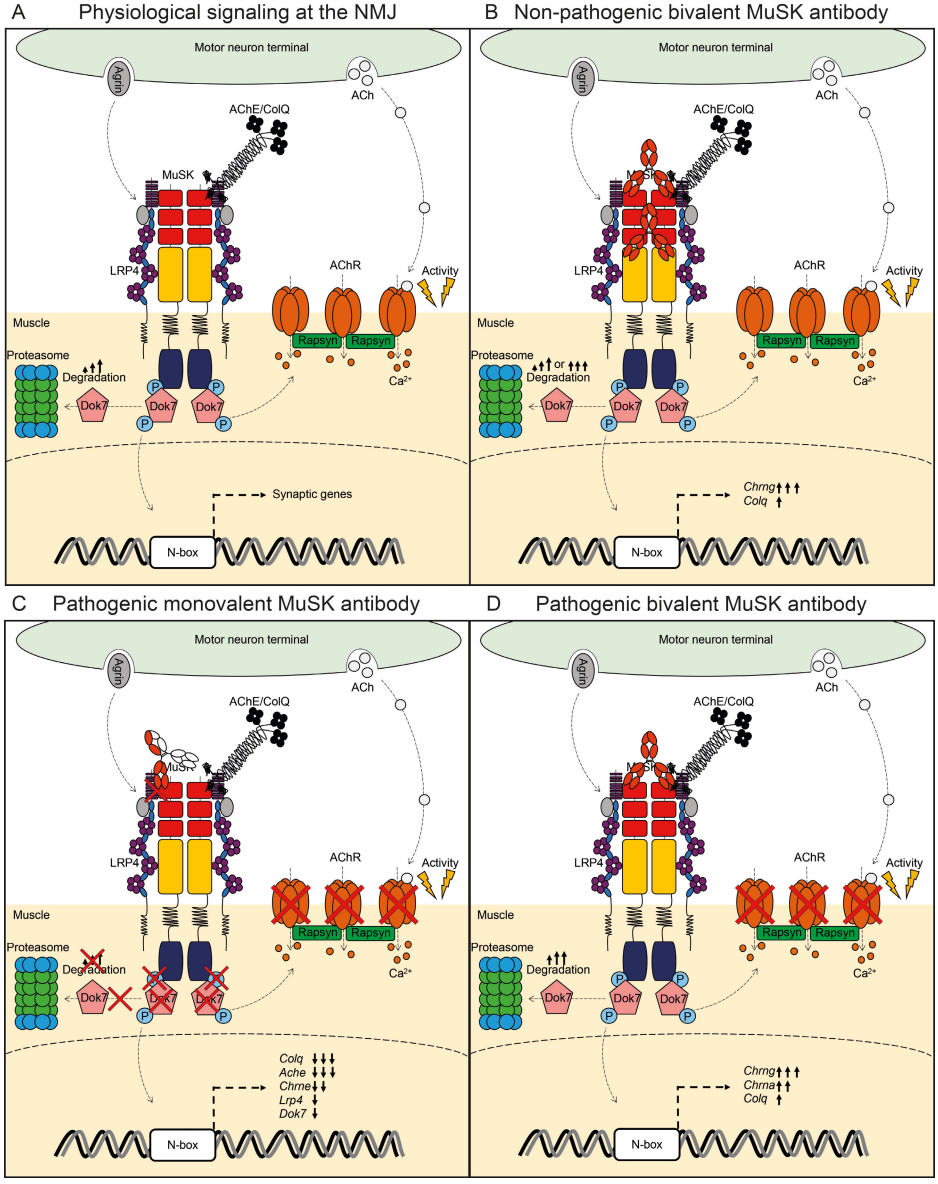


*Figure 5: Model of how MuSK antibodies with different functional characteristics impact signaling at the NMJ. A. During physiological signaling agrin-Lrp4 dimerizes and autophosphorylates MuSK. Dok7 binds to phosphorylated MuSK and is phosphorylated itself initiating a signaling cascade leading to clustering of AChR. In addition, MuSK binds ColQ-AChE complex, tethering it to the synapse, and MuSK is involved in the expression of synaptic genes. Activity-dependent degradation of Dok7 over time may be the break of this signaling cascade. B. Non-pathogenic bivalent MuSK antibodies have some effects on MuSK signaling, but these are not sufficient to cause dysfunction of neurotransmission. Pathogenic monovalent (C) and bivalent (D) MuSK antibodies have completely different effects on MuSK functioning, though both ultimately cause declustering of AChRs and myasthenic symptoms.*

The antagonistic effects of monovalent MuSK antibodies on agrin-induced MuSK signaling seem epitope-dependent. All monovalent anti-MuSK clones binding the Ig-like 1 domain inhibited agrin-induced MuSK phosphorylation, while monovalent mAb13xb12 binding to the Fz domain did not. Previously, monovalent Fabs of MuSK antibodies binding the Ig-like 2 domain fully inhibited agrin-induced AChR clustering, while their bivalent equivalents partially inhibited AChR clustering and did not inhibit agrin-induced MuSK phosphorylation, similar to Ig-like 1 domain binders [15, 16, 29, 30]. Polyclonal patient IgG4 mainly targets the N-terminal Ig-like 1 and Ig-like 2 domains of MuSK, is estimated to be up to 99% monovalent and also blocks agrin-induced MuSK activation and AChR clustering [11, 13, 18, 31]. Congruent with these *in vitro* effects, polyclonal patient IgG(4) and monovalent MuSK monoclonal antibodies targeting the MuSK Ig-like 1 domain cause myasthenia in experimental animals [16, 32, 33]. Monovalent Fz-domain binders are expected to not be pathogenic *in vivo* because they do not inhibit agrin-induced MuSK activation, indicating epitope-dependent antibody pathogenicity of monovalent MuSK antibodies.

Bivalent MuSK antibodies binding the Ig-like 1 and Fz domain appear to differentially regulate cellular Dok7 levels, despite similar effects on MuSK activation and Dok7 binding to MuSK. Upon agrin-induced signaling, Dok7 is ubiquitinated and degraded in the proteosome, reducing cellular Dok7 levels [17, 34]. This is thought to be a crucial negative feedback loop in regulating agrin-induced MuSK signaling. Bivalent MuSK antibodies to the Ig-like 1 domain appear to reduce Dok7 levels faster compared to bivalent mAb13 binding the Fz domain and agrin, suggesting Dok7 is more rapidly degraded. Less Dok7 thus seems to be available soon after initiation of MuSK activation, potentially quickly dampening further downstream signaling. In addition, bivalent 13-3B5 resulted in the largest reduction of Dok7 compared to the other Ig-like 1 domain binding clones at all time points. Since bivalent 13-3B5 binds a non-overlapping epitope on the Ig-like 1 domain compared to Ig-like 1 domain binders, this suggests that differences in epitope within a structural domain of MuSK can also have functional consequences [20]. In sum, these results support MuSK can be dimerized and activated differently depending on antibody-epitope between and within structural MuSK domains, influencing the kinetics of Dok7 degradation and potentially further downstream signaling.

These valency- and epitope-dependent effects on MuSK signaling confirm earlier studies that inhibition of agrin-induced MuSK activation, by blocking the interaction between Lrp4 and MuSK, is the driving

pathomechanism of monovalent MuSK antibodies (Figure 5) [13, 18, 35]. The strong reduction in *Chrne*, *Colq* and *Ache* expression levels by monovalent MuSK antibodies in addition suggests a production shortage of both mature AChRs and the ColQ-AChE complex. The latter may contribute to the deleterious effects of AChE inhibitors in MuSK MG patients and passive transfer models [36, 37]. Monovalent MuSK antibodies binding the Ig-like domain 1 may thus interfere with ColQ-AChE functioning by reducing the amount of the ColQ-AChE complex in the NMJ on one hand, and through inhibiting its tethering to the postsynaptic structure by blocking MuSK-ColQ interaction on the other hand [38]. The inhibition of MuSK thus has consequences throughout the NMJ that determine clinical symptoms and treatment response.

The driving mechanisms of pathogenic bivalent MuSK antibodies are still less clear. The strong upregulation of *Chrng* expression after 3 weeks may be a sign of presynaptic denervation of the NMJs [39, 40]. However, abnormalities in presynaptic morphology were not observed at 11 days (congruent with the lack of *Chrng* expression at that time), while postsynaptic pathology was already present in both bulbar and a limb muscle [16]. In addition, *Chrng* expression was also upregulated by non-pathogenic 11-3F6, suggesting any potential presynaptic pathology is unlikely to drive the pathogenicity of bivalent MuSK antibodies. The early and relatively large reduction of Dok7 levels in C2C12s by pathogenic 13-3B5 may indicate Dok7 is degraded too much too quickly upon activation, hampering further downstream signaling and supporting previous findings with polyclonal bivalent anti-MuSK IgG from rabbits (Figure 5) [17]. Fast depletion of Dok7 may contribute to the pathogenicity of bivalent MuSK antibodies, since Dok7 is essential for the activation of MuSK and proper downstream signaling [5, 17]. However, the differences between pathogenic 13-3B5 and the non-pathogenic bivalent MuSK antibodies or agrin were not all statistically significant (after correction for multiple comparisons) at the earliest time-point, and 13-3B5 and agrin are very similar at the later timepoints. The effects are thus time-dependent and the consequences for further downstream signaling should be investigated further to understand if Dok7 depletion contributes to mechanisms underlying pathogenic bivalent MuSK antibodies.

Endogenous MuSK did not deplete from the surface membrane after either monovalent or bivalent MuSK antibody binding or activation in C2C12s. Recently, agrin caused a slow decrease leading to ~30% loss of surface MuSK when protein synthesis was inhibited [41]. Protein synthesis was not inhibited in our assay. Thus, continuous synthesis and integration of

MuSK into the membrane may compensate this loss of MuSK, stabilizing the amount of MuSK on the membrane surface. This may explain why we could not reproduce loss of surface MuSK observation, despite using similar experimental settings. Gemza et al. furthermore elegantly discuss this slow rate of MuSK endocytosis in relation to previous studies and other receptor tyrosine kinases may be due to 1) the indirect activation of MuSK through Lrp4 and Dok7 and 2) anchoring of MuSK in the dense cytoskeletal structure of the post-synapse [25, 41, 42]. Furthermore, internalization of exogenous MuSK by MuSK patient-purified IgG(4) has not been consistently found [18, 43]. This may depend on the activating vs inhibiting properties of the material used and/or overestimation of internalization due to overexpression of MuSK and/or the absence of the relevant muscle-specific cytoskeletal structure. Taken together, rapid antibody-mediated or activity-dependent surface depletion of MuSK is unlikely to be a major part of the mechanism of either monovalent or bivalent MuSK antibody pathogenicity in muscle.

Of note, MuSK signaling or MuSK antibodies also did not directly alter (NMJ) gene expression in C2C12 myotubes. Previously, agrin-induced signaling was found to activate the *Musk* promotor directly through MKK7, JNK and GABP signaling [26]. In that study, agrin was coated onto the culture dish in combination with laminin from the beginning of myotube differentiation, as opposed to supplied in the media to differentiated myotubes. This suggests that this signaling pathway is not directly activated upon agrin-induced MuSK activation but requires longer. This idea is supported by the absence of MKK7, JNK and GABP phosphorylation 2 hours after stimulation, when MuSK, Dok7 and AChR phosphorylation are already present [44]. Although, MuSK antibodies affect NMJ gene expression in the masseter muscle at end-stage disease, the signaling responsible for this may require longer and be more complex downstream of MuSK activation.

Taken together, MuSK MG patients have a polyclonal antibody response resulting in a mixture of MuSK antibodies against different epitopes, and of subclasses differing in valency and complement activating capacity. The net effect of MuSK autoantibodies in individual MuSK MG patients will be determined by the unique composition of MuSK antibodies and their characteristics, in combination with their relative titres. It will be interesting to get a more in dept view on the heterogeneity and clonality of the MuSK autoantibody response in individual patients and to study if these characteristics can explain the severity of the clinical symptoms.

## Methods

*mAb production and cFAE*

Anti-MuSK clones binding to the Ig-like domain 1 of MuSK were previously isolated from a MuSK MG patient [15]. Together with mAb13 as a MuSK Fz domain binder, these clones were produced in an IgG4 Fc tail with the S228P amino-acid change and their original light chain (GeneArt, [16, 21, 22]). The b12 antibody suitable for cFAE was used as an exchange partner and control antibody [16]. Recombinant bivalent and monovalent monoclonal antibodies (mAbs) were produced, quantified and assessed on quality as described previously [15, 16]. Recombinant antibodies 11-3F6, 13-3B5 and b12 were produced in CHO cells. The other MuSK mAbs were produced in HEK cells.

*C2C12 culturing and treatment conditions*

C2C12 myoblasts were obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany), tested for mycoplasma contamination and maintained for maximum 5 passages after thawing. Myoblasts were grown in proliferation medium (DMEM Glutamax (10566016, Thermo Fisher) supplemented with 10% FBS (S1810-500, Biowest) and 1% penicillin/streptomycin (15140122, Gibco). Cells were plated at $1.25e^4$ – $2e^4$ cells per $cm^2$ in proliferation medium. Once cells reached 90 – 95 % confluency, differentiation was induced by DMEM Glutamax supplemented with 2% HS and 1% penicillin/streptomycin, refreshed every 2-3 days. Experiments were done on day 5 of differentiation. All mAbs were used at 7.7nM, neural agrin (550-AG-100, R&D systems) at 0.1nM, neuregulin (396-HB-050, R&D systems) at 4.9nM, EGF at 200ng/mL (236-EG-200, R&D systems), unless otherwise specified.

*MuSK immunoprecipitation*

MuSK was immunoprecipitated (IP) as described previously [16]. Briefly, differentiated C2C12 myotubes were cultured in 10cm dishes, treated for 30 min and lysed in phosphate lysis buffer (30 mM triethanolamine, 1% NP 40, 50mM NaF, 2mM sodium orthovanadate, 1mM sodium tetrathionate, 5 mM EDTA, 5 mM EGTA, 1mM N-ethylmaleimide, 50mM NaCl, 1× protease inhibitor cocktail, 1× phosphatase inhibitor cocktail). Lysates were cleared by centrifuging for 20 min at 5.000 g, adjusted to ensure equal protein input over all conditions and combined with 1µg/sample 11-3F6 IgG1 to IP MuSK. Antibody-protein complexes were captured with protein a agarose beads (11134515001, Roche) and eluted with 40-50µL 2x sample buffer (40mM Tris-HCl pH6.8, 3.3% SDS, 16.5% glycerol, 0.005% Bromophenol blue, 0.2M DTT) and incubated at 95 degrees for 5 min.

*Protein isolation*

To assess Dok7 levels, C2C12 myotubes were cultured in 6-well plates. After treatment, myotubes were harvested in ice-cold PBS and stored as cell pellets at -80 prior to protein isolation. Pellets were lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1x protease inhibitor cocktail) and cleared by centrifuging for 20 min at 5.000 g. Protein content of lysate was determined using BCA protein assay kit (23225, Thermo Scientific) and used to prepare samples with equal protein content in sample buffer.

*Surface depletion assay*

Immediately after treatment exposure, cells were put and kept on ice in the cold room (4°C) until lysis. Cells were thoroughly washed with ice cold $PBS^{2+}$ (1.5mM $MgCl_2$; 0.2mM $CaCl_2$ in PBS, pH 7.4). Freshly prepared 1mg/mL Sulfo-NHS-SS-Biotin (PG82077, Thermo Fisher) in $PBS^{2+}$ was added to each plate and incubated shaking vigorously (~300 rpm) for 30 min. Unbound Sulfo-NHS-SS-Biotin was washed away with quenching buffer (100mM Glycine in $PBS^{2+}$) by rinsing three times and incubating shaking for 2x 15 minutes. All cells were washed three times with ice cold $PBS^{2+}$before being lysed with RIPA buffer (10 mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% SDS; 1.0% triton X-100; 1.0% sodium deoxycholate, 1x protease inhibitor cocktail). Lysates of all samples were adjusted to ensure equal protein input over all conditions based on BCA quantification. Adjusted lysates were incubated with streptavidin beads (20349, Thermo Scientific) rotating ON (4°C). Protein was eluted from the beads using 2x sample buffer.

*Western blotting*

Protein samples were ran on SDS-PAGE gel and transferred to PDVF membrane. Western blot conditions for different samples types can be found in Table S1. Chemiluminescence was measured on the Amersham Imager 600 (Cytiva). Immunofluorescence was measured with the Odyssey (Licor).

*RNA isolation*

C2C12 myotubes or frozen muscle tissue were lysed and homogenized in QIAzol lysis reagent (Qiagen). Total RNA was extracted and purified with the miRNeasy Mini Kit according to manufacturer's instructions (Qiagen, 1038703). RNA was treated with DNase (Qiagen) on column for 30 min at room temperature. RNA concentration was quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

*Quantitative Real-time PCR*

First strand cDNA was synthesized from 1000-3000 ng total RNA with the RevertAid H Minus First Strand cDNA Synthesis kit using oligo(dT) primers (Thermo Fisher Scientific, K1632). Relative gene expression levels were determined with iQ SYBR Green Supermix (Bio-Rad, #1708886) and 1 pM forward and reverse primers (Table S2) on CFX384 Touch Real-Time PCR Detection System (Bio-Rad) with the following program: 95°C for 3 min, 40 cycles of 10 s at 95°C and a melting temperature of 60°C for 30 s, followed by a melting curve analysis from 65°C to 95°C (temperature increments of 0.5°C). Quantification cycle (Cq) values were obtained from CFx manager or maestro software (BioRad). All samples were run in triplicate and *Gapdh* and *Rpl13a* were used as housekeeping genes. Technical replicates that differed >0,5 in Cq from the others in the triplicate were excluded. Normalized fold changes were calculated compared to untreated or b12 using the validated efficiency of each primer (Table S2).

*Bulk RNA sequencing and analysis*

Total RNA integrity of the 16 h-treated C2C12 myotube samples was analyzed with the Agilent BioAnalyzer RNA Nano 6000 chip and all had an RNA Integrity Number of > 9.5 (Agilent Technologies, Amstelveen, the Netherlands). The library was prepared with the TruSeq Stranded Total RNA with Ribo-Zero H/M/R kit and 30 million reads were sequenced with the NovaSeq 6000 PE 150 system (Illumina) by Macrogen. Reads were trimmed and quality filtered by TrimGalore (v.0.6.6, Cutadapt v.2.10), using default parameters to remove low-quality nucleotides. Consequently, reads were mapped to the Genome Reference Consortium Mouse Build 38, using STAR Aligner (v.2.7.6a). A gene expression counts table was generated with HTSeq (v.0.12.4). Data were normalized for sequencing depth with the median of ratios method and consequently analyzed for differential expression in the DESeq2 R package (v.1.32.0). Genes with an adjusted p-value < 0.05 (Benjamini-Hochberg) were considered significant.

*Statistics*

Statistical analyses were done in GraphPad Prism software (version 9.3.1). Which statistical test was used per experiment is described in the figure legends. Data is presented as (geometric) mean with (geometric) standard error of the mean. p values <0.05 were considered significant. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

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## Supplementary information

A

| Clone |  |  |  |
|---|---|---|---|
| 13-4D3 | 2.1% | 82.2% | 15.7% |
| 13-3D10 | 1.1% | 97.1% | 1.8% |
| mAb13 | 1.7% | 97.0% | 1.1% |

B

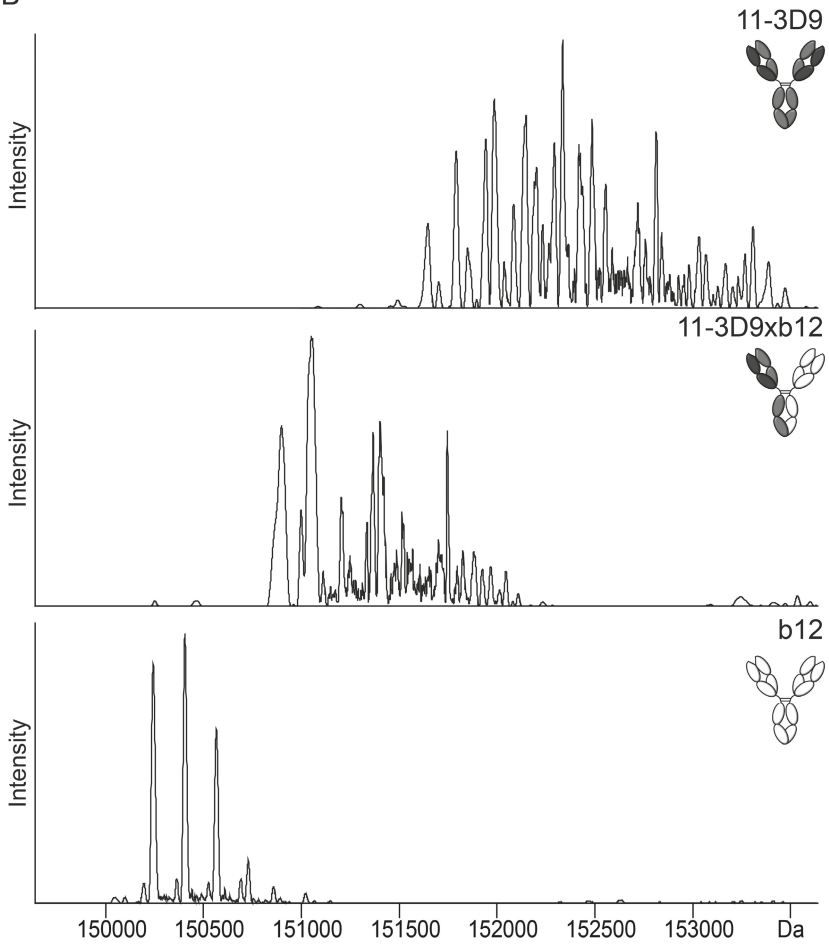


*Figure S1: Exchange efficiency of monovalent MuSK antibodies. A. Relative amounts of bivalent monospecific and monovalent bispecific antibody variants after the controlled Fab-arm exchange (cFAE) reaction measured by capillary electrophoresis. B. Deconvoluted mass spectra show generation of monovalent bispecific 11-3D9xb12 after cFAE.*

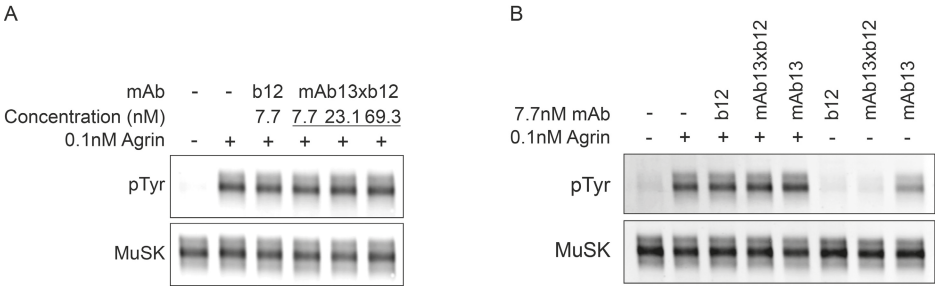


*Figure S2: Monovalent mAb13xb12 does not inhibit agrin-induced MuSK phosphorylation. A. Agrin-induced MuSK phosphorylation in combination with increasing concentrations of mAb13xb12. B. MuSK phosphorylation upon addition of monovalent mAb13xb12 or bivalent mAb13 in the absence or presence of agrin.*

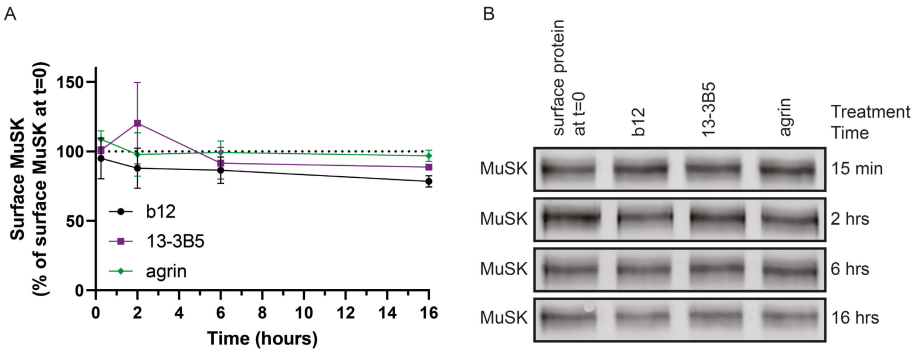


*Figure S3: Longer exposure to agrin or bivalent 13-3B5 does not reduce surface MuSK. A. Surface MuSK does no significantly differ over time upon exposure to agrin or bivalent 13-3B5. B. Representative blot of surface MuSK after 15 min, 2 h, 6 h or 16 h exposure. Data represent mean ± SEM over n=2 (15min, 2h and 6h) or n=4 (16h) experiments.*

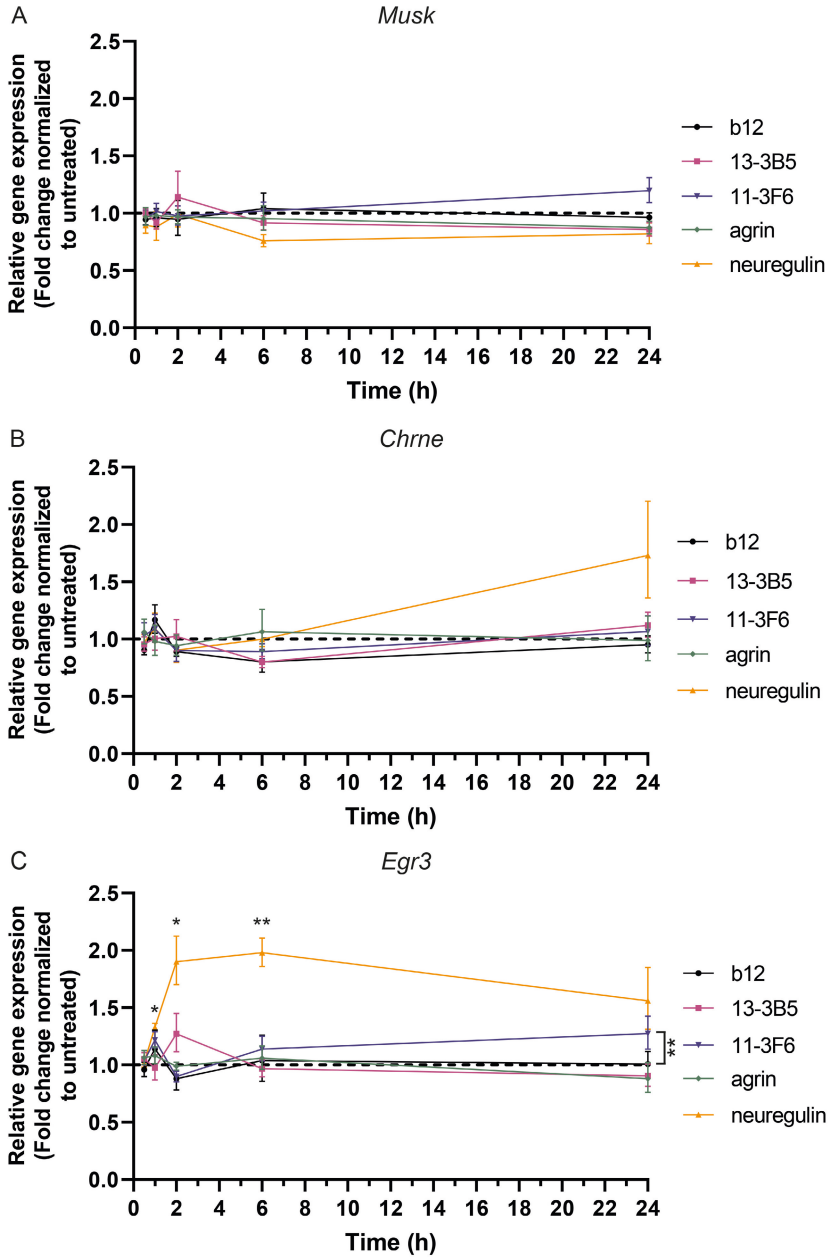


*Figure S4: MuSK signalling does not directly induce gene expression of Musk or Chrne in C2C12 myotubes. Normalised gene expression of Musk (A), Chrne (B) and Egr3 (C). Egr3 expression is regulated by neuregulin and serves as a positive control for the method. 30 min (n=5), 60 min (n=4), 2 h (n=5), 6 h (n=5) and 24 h (n=6). Data depicts geometric mean ± geometric SEM. Paired t-test on log2-transformed data with Benjamini-Hochberg false discovery rate correction. *p < 0.05, **p < 0.01.*

*Table S1: Antibody conditions for western blot*

| Antigen | Block | Antibody buffer | Primary antibody | Secondary antibody |
|---|---|---|---|---|
| MuSK IP samples | | | | |
| MuSK | 3% BSA | Immunobooster | AF562<br>R&D systems<br>0.2µg/mL | 926-32214<br>Licor<br>0.2 µg/mL |
| Phospho-MuSK | 3% BSA | Immunobooster | 05-321<br>Millipore<br>1µg/mL | 926-68072<br>Licor<br>0.2 µg/mL |
| Dok7 | 3% BSA | 0.5% BSA | AF6398<br>R&D systems<br>0.5µg/mL | 205-032-176<br>Jackson<br>ImmunoResearch<br>1:10.000 |
| Whole cell lysate | | | | |
| Dok7 | 5% milk | 2% milk | AF6398<br>R&D systems<br>0.5µg/mL | 205-032-176<br>Jackson<br>ImmunoResearch<br>1:10.000 |
| Tubulin | 5% milk | 2% milk | T6199<br>Sigma-Aldrich<br>0.2µg/mL | 926-68072<br>Licor<br>0.2 µg/mL |
| Surface depletion assay | | | | |
| MuSK | Odyssey Blocking Buffer | Immunobooster | AF562<br>R&D systems<br>0.2µg/mL | 926-32214<br>Licor<br>0.1 µg/mL |
| EGFR | Odyssey Blocking Buffer | Immunobooster | 51091-T52<br>Bioconnect<br>1:1000 | 926-32213<br>Licor<br>0.1 µg/mL |
| β-actin | Odyssey Blocking Buffer | Immunobooster | ab8226<br>Abcam<br>0.2µg/mL | 926-68072<br>Licor<br>0.1 µg/mL |

*Table S2: Primers with validated amplification efficiency in C2C12 myotubes and NOD/SCID masseter muscle*

| Gene | Accession number | Primer sequence | Amplification size (bp) | Amplification efficiency (%) C2C12 | Correlation coefficient ($R^2$) C2C12 | Amplification efficiency (%) Masseter | Correlation coefficient ($R^2$) Masseter |
|---|---|---|---|---|---|---|---|
| *Gapdh* | MGI:95640 | Fw: TCCATGACAACTTTGGCATTG<br>Rv: TCACGCCACAGCTTTCCA | 103 | 104,3 | 0,998 | 108,8 | 0,996 |
| *Rpl13a* | MGI:1351455 | Fw: TGCTGCTCTCAAGGTTGTTC<br>Rv: TTCTCCTCCAGAGTGGCTGT | 114 | 98,1 | 1,000 | 104,9 | 0,994 |
| *Egr3* | MGI:1306780 | Fw: CTGACAATCTGTACCCCGAGGA<br>Rv: GCTTCTCGTTGGTCAGACCGAT | 129 | 105,2 | 0,997 | | |
| *Musk* | MGI:103581 | Fw: AACCCCAAACCATCTGTGTC<br>Rv: GTCCTGCATCTTCCTTTTGC | 121 | 101,1 | 0,991 | 104,6 | 0,991 |
| *Chrne* | MGI:87894 | Fw: GAACTCGTGTTTGAGGGTCAG<br>Rv: TCAGCCACAAAGTTCACAGC | 125 | 103,8 | 0,993 | 96,9 | 0,994 |
| *Chrna1* | MGI:87885 | Fw: AAGCACCCTGAGGTGAAAAG<br>Rv: CCATCACCATGGCAACATAC | 118 | | | 96,6 | 0,992 |
| *Chrng* | MGI:87895 | Fw: ATCGTCGTGAACTCTGTGGT<br>Rv: CCTTCCTCTCGAGCCATGAT | 215 | | | 98,1 | 0,983 |
| *Lrp4* | MGI:2442252 | Fw: ATGGGTCTATGCGGAAAGTG<br>Rv: CGCTCTAATTTGGCGTTCTC | 121 | | | 103,4 | 0,991 |
| *Dok7* | MGI:3584043 | Fw: TCAGCCTCAGAAGAGCGTGTTG<br>Rv: GCCTCAGAAGAGGAACTGGATAG | 137 | | | 108,7 | 0,986 |
| *Colq* | MGI:1338761 | Fw: TGTGGTCAACAACCAGGAAG<br>Rv: AAAGATCGCTGGTCTCTTCG | 78 | | | 100,4 | 0,991 |
| *Ache* | MGI:87876 | Fw: GGGCTCCTACTTTCTGGTTTACG<br>Rv: GGGCCCGGCTGATGAG | 71 | | | 102,4 | 0,997 |
| *Rapsn* | MGI:99422 | Fw: AGGCTGGAGCCTCAAATATC<br>Rv: AGGGCAATCTTCATGGACTC | 117 | | | 107,4 | 0,997 |

# 6

# Timing and localization of myasthenia gravis-related gene expression

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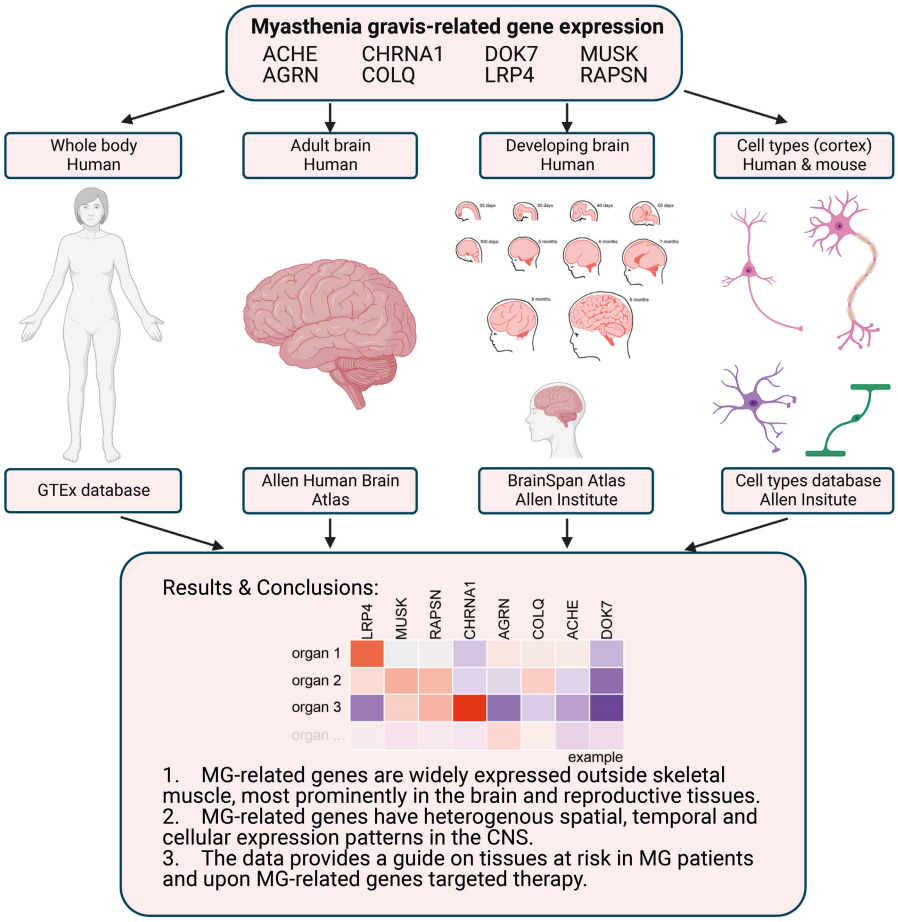
## Abstract

Myasthenia gravis (MG) is an acquired autoimmune disorder caused by autoantibodies binding acetylcholine receptors (AChR), muscle-specific kinase (MuSK), agrin or low-density lipoprotein receptor-related protein 4 (Lrp4). These autoantibodies inhibit neuromuscular transmission by blocking the function of these proteins, and thereby cause fluctuating skeletal muscle weakness. Several reports suggest that these autoantibodies might also affect the central nervous system (CNS) in MG patients. A comprehensive overview of the timing and localization of the expression of MG-related antigens in other organs is currently lacking. To investigate the spatio-temporal expression of MG-related genes outside skeletal muscle, we used *in silico* tools to assess public expression databases. Acetylcholine esterase, nicotinic AChR a1 subunit, agrin, collagen Q, downstream of kinase-7, Lrp4, MuSK and rapsyn were included as MG-related genes because of their well-known involvement in either congenital or autoimmune MG. We investigated expression of MG-related genes in 1) all human tissues using GTEx data, 2) specific brain regions, 3) neurodevelopmental stages, and 4) cell types using datasets from the Allen Institute for Brain Sciences. MG-related genes show heterogenous spatio-temporal expression patterns in the human body as well as in the CNS. For each of these genes several (new) tissues, brain areas and cortical cell types with (relatively) high expression were identified suggesting a potential role for these genes outside skeletal muscle. The possible presence of MG-related antigens outside skeletal muscle suggests that autoimmune MG, congenital MG or treatments targeting the same proteins may affect MG-related protein function in other organs.


Myasthenia gravis-related gene expression
ACHE CHRNA1 DOK7 MUSK
AGRN COLQ LRP4 RAPSN
Whole body Human
Adult brain Human
Developing brain Human
Cell types (cortex) Human & mouse
GTEx database
Allen Human Brain Atlas
BrainSpan Atlas Allen Institute
Cell types database Allen Insitute
Results & Conclusions:
LRP4
MUSK
RAPSN
CHRNA1
AGRN
COLQ
ACHE
DOK7
organ 1
organ 2
organ 3
organ ...
example
1. MG-related genes are widely expressed outside skeletal muscle, most prominently in the brain and reproductive tissues.
2. MG-related genes have heterogenous spatial, temporal and cellular expression patterns in the CNS.
3. The data provides a guide on tissues at risk in MG patients and upon MG-related genes targeted therapy.

## Introduction

Myasthenia gravis (MG) is an antibody-mediated autoimmune disorder hallmarked by fatigable skeletal muscle weakness. This muscle weakness results from autoantibodies targeting essential proteins at the neuromuscular junction (NMJ). Till date, four antigens have been described: acetylcholine receptors (AChR), muscle-specific kinase (MuSK), low-density lipoprotein receptor-related protein 4 (Lrp4) and agrin [1]. These proteins converge on a single pathway essential for establishing and maintaining NMJs and facilitating neuromuscular transmission [2]. Consequently, binding of autoantibodies interferes with the function of these proteins, resulting in failure of neuromuscular transmission and subsequent muscle fatigue and paralysis.

Even though most knowledge on these proteins relates to their function in the NMJ, they are also expressed in tissues other than skeletal muscle, like retina, lung and brain [2-5]. Insight in the localization of expression of these genes/proteins and their roles in other organs is important because: 1) Other tissues may also be affected by the autoantibodies in MG patients and 2) new therapeutic strategies targeting these MG-related genes/proteins are emerging and therefore knowledge on their localization may identify potential off-target effects. A comprehensive overview of MG-related gene expression in different human tissues however is lacking.

Evidence of other organs being affected by MG autoantibodies is mostly focused on the central nervous system (CNS). The non-motor symptoms include pain, cognitive dysfunction, fatigue and sleep disturbances [6-10]. Although AChR MG serum antibodies were reported not to bind neuronal AChR [11] and immunostaining with MuSK autoantibodies seems challenging on brain sections (personal observation), passive transfer of patient-derived MuSK and AChR antibodies resulted in behavioural deficits and EEG abnormalities in mice [12, 13]. Moreover, AChR autoantibodies have been detected in cerebrospinal fluid [14-16]. Mutations in NMJ genes may furthermore result in congenital myasthenic syndrome (CMS) and sometimes give CNS abnormalities [17]. Although CNS defects are not at the foreground of clinical symptoms in MG patients, these observations suggest that autoantibodies may have detrimental effects in the CNS when they are able to cross the blood-brain barrier.

If indeed MG-related autoantibodies are able to affect their antigens in other organs several important questions arise: e.g. 1) do these proteins, like in the NMJ, converge on a similar pathway in these organs and 2)

which cells are responsible for this expression. We therefore investigated the spatio-temporal expression patterns of MG-related genes in healthy human tissues, with a focus on the CNS, using a range of publicly-available expression databases. We selected the four NMJ genes which encode known antigens for autoantibodies in MG (*AGRN, CHRNA1, LRP4, MUSK)* and four other NMJ genes involved in maintaining NMJ neurotransmission and where mutations can cause CMS (acetylcholine esterase (*ACHE),* collagen Q *(COLQ),* downstream of kinase-7 *(DOK7),* and rapsyn *(RAPSN)*) (Table 1) [17]. We will refer to these eight genes and their gene products as MG-related genes.

*Table 1. The MG-related genes, their function at the NMJ and association with autoimmune MG or CMS [1, 2, 17, 47].*

| Gene symbol | Encoded Protein | Function at NMJ | Role in CMS or autoimmune MG |
|---|---|---|---|
| *ACHE* | Acetylcholine esterase | Breaks down the neurotransmitter acetylcholine (ACh), thereby halting neuromuscular transmission and muscle contraction. | 12.53% of CMS patients have a AChE deficiency. AChE is the main target for symptomatic treatment of autoimmune MG patients. |
| *AGRN* | Agrin | Trophic signalling molecule released by the presynaptic motor nerve terminal to induce and maintain post-synaptic differentiation. Agrin lingers in the basal lamina, can bind Lrp4 and thereby activates MuSK and AChR clustering. | Mutations in *AGRN* are a rare cause of CMS (0.58%). Autoantibodies to agrin are thought to cause autoimmune MG in a small subset of patients. |
| *CHRNA1* | Nicotinic acetylcholine receptor α subunit | Muscle-specific subunit of the AChR required for neuromuscular transmission and location for ACh binding. Upon binding of ACh to the AChR, its ion channel opens which depolarizes the muscle end plate, and may induce an action potential and muscle contraction. | ~50% of CMS patients have an AChR deficiency caused by mutations in one of the AChR subunits leading to kinetic defects and myasthenic symptoms. ~80% of autoimmune MG patients have autoantibodies to the AChR. The α subunit contains the main immunogenic region. |
| *COLQ* | Collagen Q | Collagenic subunit anchoring AChE to the basal lamina and thereby responsible for ACh breakdown. Also interacts with MuSK. | The pathomechanism of AChE and MuSK CMS as well as autoimmune MG are known to affect ColQ function. |

*Table 1. Continued*

| Gene symbol | Encoded Protein | Function at NMJ | Role in CMS or autoimmune MG |
|---|---|---|---|
| *DOK7* | Downstream of kinase 7 | Cytoplasmic adaptor of MuSK required for regulating the kinase activity of MuSK, subsequent AChR clustering and NMJ formation. | 9.75% of CMS patients have a mutation in *DOK7*. *DOK7* gene therapy rescued the phenotype of a Dok7 CMS mouse model. |
| *LRP4* | low-density lipoprotein receptor-related protein 4 | The agrin receptor that directly interacts with MuSK and further activates AChR clustering and postsynaptic differentiation. Lrp4 is also critical for presynaptic differentiation. | 0.56% of CMS patients have mutations in *LRP4*. 1-2% of MG patients have Lrp4 autoantibodies. MuSK autoantibodies inhibit MuSK-Lrp4 interaction, obstructing normal trophic signalling at the NMJ and induce myasthenia. |
| *MUSK* | Muscle-specific kinase | Orchestrates anterograde trophic signalling at the NMJ, resulting in AChR clustering. It is also required for retrograde signalling towards presynaptic differentiation of the motor neuron end plate. | 0.28% of CMS patients have a mutation in *MUSK*. Autoantibodies to MuSK cause autoimmune MG in 5-8% of patients. |
| *RAPSN* | Rapsyn | Cytoplasmic anchor that facilitates AChR clustering. | 14.21% of CMS patients have a rapsyn deficiency. |

## Materials and methods

### *Genotype-Tissue expression consortium (GTEx)*

The expression of MG-related genes was analysed across 54 non-diseased human tissues using RNA sequencing data of the nearly 1000 individuals from the open access Genotype-Tissue expression consortium (GTEx) version 8. For *ACHE* (ENSG00000087085) and *AGRN* (ENSG00000188157), all isoforms were annotated using the Ensembl database. The median gene-level transcripts per million (TPM) for all MG-related genes were downloaded per tissue. Because it is known that *ACHE* and *AGRN* produce different splice variants, of which only one is relevant for NMJs, *ACHE-207* (ENST00000428317.5) and *AGRN-208* (ENST00000620552.4) isoform data were downloaded separately. The anterior cingulate cortex, frontal cortex and cerebellar hemisphere were removed because they were covered in the cortex or cerebellum respectively. EBV-transformed lymphocytes and cultured fibroblasts were removed, because they do not naturally occur in healthy humans. Tissues (rows) and genes (columns)

were hierarchically clustered using hclust function in R with Eucledian distance and average linkage. Qualitative expression levels are used as follows: low (Log10 <1), moderate (Log10 1-1.5), high (Log10 >1.5).

*Allen Human Brain Atlas*

The Allen Human Brain Atlas (AHBA) includes anatomically-mapped Oligo-dT primed microarray data of 3,702 samples from six neurotypical individuals (5 males and 1 female, mean age 42, range 24–57 years) [18]. The expression of the MG-related genes was mapped across regions of the human brain using BrainScope [19]. The MG-related gene-probes used are marked in Supplemental Table 1. All probes were confirmed to align to their respective MG-related gene using the Ensembl database. *AGRN* probe A_24_P358462 (#) detected the NMJ-specific *AGRN-208* isoform, able to induce AChR clustering and clusters, separately from the probe used in the other analyses (*) (Supplemental Fig. 3a) [20]. For *ACHE*, the available probe did not selectively bind to *ACHE-207*. The expression of MG-related genes was downloaded from http://human.brain-map.org/. Log2-transformed expression values were converted into z-scores normalized per donor. The median z-score of each gene was taken across the set of 22 non-overlapping brain regions. Finally, the median z-score of every MG gene across the six donors was taken. Hierarchical clustering was done as described above. The nature of this data only allowed comparison of z-scores across anatomical brain areas within one gene, which is why the term relative expression is used. Qualitative expression levels are used as follows: low (Z < 0), moderate (Z 0-1), high (Z >1).

*BrainSpan atlas of the developing human brain*

The BrainSpan atlas of the developing human brain includes 42 healthy human brains, ranging in age from 8 weeks post-conception to 40 years old, from which a total of 524 anatomically annotated samples were taken [21]. Gene expression was determined using RNA sequencing and visualized using BrainScope [19]. *ACHE-207* and *AGRN-208* isoforms could not be distinguished in this dataset. MG-related genes *CHRNA1*, *DOK7*, *MUSK* and *RAPSN* were not present in BrainScope, since genes with RPKM-value above 1 in less than 20% of all samples were removed. For those genes, we used the brain-map.org portal to plot their expression (Supplemental Fig. 4).

*Single-cell analyses*

Single-nucleus RNA sequencing (snRNA-seq) data from multiple human cortical regions and single-cell RNA sequencing data from the whole mouse cortex were downloaded from the Allen Institute (https://portal.brain-map.org/atlases-and-data/rnaseq). For the human dataset, the donors included 4 males and 4 females (age 24-66) without a history of neuropsychiatric or neurological conditions [22]. From 4 donors, multiple cortical areas were sampled post-mortem and from 4 donors the medial temporal gyrus was removed during neurosurgery. For the mouse dataset, 538 animals were used from multiple transgenic lines to enrich for rare cell types, all on a C57BL/6J background [23].

Sample processing and analysis methods have been described previously [22, 23]. Briefly, the SMART-seq method yielded transcriptome profiles for 10,708 glutamatergic neurons, 4,297 GABAergic neurons and 923 non-neuronal cells for the human dataset and 40,276 glutamatergic neurons, 22,573 GABAergic neurons and 1,958 non-neuronal cells for the mouse dataset. The trimmed-mean gene expression (counts per million reads mapped (CPM)) data per cluster was downloaded for 8 MG-related genes and grouped in the cell type subclasses defined in the sampling strategy. In the mouse dataset, we selected the analogous cell type subclasses that are present in the human dataset [22].

*Code and Data Availability*

The data that support the findings of this study are available from GTEx version 8 and the Allen Institute as described under the relevant subheadings of the methods section. Scripts to generate all the results presented in this manuscript can be found online at: https://github.com/ahmedmahfouz/MG-analysis.

## Results

*Expression of MG-related genes in muscle and brain regions*

Expression of MG-related genes was assessed in human tissues using GTEx data. For *ACHE* and *AGRN*, specific splice variants (*ACHE-207* and *AGRN-208*) are known to be (NMJ) synapse-specific [20, 24]. *ACHE-207* and *AGRN-208* were indeed specifically expressed in skeletal muscle and/or brain regions, in contrast to other isoforms (Supplemental Fig. 1). Because of their relevance for the NMJ and association with autoimmune MG, *ACHE-207* and *AGRN-208* were selected for further analysis (Fig. 1). For completeness, the analysis was also performed using gene counts (i.e. including all isoforms) in Supplemental Fig. 2.

Hierarchical clustering revealed that skeletal muscle segregates from all other tissues based on expression of MG-related genes, supporting the unique combined role of these genes in the NMJ (Fig. 1). Agrin is secreted by motor neurons, explaining the absence of *AGRN-208* expression in skeletal muscle. The absence of *AGRN-208* in the tibial nerve may be explained by localization of mRNA to subcellular compartments in long axons [25]. Such transcripts are missed when sampling the nerve nucleus.

Cerebral brain areas clustered together close to skeletal muscle with expression of nearly all MG-related genes, confirming expression of these genes in healthy adult human brain. All MG-related genes could be detected in areas such as hippocampus and basal ganglia, although the level of expression differed compared to skeletal muscle. The cerebellum clustered separately from the cerebrum with moderate expression of *DOK7, LRP4* and *COLQ*. Transcriptomic separation of cerebellum and cerebrum is observed for many other genes [19]. Outside the brain, almost all MG-related genes are found in the testis and the ectocervix. Other components of the male and female reproductive systems cluster together with moderate expression of *LRP4* and *COLQ* and low expression of *DOK7, MUSK* and/or *RAPSN*. Varying expression for subsets of MG-related genes could be detected in the remaining tissues.

We observed three clusters of the MG-related genes (Fig. 1). *LRP4*, *COLQ* and *DOK7* have relatively ubiquitous expression across all tissues. Notably, *LRP4* was most expressed in the brain and the skin, *COLQ* in the cerebellum, pituitary gland, testis and heart, and *DOK7* in the heart and pituitary gland. In contrast, *MUSK*, *RAPSN* and *CHRNA1* showed low expression in a more limited subset of tissues. *MUSK* expression is highest in the small intestine, bladder and testis. *RAPSN* and *CHRNA1* expression was largely restricted to skeletal muscle, with some additional expression in the tibial nerve, and testis and pituitary gland respectively. Finally, the *ACHE-207* and *AGRN-208* isoforms clustered together based on their expression in the brain and ectocervix. Taken together, the expression of MG-related genes is prominent in skeletal muscle and the brain, but individual genes are also expressed in other tissues of the human body.

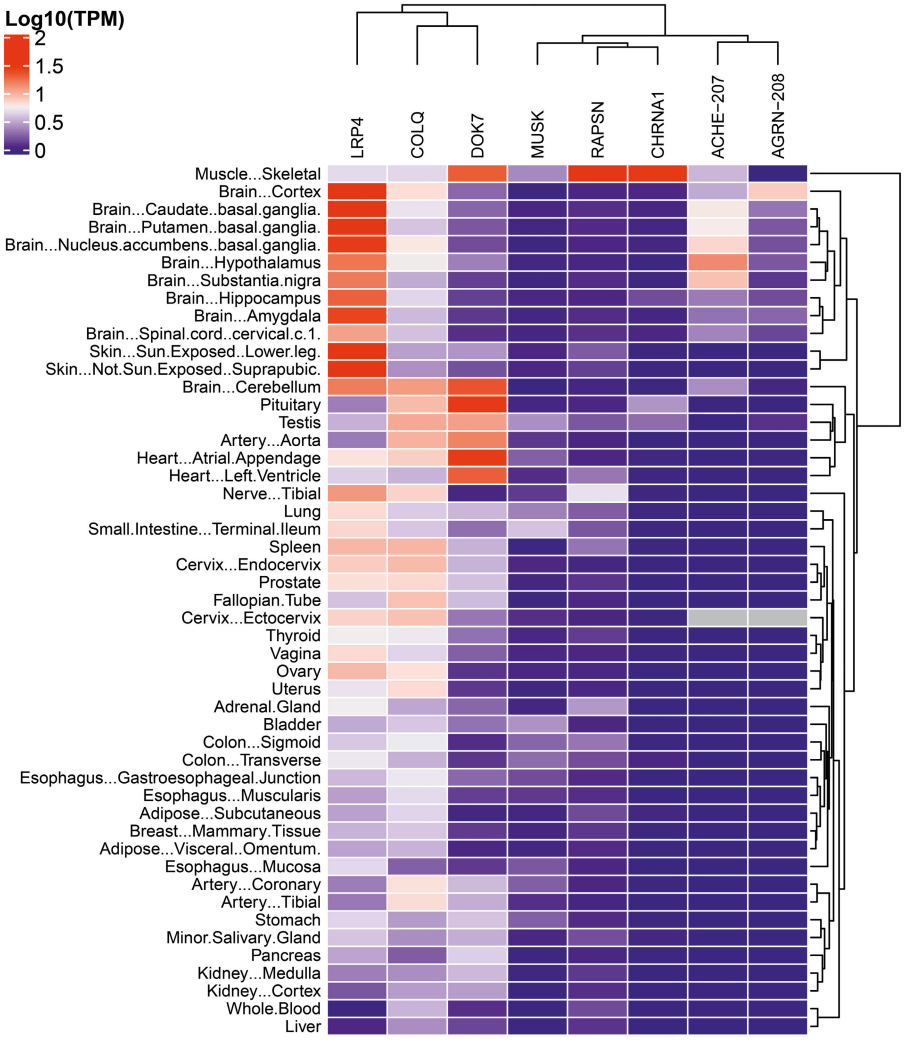



*Fig. 1. Overview of tissue-specific expression of MG-related genes reveals most co-expression in skeletal muscle, the CNS and parts of the male and female reproductive system. The heatmap shows the median expression of each MG-related gene (column) in TPM across human tissues (rows) using data from GTEx V8. Average expression is reported as Log10(TPM+1).*

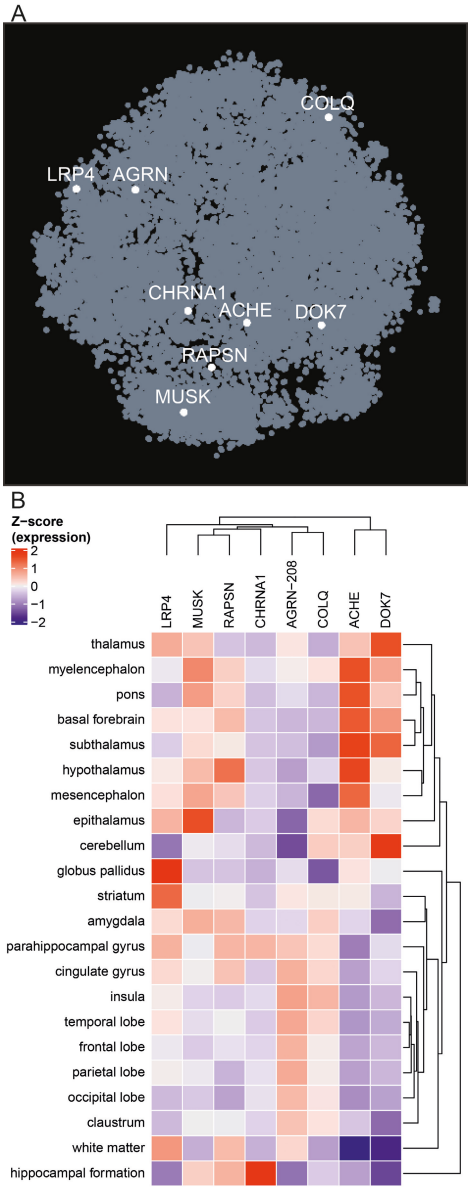



*Fig. 2. Spatial expression of MG-related genes in the adult human brain. Co-expression analysis of MG-related genes, based on their distribution in a t-SNE embedding, reveals that they do not share spatial co-expression (a). The t-SNE from BrainScope shows an embedding of all genes (dots) based on their expression pattern across the whole brain. (b) A heatmap showing the average expression of MG genes across distinct (non-overlapping) brain regions. Expression values represent the average z-score normalized expression per gene (i.e. relative expression across brain regions).*

*NMJ genes do not share anatomical expression patterns in the brain*

Compared to the ten brain regions in the GTEx database, the AHBA provides a high-resolution map of relative gene expression across the adult human brain. Strong correlation between spatial expression patterns would become apparent when genes cluster in the same region of the t-SNE plot (Fig. 2a). However, MG-related genes are scattered across the t-SNE plot, suggesting they do not share spatial expression patterns across the brain.

For hierarchical clustering analysis of distinct brain regions, the NMJ-specific isoform of agrin (*AGRN-208)* was included (Fig. 2b) while a heatmap including all *AGRN* isoforms can be found in Supplemental Fig. 3b. The hippocampus clustered separately from all other brain regions with the unique pattern of high relative expression of *CHRNA1*, *RAPSN* and *MUSK* (Fig. 2b). The remaining anatomical brain regions separated in two large clusters enriched in the developmentally early and late regions respectively. The early developmental cluster with hindbrain, midbrain and diencephalon was characterized by high relative expression of *DOK7* and *ACHE* and predominant low relative expression of *COLQ* and *AGRN-208*. In contrast, the late developmental cluster, with the basal ganglia, limbic system and cortical regions, was characterized by moderate relative expression of *COLQ* and *AGRN-208* and low relative expression of *DOK7* and *ACHE*. Furthermore, expression is highest in a unique structure for most genes. *DOK7* in cerebellum, *CHRNA1* in hippocampus, *MUSK* in epithalamus, *LRP4* in globus pallidus and *RAPSN* in the hypothalamus. Taken together, this suggests that MG-related genes are likely not active in similar pathways in the CNS as they do not share spatial expression patterns. However, subsets of these genes may be involved in signalling in similar structures.

*NMJ genes do not co-segregate in developmental time in the human brain*

To investigate whether expression of these genes follows a temporal pattern in brain development, we used the BrainSpan atlas visualized in the BrainScope browser [19, 21]. The expression of *ACHE, AGRN, COLQ* and *LRP4* did not follow the same pattern across human brain development (Fig. 3). *AGRN* was particularly expressed until early childhood while *LRP4* was expressed from the 3rd prenatal stage into adulthood. *ACHE* expression was predominantly restricted to the cerebellum, thalamus, amygdala and striatum from birth until adulthood. *COLQ* is highest across the entire brain in adult life, although various brain regions also show high expression in the last prenatal stage. *CHRNA1*, *DOK7*, *MUSK* and *RAPSN* could not be studied with BrainScope, but the brain-map.org

portal confirms the low and unique expression patterns of these genes (Supplemental Fig. 4). In sum, spatio-temporal expression of MG-related genes is heterogenous in the human brain.

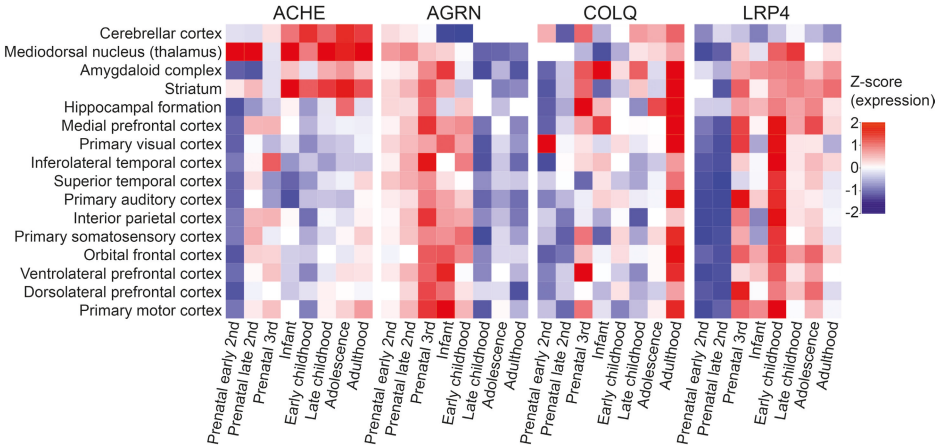



*Fig. 3. Overview of the expression patterns of four MG-related genes throughout human brain development. ACHE, AGRN, COLQ and LRP4 are expressed during different phases of human brain development.*

### *MG-related genes do not co-express in the same cell types in the cortex*

To understand which cell types are responsible for the regional expression of MG-related genes in the CNS, we explored a snRNAseq dataset [22]. The expression of *CHRNA1* and *RAPSN* could not be detected in cortical cell types (Fig. 4a). This is in line with the very low to absent expression in the bulk RNAseq datasets of the cortical regions (Fig. 1 and Supplemental Fig. 4). Interestingly, *MUSK* and *LRP4* were uniquely expressed in non-neuronal cell types, with *MUSK* solely in vascular leptomeningeal cells (VLMC) and oligodendrocytes and *LRP4* predominantly in astrocytes and oligodendrocyte progenitor cells (Fig. 4a). *ACHE*, *AGRN*, *COLQ* and *DOK7* were predominantly expressed in glutamatergic and/or GABAergic neurons; although, *AGRN* was also expressed in pericytes. Overall, a variety of cell types seem to be responsible for expression of MG-related genes in the cortex.

As many preclinical studies related to MG are done in rodents, it is relevant to know how the expression of MG-related genes compares between humans and rodents. In mouse cortical regions, similar cell types expressed *MUSK*, *LRP4*, *CHRNA1* and *RAPSN*, suggesting adequate translatability of results (Fig. 4b). In contrast, *ACHE* and *AGRN* expression was ubiquitously high in mouse neuronal cell types, but much more

restricted to GABAergic or glutamatergic cell types respectively in the human cortex. For *COLQ*, the expression is ubiquitously high in human neuronal cell types, but very much restricted to a single glutamatergic subclass in the mouse. For these genes, translatability of functional studies to humans may thus be limited.

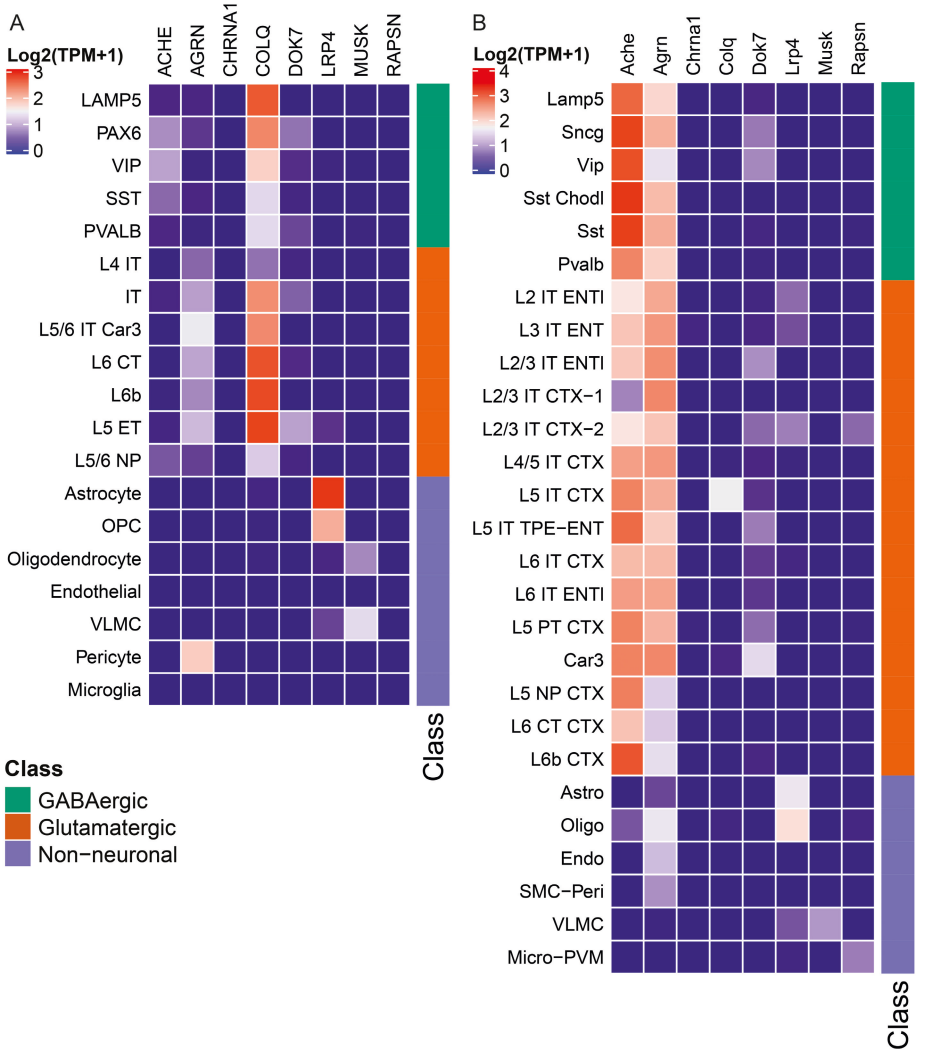



*Fig. 4. Cortical cell type expression of MG-related genes. Heatmap of adult human cortical cell types (a). Heatmap of adult mouse cortical cell types (b). CT = corticothalamic, CTX = isocortex, ENTI/ENT = entorhinal area, ET = extratelencephalic, IT = intratelencephalic, Micro-PVM = microglia - perivascular macrophages, NP = near projecting, OPC = oligodendrocyte progenitor cells, PT = pyramidal tract, SMC-Peri = smooth muscle cell – pericyte, TPE = temporal, perirhinal and ectorhinal, VLMC = vascular leptomeningeal cells. Average expression is reported as Log10(TPM+1).*

## Discussion

This study confirms that many of the MG-related genes are widely expressed outside skeletal muscle, with the exact pattern of involved organs varying per gene. Within the CNS, these genes furthermore show heterogenous spatial, temporal and cellular expression patterns. This suggests that these genes do not converge on a single pathway in the brain or other human tissues. It furthermore provides valuable insights for new research questions and hypotheses, explaining possible non-motor symptoms in myasthenic syndromes or off-target effects of MG-related targeted treatments.

Validity of these expression data is supported by studies confirming the presence of these MG-related proteins outside skeletal muscle. Congruent with the observed expression in testis, agrin, MuSK and rapsyn are present in human sperm, [26, 27]. Dok7 protein was detected in heart and brain, and MuSK in bladder, heart, lung and liver [28, 29]. Agrin is present in glutamatergic neurons and increasingly surrounding brain microvasculature during development of the blood-brain barrier, validating the expression in pericytes and during prenatal development [30, 31]. Lrp4 is indeed abundantly present in the brain, most prominently in astrocytes [3, 32]. Not all observations however match with previous studies. Dok7 was not detected in liver and spleen before [28, 29] and MuSK protein was detected in spleen, but is not present in our dataset. MuSK and agrin were found in glutamatergic neurons in the mouse cortex, while *MUSK* is only expressed in VLMCs and *AGRN* also in GABAergic neurons in the cell-type analysis [33]. It is well established that RNA levels do not always translate to protein; however, discrepancies may also be explained by sensitivity and type of detection method, timing or location of sampling, or differences between rodents and humans. The Allen Institute databases furthermore cover a limited number of individuals, making it difficult to translate these observations to a larger heterogenous population. Cell type-specific expression is currently only available for the cortex, covering a fraction of the cellular complexity in the CNS. More single cell data is anticipated as this field is rapidly expanding. Keeping these limitations in mind, our gene expression analysis method provides biological rationale to further investigate a possible role for candidate genes in newly identified target tissues. This is particularly important for MG-related genes like *Musk*, *Agrin, Dok7* and *Lrp4,* for which the role in other organs may be overlooked, because null mice die at birth of impaired breathing due to dysfunctional NMJs [28, 34-36].

Surprisingly, many of the MG-related genes were found in parts of the male and female reproductive system. Their role there remains to be uncovered. However, fertility issues have not been described in MG patients nor in mouse models lacking these genes. Interestingly, quite a number of MG-related genes have prominent expression in non-neuronal cell types in the cortex, suggesting a role for these genes outside a synaptic context. Expression of *MUSK* in VLMCs has not been described before and provides an important clue for further research into the role of MuSK in the brain. Till date, for a limited number of MG-related genes the function was studied in the CNS. Reduced agrin, Lrp4 or MuSK levels impaired cortical synaptogenesis and/or hippocampal functioning in mice [29, 32, 33, 37-39]. This fits the observation that all three genes are expressed in the hippocampus, although the prominent effects of reducing MuSK or agrin were surprising considering the relatively low expression of *MUSK* and *AGRN-208*. Whether these three genes converge on the same pathway in the hippocampus is uncertain. Agrin was shown to induce MuSK signalling through Lrp4 in hippocampal astrocytes [39]. However, the role of agrin and Lrp4 in hippocampal neurogenesis seems to be mediated by Ror2, instead of MuSK, supporting that subsets of MG-related genes may also work together with other proteins [38]. Taken together, MG-related genes may participate in similar pathways under certain condition in the hippocampus, but little is known about their role in the rest of the brain. The spatial, temporal and cellular resolution of our analysis can guide future studies to specific anatomical regions, developmental phase and cell types in the brain.

The expression of MG-related genes outside skeletal muscle suggests that other organs may be at risk for impairment by autoantibodies. In autoimmune MG and CMS patients, skeletal muscle weakness is clearly at the foreground of symptoms. The presence of these genes in the CNS suggests that the reported CNS-related symptoms in subsets of MG patients may be due to autoantibodies binding their target if they cross the blood-brain barrier, although this is not expected in patients with an intact blood-brain barrier [6-10]. In CMS patients, a mutational bias may occur as observed genetic defects in these genes are likely to result in a sufficiently mild phenotype to allow development. Absence of other symptoms may be explained by MG-related gene products 1) having low levels of expression, 2) being inaccessible to autoantibodies, 3) not fulfilling an essential function, 4) having alternative splice variants or 5) being differentially post-translationally modified masking relevant epitopes. Furthermore, observed non-motor symptoms or comorbidities may also be due to underlying immune dysfunction, thymoma's or

treatment side-effects. Future studies are needed to investigate whether CNS-related or other non-motor symptoms in MG patients are due to autoantibody binding or gene dysfunction not yet recognized.

MG-related genes or proteins are interesting targets for treatment of neuromuscular diseases through strengthening NMJs and improving or maintaining muscle function [40]. *DOK7* gene therapy, agonistic MuSK antibodies and agrin biologicals have proven beneficial in several mouse models for ALS, Dok7 CMS, Emery Dreifuss muscular dystrophy, spinal muscular atrophy or sarcopenia [41-46]. Off-target effects in other organs have not been reported, but also not specifically investigated. Our data furthermore identified some differences between mouse and human MG-related gene expression which emphasizes where caution is needed for accurate interpretation and translatability of studies using mouse models. For further clinical development, our data provides clear guidance as to which organs may be at risk for off-target effects. Since many of the MG-related genes are expressed in the brain, a treatment strategy that does not cross the blood-brain barrier is recommended.

This hypothesis-free approach to study timing and localization of MG-related gene expression suggests wide-spread expression of these genes outside skeletal muscle. These insights can guide future studies to uncover their role in other organs and guide pre-clinical development of related novel therapeutics.



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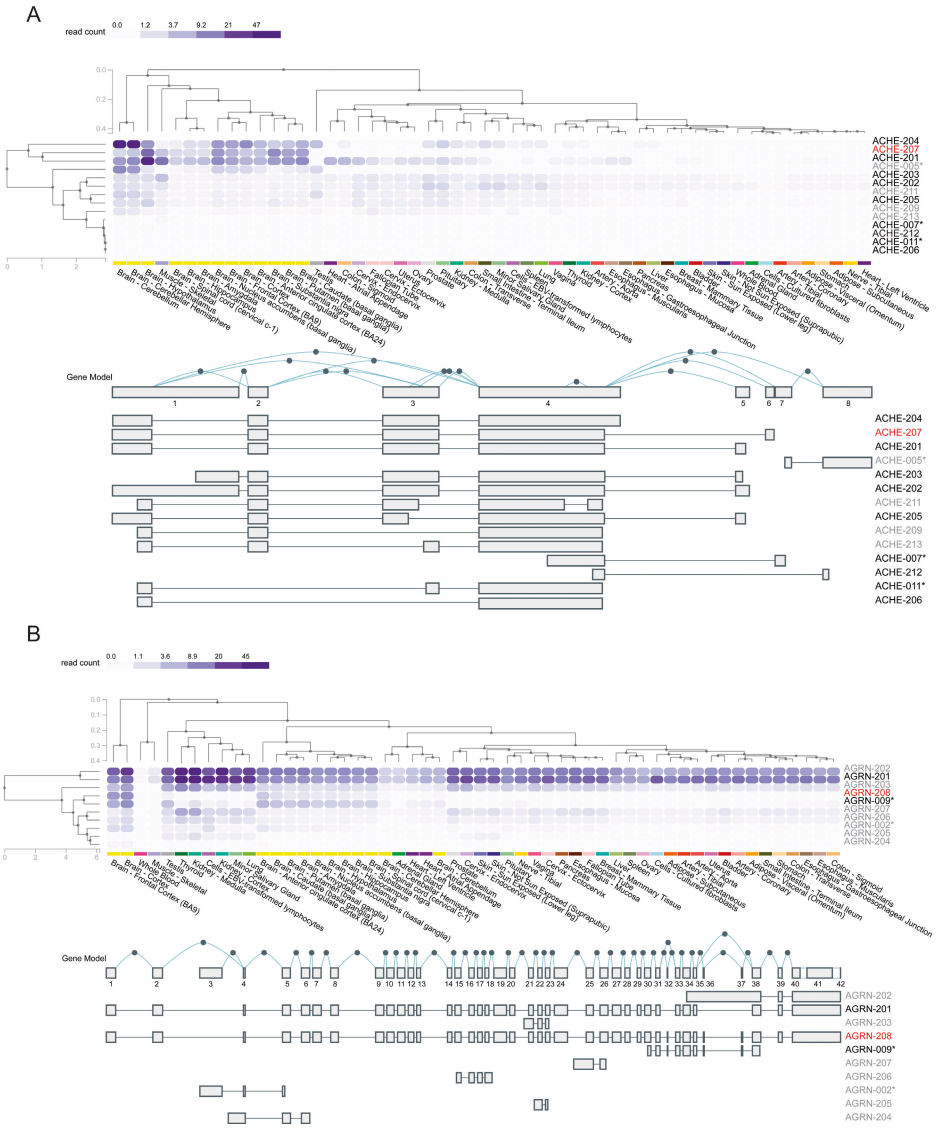
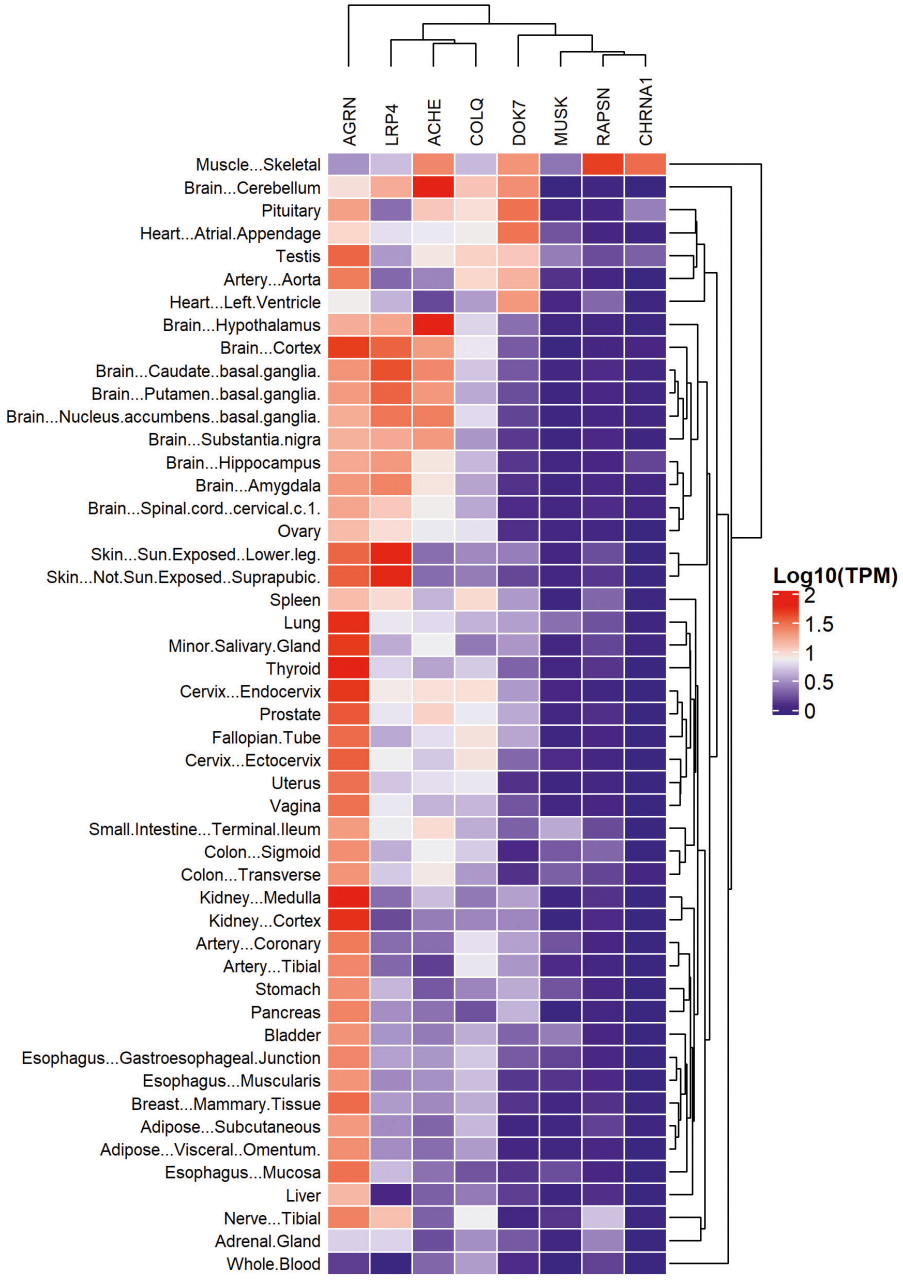
## Supplemental material

*Supplemental Table 1. Genes and probes used for MG-related genes from the AHBA. * used for analysis, unless otherwise specified. # detects the AGRN-208 isoform able to induce AChR clustering at the NMJ.*

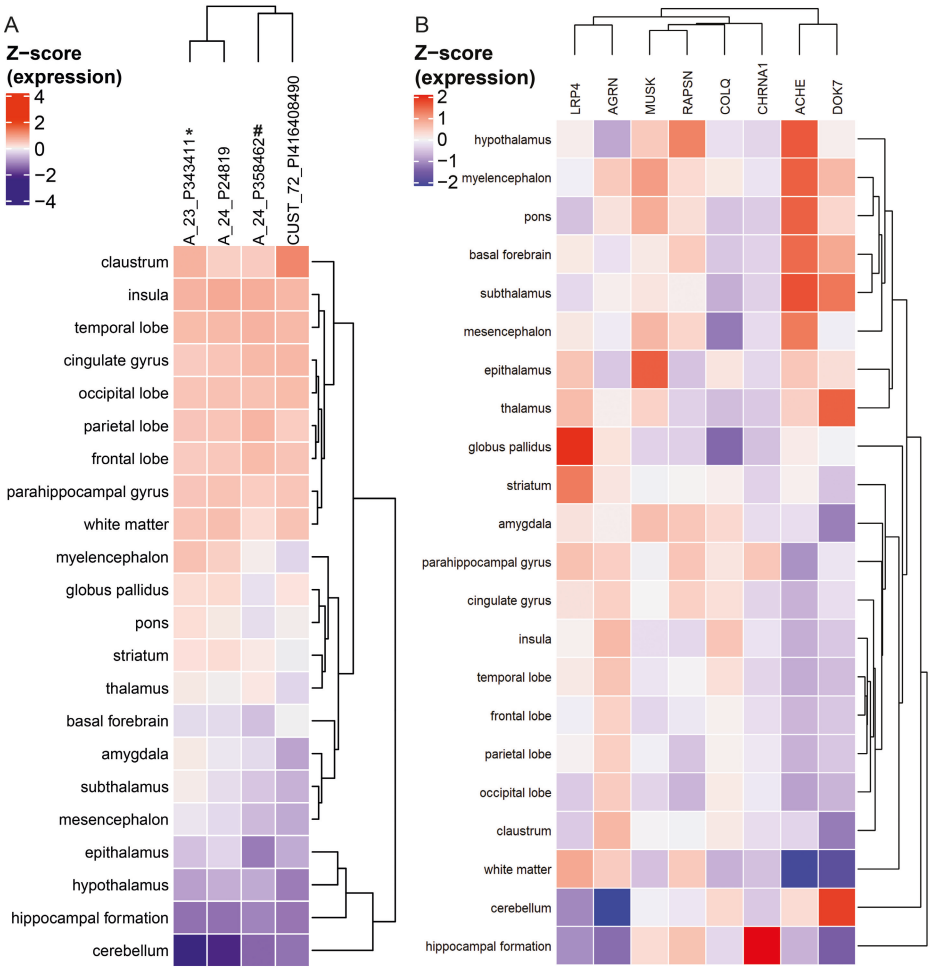
| Gene names | Allen Brain Atlas probes |
|---|---|
| *ACHE* | A_24_P60845* |
| *AGRN* | A_23_P343411*<br>A_24_P24819<br>A_24_P358462#<br>CUST_72_PI416408490 |
| *CHRNA1* | A_23_P90888*<br>CUST_14430_PI416261804 |
| *COLQ* | A_23_P212126*<br>A_24_P26160 |
| *DOK7* | A_23_P39885*<br>CUST_10065_PI416261804 |
| *LRP4* | A_24_P403561*<br>CUST_9952_PI416261804 |
| *MUSK* | A_23_P71649*<br>A_24_P129536 |
| *RAPSN* | A_23_P86801<br>CUST_899_PI416261804* |



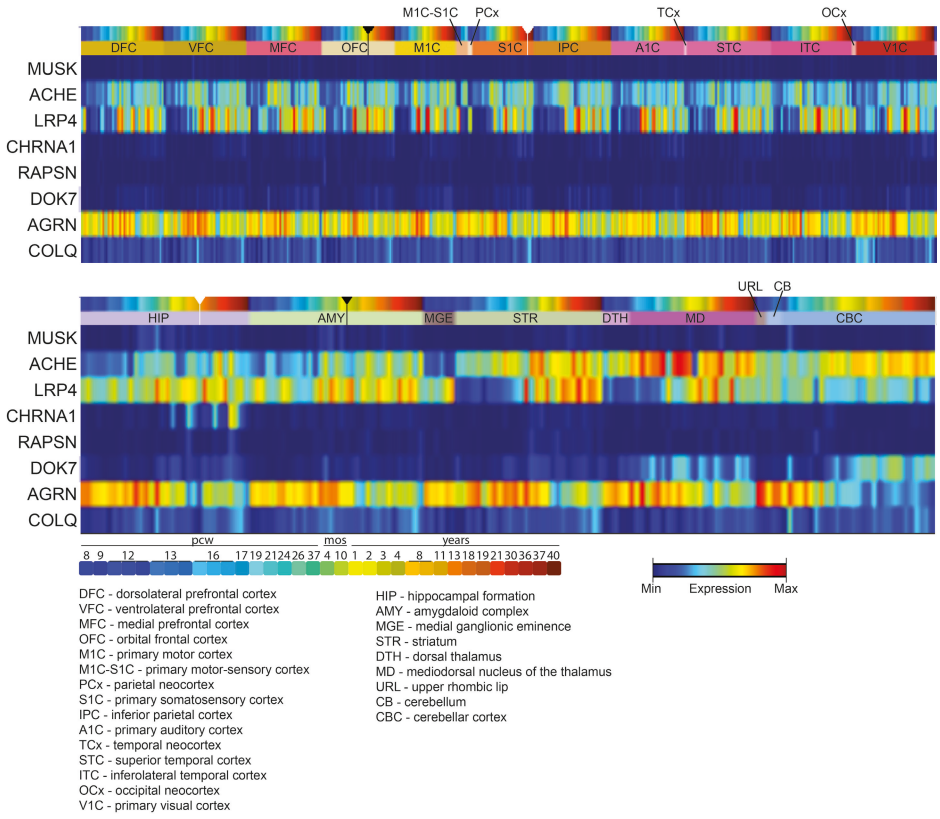

*Supplemental Fig. 1. Expression of ACHE isoforms (A) and AGRN isoforms (B) extracted from the GTEx database. Expression of synapse-specific ACHE-207 isoform (red) is limited to brain areas and skeletal muscle. Expression of NMJ-specific AGRN-208 isoform (red) is limited to brain areas. Grey isoforms are non-protein coding according to Ensembl (GRCh38). * isoforms identified in earlier assemblies of the human genome.*




*Supplemental Fig. 2. Hierarchical clustering of gene expression in the GTEx database using all isoforms of ACHE and AGRN.*




*Supplemental Fig. 3. Heatmaps from Allen human brain atlas. Relative expression of different agrin probes across distinct brain areas (A). # probe that selectively detects NMJ-relevant isoform of agrin. * probe for AGRN used in the BrainScope tool detecting all AGRN isoforms. Hierarchical clustering using probe for AGRN that does not discriminate between isoforms (*) (B).*




*Supplemental Fig. 4. Gene expression of MG-related genes during human development do not show the same pattern across development. CHRNA1 expression is increased across development restricted to the hippocampus. DOK7 shows a pattern of increasing expression across development, with highest expression in the cerebellum and thalamus. MUSK is lowly expressed during prenatal development in the hippocampus, amygdala and cerebellum. RAPSN shows very limited expression across development, restricted to some subcortical regions of the brain. Visualized using the BrainSpan portal.*

# 7

# General Discussion

## General Discussion

Muscle-specific kinase (MuSK) myasthenia gravis (MG) is an autoimmune disease (AID) where the neuromuscular junction (NMJ) protein MuSK is targeted by antibodies. MuSK is a key regulator of establishing and actively maintaining skeletal muscle NMJs [1]. Disruption of its function by antibodies impairs neurotransmission, resulting in fatigable muscle weakness [2, 3]. MuSK MG is part of the expanding group of IgG4-AIDs, because the majority of MuSK antibodies in these patients are IgG4 and these are sufficient to cause disease [3-8]. IgG4 is considered anti-inflammatory due to its inability to activate complement and antibody-dependent cytotoxicity [9]. In addition, IgG4 has the unique ability to exchange half-molecules in a stochastic process called Fab-arm exchange [10]. The majority of IgG4 will undergo Fab-arm exchange within one day [11, 12]. Consequently, these antibodies become bispecific and functionally monovalent in antigen-binding capacity, losing their ability to internalize antigens through cross-linking. Why MuSK antibodies are predominantly of the IgG4 subclass and whether that matters for their pathogenicity was still largely unknown. The studies in this thesis aim to elucidate the cause and consequences of MuSK antibody pathogenicity related to antibody subclass and characteristics. Here, we will discuss what may be predisposing factors for developing an IgG4 response in IgG4-AIDs, how antibody valency influences the pathogenicity of IgG4 autoantibodies, what we have learned on the pathophysiology of MuSK MG and how this knowledge is contributing to development of novel treatments for neuromuscular disorders. In this discussion, the term "monovalent antibodies" will be used for bispecific antibodies with functionally monovalent antigen-binding capacity and "bivalent antibodies" for monospecific antibodies with functionally bivalent antigen-binding capacity.

### Drivers of the IgG4 response in IgG4-AIDs

The dominance of IgG4 in autoimmune diseases is rather surprising given the anti-inflammatory characteristics of IgG4 and its relative low abundance in serum. Several hypotheses may explain the onset of an IgG4 response in autoimmune disease. We used MuSK MG as a model IgG4-AID and hypothesized that MuSK MG patients may have a general predisposition to generate IgG4 responses. Therefore, in **chapter 2** we investigated if these patients have generally elevated serum IgG4 levels. MuSK MG patients (not under recent immunosuppression) have higher serum IgG4 compared to healthy donors and acetylcholine receptor (AChR) MG patients, but for most patients the IgG4 levels fall within

the normal range. Serum IgG4 is also enriched in pemphigus patients, another IgG4-AID [13]. In both diseases, the higher IgG4 levels are partly due to autoantigen-specific IgG4 antibodies. Since the IgG4 levels in these patients generally fall within the range of normal, it is unlikely they have a predisposition to develop IgG4 responses to many antigens that also drives the IgG4 response against the autoantigen. Rather, the increase in serum IgG4 levels may be a bystander effect of the cytokines and other immune signaling factors involved in the class-switch to IgG4 of the autoantigen-specific B-cells, such as IL-10 [9]. In support of this, (trends for) elevated IL-10 levels have been found in MuSK MG, and also in the IgG4-AIDs pemphigus and thrombotic thrombocytopenic purpura [14-17]. In conclusion, other factors likely contribute to the development of IgG4 responses in IgG4-AIDs, such as genetic variants in human leukocyte antigen (HLA) genes, antigen characteristics and environmental triggers.

*HLA*

Many autoimmune diseases have been associated with specific HLA class II genetic variants, suggesting a role for these genes in their etiology. HLA class II genes encode the major histocompatibility complex (MHC) II responsible for antigen-presentation to T-helper cells by immune antigen-presenting cells such as B-cells and dendritic cells. A recent meta-analysis including all studies investigating HLA class II associations in known IgG4-AIDs found an increased frequency of HLA-DQB1*05, HLA-DRB1*14 alleles and the HLA-DQB1*05-HLA-DRB1*14 combined haplotype in the IgG4-AIDs combined [18]. For example, the HLA-DQB1*05 allele occurs approximately 2-3 times more often and the HLA-DRB1*14 allele 3-4 times more often in patients with pemphigus or MuSK MG compared to controls [18]. Increased HLA-DQB1*05 has not been found in non-IgG4 autoimmunity and may thus confer a specific genetic predisposition for developing IgG4 autoantibodies [18]. In addition, HLA-DRB1*14 was associated with higher anti-MuSK IgG4 titers and IL-10 in the serum of MuSK MG patients [19]. HLA genes may thus influence the development of an IgG4 response, for example through structural properties which allow a good fit for IgG4-inducing peptides, or through regulation of T-cell response facilitating IgG4 responses [20]. Taken together, from the frequency of certain HLA class II haplotypes in IgG4-AIDs, it is clear that proper antigen presentation is one of the factors permitting the development of an IgG4 (autoimmune) response.

*Antigen characteristics*

Class-switch to IgG4 does not occur in response to all antigens. Rather IgG4 responses have been linked to specific types of antigens associated

with food, therapeutic proteins, autoantigens and helminth infections [9]. Furthermore, the IgG4 response often only develops upon prolonged exposure to these antigens and is thought to be driven by cytokines related to a Th2 response [9]. Active immunization with MuSK in mice results in mouse IgG1 (mouse equivalent of IgG4) antibodies against MuSK with an increase in Th2 cytokines, suggesting the autoantigen itself may drive the Th2-like response [21]. Extracellular autoantigens are generally exposed and accessible to antibodies. After initiation of an autoantibody response this might contribute to prolonged chronic exposure to the immune system. However, not all (partly) extracellular autoantigens causing antibody-mediated autoimmunity induce IgG4 responses. For example, the AChR also has a prominent extracellular part, but AChR MG is mainly mediated by IgG1 AChR antibodies. Interestingly, the autoantigens in IgG4-AIDs are all soluble or single-pass membrane proteins [7,8]. In contrast, the antigens targeted in autoimmune diseases with a striking IgG1/3 dominance (e.g., AChR MG, neuromyelitis optica or systemic lupus erythematosus) are receptors spanning the membrane multiple times or located in the nucleus. This suggests specific characteristics related to such proteins may be needed in combination with chronic exposure and permissive HLA variants. Such characteristics may be of biochemical nature e.g., charge, hydrophobicity or functional groups. Similar biochemical properties may be the result from different combinations of post-translational modifications, amino acid sequences or tertiary structures. Commonalities may thus not lie in specific sequences or modifications, but in the combined properties they have. Therefore, it will be interesting to compare the different IgG4 autoantigens from a high-level structural biochemical perspective to identify commonalities. Alternatively, it may be the way the immune system is exposed to these types of antigens, for example mostly in the absence of infection or damage that may ultimately lead to an anti-inflammatory IgG4 response [9]. Taken together, how antigen characteristics and the immune response against them interact and influence each other may be an important factor contributing to IgG4 responses in AIDs.

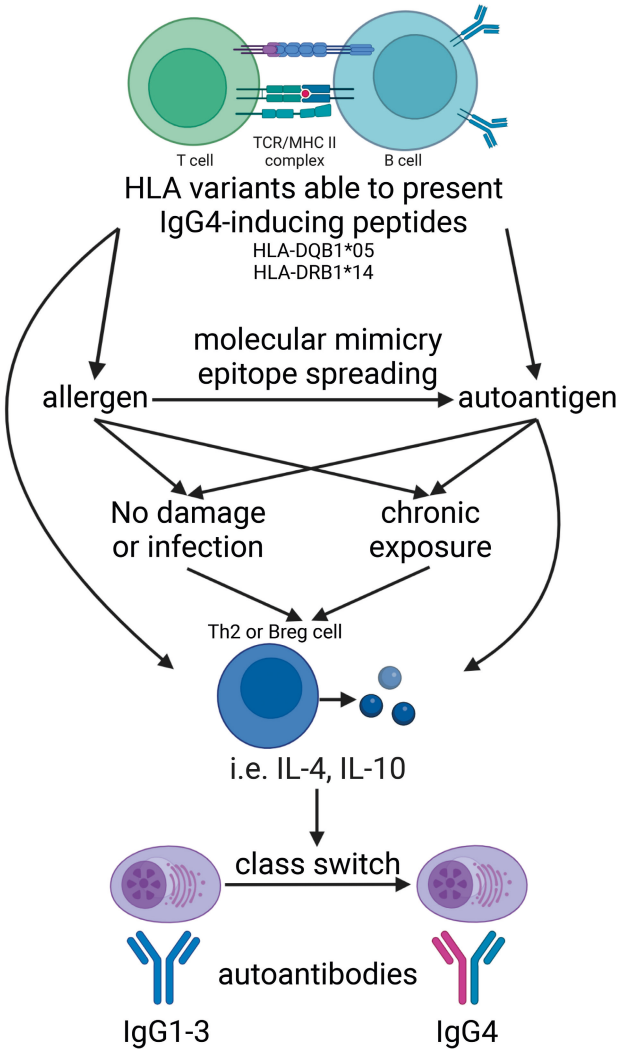



*Figure 1. Factors contributing to the IgG4 response in IgG4-AID. The class-switch to IgG4 autoantibodies in IgG4-AIDs likely requires a combination of patient and antigen-dependent stimulating and permissive factors. The structure of the MHC class II complex is determined by genetic HLA variants and needs to match the (auto)antigen peptide. This can either be an environmental antigen such as allergens, which through molecular mimicry or epitope spreading can lead to an antibody response against the autoantigen, or the autoantigen itself. Chronic exposure and a no-damage or infection environment combined with (yet unknown) characteristics of the (auto)antigen and the HLA variants can stimulate cytokines secretion (such as IL-4 or IL-10) that induce the class switch to IgG4 autoantibodies. Created with BioRender.com.*

*Environmental triggers*

Sometimes environmental antigens trigger an autoimmune reaction through molecular mimicry or epitope spreading. Sand-fly saliva or walnut antigens have been identified as potential environmental antigens triggering different forms of pemphigus, which is mediated by antibodies against skin proteins desmoglein (dsg) 1 or 3 [22, 23]. Both environmental antigens are allergens, fitting with other known IgG4-inducing antigens. Healthy individuals in regions with endemic forms of pemphigus were found to have (IgG1) antibodies against non-blocking epitopes of dsg-1 and cross-reactive with sandfly antigens, while pemphigus patients in these regions have high levels of IgG4 antibodies against blocking epitopes of dsg-1 [22, 24]. Molecular mimicry and epitope spreading both between the environmental and autoantigen, and within the autoantigens appears to be involved in the evolution to pathogenic dsg-1 or -3 antibodies [22]. Taken together, this suggests the environmental antigen may be the initial trigger of the autoantibodies, but symptoms will only develop upon epitope spreading to blocking epitopes driven by the autoantigen. It will be exciting to investigate whether for other IgG4-AIDs cross-reactive allergens can be identified. This would suggest that IgG4-inducing environmental factors may play a critical role in the etiology of IgG4-AIDs.

In sum, the IgG4 response in IgG4-AIDs is likely driven by a combination of patient, environmental and antigen-dependent characteristics. Since IgG4-AIDs are rare, a multiple-hit model for the etiology of the IgG4 response in IgG4-AIDs can be hypothesized. This model may include a permissive HLA class II to properly present an (environmental) antigen with IgG4-inducing properties. Antibodies against the environmental antigen may cross-react with an autoantigen or epitope spreading will generate auto-reactive antibodies. Continuous exposure to the autoantigen may further shape an autoimmune response dominated by IgG4. Some of the critical steps currently hypothesized to contribute to the development of an IgG4 autoimmune response are summarized in Figure 1. It is unlikely these are the only factors driving the IgG4 (autoimmune) response, because not all individuals carrying these HLA variants develop IgG4-AID after exposure to specific environmental antigens. It is thus expected the model will become more complex in the future.

## Role of antibody valency in MuSK MG

Several observations from the clinic suggest MuSK antibodies of the IgG4 subclass are critical contributors to disease: 1. IgG4, but not IgG1,

MuSK antibody titers correlate with disease severity in MuSK MG patients [25, 2]. In one MuSK MG patient clinical remission was associated with a switch of serum MuSK antibodies from predominantly IgG4 to IgG1 [25, 3]. Symptomatic MuSK MG patients without anti-MuSK IgG4 have not been described until date. In addition, passive transfer of MuSK MG patient IgG4 caused muscle weakness in mice, while IgG1-3 fractions of the same patients did not [3]. Monovalent antigen binding is unique to IgG4, and it has been estimated that up to 99% of anti-MuSK IgG4 in the serum is monovalent [26]. Functional monovalency of IgG4 MuSK antibodies may thus contribute to their clinical relevance in MuSK MG.

To investigate the role of MuSK antibody valency in MuSK MG, we generated patient-derived monoclonal MuSK antibodies from B-cell receptor sequences (BCR) from individual MuSK-positive memory B-cells (**Chapter 3** [27]). We discovered that monovalent MuSK Fab fragments are antagonists of agrin-induced MuSK activation, while their bivalent equivalents are (partial) agonists. This supports the previously found valency-dependent effects of anti-MuSK rabbit IgG and Fab fragments also apply to MuSK antibodies from patients [28, 29]. Next, we adapted the controlled Fab-arm exchange method to IgG4 to generate pure and stable monovalent MuSK antibodies (**chapter 4** [30-32]). Passive transfer of monovalent MuSK antibodies caused rapid-onset muscle weakness, while their bivalent equivalents did not cause muscle weakness or muscle weakness with later onset and slower progression. Functional monovalency can thus exacerbate the pathogenicity of MuSK antibodies, but bivalent MuSK antibodies can be pathogenic in a clone-dependent manner. Isotype switching to IgG4 and subsequent Fab-arm exchange-induced functional monovalency of IgG4 is thus clinically relevant for the development of MuSK MG.

Depending on valency, MuSK antibodies have opposing effects on MuSK. Dimerization of MuSK upon agrin and Lrp4 binding is crucial for MuSK autophosphorylation, initiating further intracellular signaling. Monovalent MuSK antibodies are thought to block the interaction between MuSK and Lrp4, thereby preventing MuSK dimerization, intracellular signaling, AChR clustering and finally neuromuscular transmission [6, 33, 34]. Since bivalent MuSK antibodies can bind two MuSK molecules simultaneously, it is hypothesized that bivalent binding to MuSK can force dimerization, initiating autophosphorylation of MuSK independent of Lrp4 and thereby maintaining MuSK functioning (**chapter 3** [27]). The fact that dimerization of MuSK is a crucial part of its function is thus likely responsible for the amplified pathogenicity of functionally monovalent MuSK antibodies.

*Relevance of antibody valency in other IgG4-AIDs*

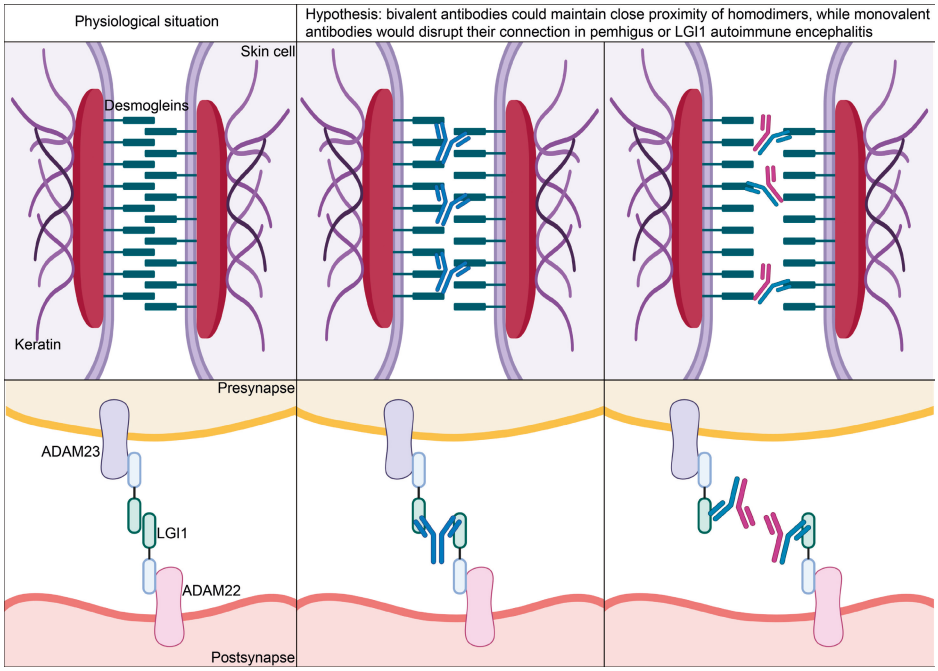



*Figure 2: Hypothesis of theoretical valency-dependent effects in IgG4-AIDs where the antigens form a homodimer. Top: in pemphigus homodimers of desmogleins contribute to the cell-cell contact between epithelial cells in the skin. Bottom: in LGI1 autoimmune encephalitis homodimers of LGI1 connect ADAM23 at the pre-synapse to ADAM22 at the post-synapse. Bivalent antibody binding might maintain the close proximity of the homodimers, while monovalent antibody binding might completely disrupt the interaction. Created with BioRender.com.*

Functional monovalency of IgG4 autoantibodies may also amplify pathogenicity for other IgG4-AIDs if dimerization of the antigen is part of its normal physiological function like in MuSK MG. In most IgG4-AIDs, the IgG4 autoantibodies block the interaction between two different proteins, making valency-dependent effects unlikely [7, 8]. However, in pemphigus, dsg-1 and dsg-3 can make homodimers between skin cells to ensure skin integrity. In leucine-rich, glioma inactivated 1 (LGI1) autoimmune encephalitis, LGI1 homodimers connect ADAM23 and ADAM22 on the pre- and post-synapse respectively. Dsg-1, dsg-3 and LGI1 make homodimers between cells in a trans-manner, in contrast to MuSK in which both MuSK molecules are in the same cellular membrane. Still, one could hypothesize that a bivalent antibody against these antigens maintains the close proximity of the antigens by binding both molecules, while monovalent antibodies would just interfere with the interaction

(Figure 2). If bivalent antibodies maintain the function of the antigen sufficiently, one expects valency-dependent effect on pathogenicity.

In an endemic form of pemphigus, healthy individuals and pemphigus patients in remission can have IgG1 dsg-1 antibodies, while patients with active disease have very high anti-dsg-1 IgG4 titers, supporting the important role of IgG4 in pemphigus similar to MuSK MG [24]. Both monovalent and bivalent dsg-1 or -3 antibodies can cause skin lesions in mice or *in vitro* human models [35-39]. Fabs from patient-purified anti-dsg-1 IgG caused skin lesions much quicker than the undigested patient-purified IgG, suggesting valency-dependent effects [40]. However, this was not the case for Fabs of patient-purified anti-dsg-3 IgG [41]. Most autoantibodies in patient-purified IgG may already be Fab-arm exchanged monovalent IgG4, possibly explaining a lack of effect upon digestion into Fab fragments. Furthermore, not all bivalent dsg-1 or -3 antibodies may be able to maintain sufficient dimerization, depending on the epitope recognized. Differences in epitopes targeted between patients may also explain differences in valency-dependent effects between patient-derived antibody preparations. So far, the evidence for valency-dependent effects in pemphigus thus remains inconclusive. To determine if functional monovalency exacerbates pathogenicity of autoantibodies in pemphigus, studies using a panel of pure (monoclonal) bivalent or monovalent dsg-1 or -3 antibodies side by side would be needed.

Two important functional domains of LGI1 are the leucine-rich repeat (LRR) domain and the epitempin-repeat (EPTP) domain. Two LGI1 molecules interact through the LRR domain, while LGI1 interacts with ADAM22 or 23 through the EPTP domain. LGI1 antibodies binding the EPTP domain block the interaction between two different proteins (LGI1 and ADAM22 or 23) [42]. Amplified pathogenicity by monovalency is thus not expected for anti-EPTP domain antibodies. Both bivalent monoclonal LGI1 antibodies and monovalent Fabs binding the LRR domain internalize the LGI1-ADAM22 complex in HEK293 cells, which is recapitulated by patient-purified IgG4 and to a lesser extent patient-purified IgG1-3 [42]. In addition, passive transfer of bivalent mAbs and patient purified IgG both caused impairments in the hippocampus, indicating both monovalent and bivalent LGI1 antibodies are pathogenic *in vivo* [42-44]. The pathogenic effects of LGI1 antibodies binding the LRR domain thus also do not seem to be amplified by functional monovalency.

Taken together, until now the functional monovalency exacerbating pathogenicity of IgG4 autoantibodies is rather unique to MuSK MG and perhaps pemphigus, but an unlikely pathogenic mechanism in most IgG4-AIDs. Importantly, functional monovalency of anti-neurofascin 155 (anti-NF155) IgG4 even reduced their pathogenicity in chronic inflammatory demyelinating polyneuropathy (CIDP), because cross-linking is an important part of antibody pathogenicity in this disease [45]. This suggests the valency-dependent effects depend both on the disease-specific pathogenic mechanism and the function of the antigen.

The study on valency-dependent effects in CIDP furthermore shows significant individual variation in the percentage of bivalent anti-NF155 IgG4 among all IgG4 NF155 antibodies [45]. Between 10 and 20% bivalent anti-NF155 IgG4 was common among these patients and the patient with the highest anti-NF155 titers even had 78% bivalent anti-NF155 IgG4. Bivalent anti-MuSK IgG4s were also detected in MuSK MG patients, but the method did not allow for the quantification of the proportion of total anti-MuSK IgG4 [26]. It thus appears that although the majority of IgG4 undergoes Fab-arm exchange, a clinically relevant amount of bivalent IgG4 autoantibodies may be present. The relative amounts of bivalent and monovalent IgG4 autoantibodies may vary between individuals due to differences in the reducing environment permitting Fab-arm exchange, or the polyclonality of the total IgG4 pool. One can imagine that if a large proportion of total IgG4 are the IgG4 autoantibodies, Fab-arm exchange between IgG4 autoantibodies targeting the same or different epitopes on one autoantigen becomes more likely. The resulting "bispecific" IgG4 autoantibody may then still be able to bind the autoantigen in a functionally bivalent manner after Fab-arm exchange. For IgG4-AIDs with valency-dependent pathogenicity, the net pathogenic effect of IgG4 autoantibodies will thus depend on the complex mixture and relative amounts of functionally monovalent and bivalent autoantibodies present in the patient.

The clinical relevance of the IgG4 subclass in IgG4-AIDs may be mediated by other factors associated with the class-switch to IgG4, such as higher titers and high affinity antibodies due to further affinity maturation [9]. These processes occur in IgG4-AIDs: in MuSK MG, pemphigus and LGI1 autoimmune encephalitis, the IgG4 autoantibody titers are much higher than the IgG1 autoantibody titers, and the BCR repertoire and patient-derived isolated clones show a large amount of mutations suggestive of affinity maturation (**chapter 3**, [4-6, 24, 25, 27, 42, 46, 47]). Furthermore, *in vitro* pathogenicity of monovalent MuSK antibodies depended on affinity

maturation due to monovalent binding. The pathogenic bivalent anti-MuSK clone in **chapter 4** also had a higher affinity than the non-pathogenic clone (unpublished observation), suggesting that high affinity might also contribute to the *in vivo* pathogenicity of bivalent MuSK antibodies. The class-switch to IgG4 may thus drive symptom manifestation in most IgG4-AIDs by inducing higher titers of high affinity autoantibodies, but in MuSK MG (and potentially pemphigus) Fab-arm exchange induced monovalency adds significantly to this pathogenicity.

## Mechanisms of MuSK antibody-mediated pathogenicity at the NMJ in MuSK MG

The overview of prior MuSK antibody research in **chapter** 1 highlights the importance of investigating how intrinsic MuSK antibody characteristics influence the consequences they have on the NMJ, to fully understand the mechanisms of disease in MuSK MG. Monoclonal MuSK antibodies are useful tools to link antibody characteristics to consequence. The MuSK clones we isolated from a MuSK MG patient in **chapter 3** bind the Ig-like 1 domain [27]. Another group generated monoclonal MuSK antibodies binding the Ig-like 2 domain of MuSK from MuSK MG patients [48]. In addition, several monoclonal antibodies binding the Fz domain of MuSK were identified in phage-display screens [49-51]. The functional effects of patient-derived monoclonal antibodies on muscles have been tested by others [47, 48, 52] and by us *in vitro* and *in vivo* in this thesis (**chapter 3, 4 & 5** [27, 31]), and are summarized in Table 1.

*Table 1: overview of consequences of MuSK antibodies on the NMJ, based on monoclonal MuSK antibodies with differences in pathogenicity, epitope and valency.*

| | Agrin | Pathogenic MuSK antibodies | | Nonpathogenic MuSK antibodies | |
|---|---|---|---|---|---|
| **Characteristics*** | | | | | |
| Functional valency | | monovalent | bivalent | monovalent | bivalent |
| Epitope | | Ig-like 1<br>Ig-like 2[#] | Ig-like 1 | Frizzled | Ig-like 1<br>Frizzled[&] |
| **C2C12 myotubes** | | | | | |
| MuSK-P activation | +++ | ---- | ++++ | = | +++ |
| AChR clustering | +++ | ---- | + | = | + |
| MuSK internalization | = | = | = | = | = |
| Dok7-MuSK interaction | + | - | + | = | + |
| Dok7 protein level | - | = | -- | | -/-- |
| (NMJ) gene expression | = | = | = | | = |
| **NOD/SCID mice** | | | | | |
| *In vivo* Muscle strength | | ---- | --- | | = |
| *In vivo* Muscle fatigability | | ---- | -- | | = |
| EMG decrement | | -- | -- | | = |
| Postsynaptic NMJ morphology | | -- | ---- | | - |
| Presynaptic NMJ morphology | | = | = | | = |
| Safety factor | | -- | --- | | - |
| NMJ gene expression | | | | | |
| *Musk* | | + | + | | = |
| *Lrp4* | | =/- | = | | = |
| *Dok7* | | =/- | = | | = |
| *Rapsn* | | = | = | | = |
| *Chrne* | | --- | + | | = |
| *Chrng* | | = | ++++ | | ++++ |
| *Chrna1* | | = | ++ | | = |
| *Ache* | | ---- | + | | = |
| *Colq* | | ---- | + | | + |

*Table 1 Continued*
*from known and tested monoclonal antibodies
[#] tested on AChR clustering only [47]
[&] tested on MuSK-P activation, AChR clustering, Dok7-MuSK interaction, Dok7 protein levels, *in vivo* muscle fatigability, EMG decrement, postsynaptic pathology (**chapter 5**, [52])

| ---- | --- | -- | - | = | + | ++ | +++ | ++++ |
|---|---|---|---|---|---|---|---|---|

Scale from decreasing/impairing (-) to increasing/activating (+) relative to healthy physiological processes. No change is denoted with the equal sign (=). The amount of – or + signs is an indication of the order of the relative effect sizes.

### *Antibody valency*

By comparing the consequences of pathogenic bivalent and monovalent MuSK antibodies side by side, extensive differences become apparent. The valency-dependent effects on *in vitro* MuSK activation were discussed earlier. *In vivo*, it took longer for signs of muscle weakness to develop and muscle fatigability was not progressive to complete loss upon the pathogenic bivalent MuSK antibody compared to its monovalent equivalent (**chapter 4** [31]). In addition, bivalent and monovalent MuSK antibodies binding the Ig-like 1 domain of MuSK affected the expression of NMJ genes in the masseter muscle differently. Monovalent MuSK antibodies reduced the gene expression levels of the AChR epsilon subunit (*Chrne*), Collagen Q (*Colq*) and acetylcholine esterase (*Ache*), while the pathogenic bivalent MuSK clone slightly increased the expression of these genes (Table 1, **chapter 5**). In contrast, bivalent MuSK antibodies binding the Ig-like 1 domain increased the expression of the gamma subunit of the AChR (*Chrng*) regardless of pathogenicity. Although pathogenic bivalent and monovalent MuSK antibodies both ultimately lead to failure of neurotransmission, the processes leading up to that appear strikingly different. These different pathomechanisms may also contribute the observed differences in onset, progression and severity of muscle fatigability.

These differential pathogenic mechanisms between monovalent and bivalent MuSK antibodies have consequences for the model systems used to study MuSK MG. Active immunization models of MuSK MG in rodents will only produce bivalent MuSK antibodies. The disease course of the pathogenic bivalent MuSK antibody is more similar to the development of weakness upon the antigen boost in active immunization models of MuSK [21, 53-56]. In addition, the NMJ gene expression changes seen upon active immunization match those seen upon passive transfer of pathogenic

bivalent MuSK antibodies, but not monovalent MuSK antibodies [54]. Active immunization models of MuSK MG thus only recapitulate a part of the disease mechanisms of MuSK MG in patients. Passive transfer of patient-purified IgG4 likely represents the dominant disease mechanism of monovalent MuSK antibodies. However, patient-purified total IgG models contain the entire complex antibody pool present in patients and thus likely have the best translational value as a pre-clinical model for (therapeutic agents targeting) the molecular mechanisms of disease in muscles.

*Epitope*

So far, only one patient-derived MuSK antibody was found to be pathogenic *in vivo* in bivalent form [31]. What drives the pathogenicity of bivalent MuSK antibodies such as the 13-3B5 clone is still unclear, because the cellular consequences of pathogenic and nonpathogenic bivalent MuSK antibodies investigated so far are quite similar to each other and agrin (Table 1). It can be hypothesized that pathogenicity of bivalent MuSK antibodies may be explained by its specific binding epitope, because that will determine how MuSK is forced to dimerize. The binding-site of pathogenic MuSK antibodies may induce such an unnatural conformation that it precludes the interaction with proteins binding to MuSK both extra- and intracellularly, beyond the antibody-binding site. In line with this, the pathogenic and nonpathogenic bivalent MuSK clones have different binding sites on the Ig-like 1 domain of MuSK, because they do not compete for binding [52]. So different binding sites, even within the same functional domain of MuSK, appear to have different consequences. In **chapter 5**, we found that Dok7, considered one of MuSK's most important downstream interactors, can bind upon forced dimerization by a pathogenic bivalent antibody. However, a growing number of interactors for MuSK is being identified with functions in downstream signaling, scaffolding and gene expression [57]. To give insights into what drives the pathogenicity of bivalent MuSK antibodies, it would thus be very interesting to 1) resolve the conformation of MuSK in complex with different MuSK antibodies, and 2) map MuSK's extracellular and intracellular interaction partners upon bivalent antibody binding.

*Complement*

The mechanisms of disease in MuSK MG investigated in this thesis are all independent of immune system interactions, such as with the complement system. Complement is not considered to play a large role in MuSK MG due to the predominance of IgG4 autoantibodies. However, co-occurring MuSK IgG1-3 antibodies may in theory bind complement.

Bivalent MuSK IgG1-3 antibodies, that are non-pathogenic in the absence of complement, might thus cause damage if complement can bind. In addition, IgG4 can activate the lectin complement pathway in a glycosylation-dependent manner in another IgG4-AID [58]. Although complement is certainly not required to develop MuSK MG, it may have a disease modifying effect for a subset of autoantibodies. So far, there is no support for complement activation in muscles of MuSK MG patients [59]. However, it is important to realize that complement deposition has been investigated on very limited human muscles. Serum analyses do suggest an active alternative complement pathway in MuSK MG patients [60]. To better understand if complement activation has any relevance in MuSK MG, it will be interesting to investigate whether MuSK IgG4 antibodies have glycosylation patterns compatible with the lectin pathway and if bivalent MuSK IgG1 antibodies can activate complement on muscles [61].

*From monoclonal back to polyclonal*

In this thesis, monoclonal antibodies were used to interrogate the mechanisms of disease in MuSK MG. However, MuSK MG patients have a pool of MuSK antibodies with different characteristics. Most patients have MuSK antibodies predominantly of the IgG4 subclass (mostly monovalent) and targeting the Ig-like 1 domain of MuSK, but co-occurring MuSK antibodies of other IgG subclasses (bivalent) and targeting other domains of MuSK are regularly found [4-6, 62]. In patients, different mechanisms thus likely contribute to their disease. Passive transfer of MuSK MG patient-purified IgG(4) induces *in vivo* muscle weakness similar to monovalent MuSK antibodies. This supports the idea that binding of monovalent IgG4 MuSK antibodies to MuSK is the most relevant pathological mechanism in patients with MuSK MG [3, 63-67]. However, in some patients purified total IgG can activate MuSK phosphorylation or induce some AChR clustering *in vitro*, suggesting bivalent MuSK antibodies can play a role [68, 69]. The unique composition and relative abundance of MuSK antibodies with different characteristics will thus determine the net effect and pathogenic mechanisms in an individual patient.

To understand the complexity of the anti-MuSK response, it will be interesting to decompose the polyclonal MuSK antibody pool in individual patients to determine if it consists of a few dominant clones or many different clones. Monoclonal MuSK antibodies can then be used to investigate how the effects of different clones influence each other. Co-occurring pathogenic bivalent MuSK antibodies may contribute to the disease pathophysiology of monovalent MuSK antibodies, while nonpathogenic bivalent MuSK antibodies may counteract pathogenic

effects of both monovalent and bivalent MuSK antibodies. In addition, it can be hypothesized that multiple antibodies binding to MuSK simultaneously may work synergistically to disrupt more processes due to steric hindrance or conformational changes. In line with this, some polyclonal preparations of bivalent MuSK antibodies could completely inhibit AChR clustering, while this was generally not the case for monoclonal bivalent MuSK antibodies [6, 27-29, 31, 47-49]. Although monoclonal antibodies are a great tool to systematically investigate the consequence of specific antibody characteristics, we ultimately need to combine them again to generate a more complex model based on multiple MuSK antibody characteristics. Such a model may be of better predictive value for disease severity and therapeutic response and thereby benefit clinical management.

## Therapeutic potential of the MuSK signaling pathway

Since nonpathogenic bivalent (agonistic) MuSK antibodies have the opposite effect of monovalent (antagonistic) MuSK antibodies, it is tempting to speculate they can also have therapeutic potential for MuSK MG by counteracting the effect of pathogenic (monovalent) MuSK antibodies. Agonistic MuSK antibodies against the Ig-like 1 domain of MuSK can partly rescue *in vitro* AChR clustering of monovalent MuSK antibodies and MuSK MG patient-purified IgG, suggesting MuSK agonists can overcome antibody-induced MuSK inhibition [52]. So far, the *in vitro* rescue by MuSK agonists binding the Ig-like 1 domain of MuSK was not able to prevent *in vivo* muscle weakness upon passive transfer of polyclonal MuSK MG patient-purified IgG4. In addition, some male Bl6 mice developed a lethal urologic syndrome. This phenomenon did not occur in NOD/SCID mice and therefore appears mouse strain-specific and may not translate to humans. Nevertheless, it complicates further pre-clinical development of monoclonal antibodies targeting the Ig-like 1 domain of MuSK as a therapeutic [52].

To understand or even anticipate unexpected (detrimental) side-effects of new therapeutics, it is important to know where the target is expressed. In **chapter 6**, we investigated the spatio-temporal expression pattern of eight "MG-related genes" involved in maintaining neurotransmission, which cause (congenital) myasthenia gravis when impaired by autoantibodies or mutations [70]. This revealed that many of these genes are widely expressed in tissues other than skeletal muscle. For example, *DOK7* was prominently expressed in the cerebellum, pituitary gland, testis and heart. The function of these tissues thus warrants monitoring upon

*DOK7* targeted therapies. *MUSK* is expressed in the testis, small intestine and bladder. This may guide further studies in light of the urologic syndrome observed in male mice discussed above.

Impaired neurotransmission is not only a problem in MG, but also in neuromuscular disorders such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), muscular dystrophies and sarcopenia. Stimulating AChR clustering through the MuSK signaling pathway may have therapeutic potential to enhance neurotransmission in multiple of these diseases [71]. The MuSK signaling pathway can be activated in different ways. Activating MuSK signaling through *DOK7* gene therapy, Lrp4 overexpression or agrin biologicals has already shown beneficial effects in mouse models for Emery Dreifuss muscular dystrophy, SMA or sarcopenia [72-76]. In addition, activating MuSK directly with agonistic MuSK antibodies binding the Fz domain of MuSK was beneficial in mouse models for ALS and Dok7 congenital myasthenic syndrome, and did not cause unwanted side-effects [50, 51, 77]. Together these studies provide proof-of-concept that stimulating MuSK signaling, e.g. by agonistic MuSK antibodies, has therapeutic potential in neuromuscular disorders.

## Concluding remarks

Taken together, the results in this thesis contributed to our understanding of the cause and consequences of (IgG4) MuSK antibodies in MuSK MG. The IgG4 response in MuSK MG is unlikely to be driven by a predisposition to generate IgG4 responses or other abnormalities in the immunoglobulin response. Rather the characteristics of (prolonged exposure to) the autoantigen or an environmental trigger, combined with antigen presentation driven by variations in the HLA genes are likely contributors to the IgG4 autoimmune response. Overlap in for example the HLA associations between different IgG4-AIDs suggest a common underlying mechanism.

The class-switch to IgG4 may be critical for symptom manifestation in MuSK MG because Fab-arm exchange-induced functional monovalency amplifies the pathogenicity of many MuSK antibodies. In addition, an IgG4 response usually consists of high titers of high affinity antibodies. Therefore, an initial IgG1 response against MuSK may not result in clinical MG because of the low titers and their sometimes non-pathogenic agonistic effects. Although the effect of functional monovalency is rather unique to MuSK antibodies, the high titers and affinity of IgG4

autoantibodies may also play a role in symptom manifestation in other IgG4-AIDs.

Monoclonal patient-derived MuSK antibodies have been key in discovering the different consequences contributing to the mechanisms of disease in MuSK MG. We have learned the consequences MuSK antibodies have on the NMJ 1) can range from causing MG by impairing MuSK function or having therapeutic potential by inducing proper MuSK signaling, 2) is determined by how they influence MuSK dimerization and 3) depend on at least a combination of antibody subclass, valency, epitope, affinity, and relative titers. In the future, when we understand the composition of antibody characteristics in individual patients and how the different consequences combine, we can translate the effects of individual MuSK antibodies back to an integrated model of the mechanisms underlying MuSK MG.

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A

# Appendix

## English summary

Myasthenia gravis (MG) is a neuromuscular autoimmune disease caused by antibodies against muscle-specific kinase (MuSK) in about 5% of MG patients. MuSK MG patients suffer from fatigable skeletal muscle weakness of mainly bulbar muscles and have a high risk of experiencing a respiratory crisis. MuSK is a critical protein in forming and actively maintaining the neuromuscular junction (NMJ). Disruption of its function by antibodies impairs neurotransmission at the NMJ. MuSK antibodies are predominantly of the IgG4 subclass and therefore have the ability to swap half-molecules with other IgG4s (Fab-arm exchange), resulting in monovalent-antigen binding. MuSK antibodies have different characteristics depending on their (species-specific) subclass or isotype. **Chapter 1** describes the lessons we learned from prior MuSK antibody research by placing these different antibody characteristics in the appropriate context, thus helping us to understand the mechanisms underlying MuSK MG and the importance of understanding the source of antibodies that was used.

One of the hypotheses regarding the cause of the IgG4 response against MuSK is addressed in **chapter 2** by investigating the serum immunoglobulin composition in MuSK MG patients (IgG4 autoimmune disease) compared to acetylcholine receptor (AChR) MG patients (IgG1/3 autoimmune disease) and healthy donors. This study shows serum IgG4 is enriched in MuSK MG patients compared to the other groups, but for most MuSK MG patients their serum IgG4 titers fall within the normal range. MuSK MG patients do not seem to have a general predisposition to generate IgG4 responses. The predominance of IgG4 MuSK antibodies thus has a different cause.

Monoclonal antibodies are great tools to systematically investigate the consequence of specific antibody characteristics. **Chapter 3** describes the isolation of monoclonal MuSK antibodies from MuSK MG patients. Six B-cell receptor sequences were determined from single MuSK-reactive B-cell cultures and produced recombinantly as monoclonal antibodies. Monovalent anti-MuSK Fab fragments are antagonists of MuSK signaling, recapitulating the effects of MuSK MG patient-purified IgG4. In contrast, bivalent anti-MuSK IgG are agonists of MuSK signaling, likely by forcing the dimerization of two MuSK molecules independent of agrin. Valency of these monoclonal MuSK antibodies thus dictates their consequences on *in vitro* MuSK signaling.

**Chapter 4** translates the valency-dependent *in vitro* effects of MuSK antibodies to *in vivo* pathogenicity. Upon passive transfer, stable monoclonal monovalent MuSK antibodies cause rapid onset fatigable muscle weakness characterized by complete loss of muscle strength, weight loss and postsynaptic loss of AChR receptors, independent of antibody clone. In contrast, the pathogenicity of the bivalent equivalents was clone-dependent and less severe: one clone was not pathogenic, while the other clone caused slower onset muscle weakness and weight loss. These differential effects are likely caused by the opposing valency-dependent effects on MuSK signaling described in chapter 3. Functional monovalency thus amplifies the pathogenicity of monoclonal MuSK antibodies, indicating that class-switch to IgG4 MuSK antibodies may be a critical step in developing MuSK MG symptoms.

To further understand the pathomechanisms underlying MuSK MG, **chapter 5** explores the consequences of MuSK antibodies with different functional characteristics on the NMJ. Antibody valency influenced the consequences on agrin-induced MuSK activation, Dok7 binding to MuSK and NMJ gene expression. In addition, monovalent MuSK antibodies binding the frizzled domain of MuSK did not inhibit agrin-induced MuSK activation, while Ig-like 1 domain binders do. Bivalent MuSK antibodies affected the kinetics of Dok7 degradation differently, possibly depending on binding epitope between and within structural domains of MuSK. The consequences and pathogenicity of MuSK antibodies thus at least depend on a combination of valency and epitope. The pathophysiology underlying MuSK MG in individual patients thus likely depends on the unique composition of their MuSK antibody pool.

To broaden the view on potential consequences of antibodies in MG beyond the NMJ, **chapter 6** gives a comprehensive overview of the timing and localization of gene expression of MG-related genes. This *in silico* analysis using existing expression databases revealed that MuSK and seven other genes affected in (congenital) myasthenia gravis are expressed in different locations outside skeletal muscles. Since the co-expression of these genes in these locations is limited, it is unlikely they converge on the same functional pathway as in skeletal muscles. However, these locations are at risk for non-muscular (side-)effects of autoantibodies and new therapeutics targeting these proteins.

## Nederlandse Samenvatting

Myasthenia gravis (MG) is een neuromusculaire auto-immuunziekte die wordt veroorzaakt door antistoffen tegen het eiwit muscle-specific kinase (MuSK) in ongeveer 5% van de MG patiënten. MuSK MG patiënten hebben last van vermoeibare spierzwakte vooral in hun bulbaire spieren en hebben een verhoogd risico op een ademhalingscrisis. MuSK is een essentieel eiwit voor het vormen en in stand houden van de zenuw-spierovergang. Verstoring van MuSK's functie door antistoffen hindert de signaaloverdracht in de zenuw-spierovergang. MuSK antistoffen zijn vooral van de IgG4 subklasse en kunnen daardoor half-moleculen uitwisselen met andere IgG4s (Fab-arm exchange). Dit resulteert in monovalente binding aan MuSK. De eigenschappen van MuSK antistoffen hangen af van de (diersoort-specifieke) subklassen en isotypes. **Hoofdstuk 1** beschrijft de lessen die we hebben geleerd uit eerder onderzoek met MuSK antistoffen door de verschillende eigenschappen van antistoffen in de juiste context te plaatsen. Dit helpt ons te begrijpen wat de veroorzakende mechanismen van MuSK antistoffen in MuSK MG zijn, en dat de bron van de gebruikte antistoffen belangrijk is.

Eén van de hypothesen met betrekking tot waarom antistoffen tegen MuSK van de IgG4 subklasse zijn, wordt behandeld in **hoofdstuk 2**. Hier wordt het onderzoek beschreven hoe de samenstelling van immunoglobulines in het serum van MuSK MG patiënten (IgG4 auto-immuunziekte) verschilt met die van acetylcholine receptor (AChR) MG patiënten (IgG1/3 auto-immuunziekte) en gezonde donoren. Deze studie laat zien dat MuSK MG patiënten relatief meer IgG4 in hun serum hebben vergeleken met de andere groepen, maar voor de meeste MuSK MG patiënten valt hun IgG4 titer binnen de normaalverdeling. MuSK MG patiënten lijken dus niet een algemene aanleg te hebben om IgG4 antistoffen te produceren. De dominantie van IgG4 MuSK antistoffen heeft dus een andere oorzaak.

Monoklonale antistoffen zijn goede hulpmiddelen om systematisch de gevolgen van specifieke eigenschappen van antistoffen te onderzoeken. **Hoofdstuk 3** beschrijft de isolatie van monoklonale MuSK antistoffen van MuSK MG patiënten. Zes sequenties van B-cel receptoren zijn bepaald uit klonale MuSK-reactieve B-cel kweken en recombinant geproduceerd als monoklonale antistoffen. De hieruit voortkomende monovalente Fab fragmenten tegen MuSK zijn antagonisten van MuSK signalering, vergelijkbaar met de effecten van gezuiverd IgG4 van MuSK MG patiënten. Bivalente MuSK antistoffen zijn daarentegen agonisten van

MuSK signalering, waarschijnlijk door het forceren van de dimerisatie van twee MuSK moleculen onafhankelijk van agrine. De valentie van deze monoklonale MuSK antistoffen bepaalt dus welke gevolgen ze hebben op de *in vitro* MuSK signalering.

**Hoofdstuk 4** vertaalt de valentie-afhankelijke *in vitro* effecten van de MuSK antistoffen naar de mate van ziekmakendheid *in vivo*. Passieve immunisatie met monoklonale monovalente MuSK antistoffen onafhankelijk van de kloon veroorzaakt snel vermoeibare spierzwakte gekarakteriseerd door compleet verlies van spierkracht, gewichtsverlies en het verlies van AChR in de zenuw-spierovergang. In tegenstelling zijn de effecten van hun equivalente bivalente antistoffen wel afhankelijk van kloon en zijn zij óf niet ziekteverwekkend óf minder ziekteverwekkend met een langzamere ontwikkeling van gewichtsverlies en spierzwakte. De valentie-afhankelijke verschillen komen waarschijnlijk door de tegenovergestelde effecten op MuSK signalering beschreven in hoofdstuk 3. De functionele monovalentie verergert dus de ziekmakendheid van monoklonale MuSK antistoffen. Dit suggereert dat de overschakeling van IgG subklasse naar IgG4 MuSK antistoffen mogelijk een kritieke stap is in het ontwikkelen van MuSK MG klachten.

Om de ziektemechanismen van MuSK MG beter te begrijpen, onderzoekt **hoofdstuk 5** de gevolgen van MuSK antistoffen met verschillende eigenschappen op de zenuw-spierovergang. Dit hoofdstuk laat zien dat de valentie van de MuSK antistoffen de gevolgen op agrine-gemedieerde MuSK activatie, Dok7 binding aan MuSK en expressie van zenuw-spierovergang genen beïnvloedt. Daarnaast verhindert een monovalente MuSK antistof tegen het frizzled domein van MuSK agrine-gemedieerde MuSK activatie niet, terwijl MuSK antistoffen tegen het Ig-like 1 domein dat wel doen. Bivalente MuSK antistoffen hebben verschillende effecten op de afbraak van Dok7, welke wellicht afhankelijk zijn van het epitoop tussen en binnen structurele domeinen van MuSK. De gevolgen en ziekteverwekkendheid van MuSK antistoffen hangen dus af van minstens een combinatie van valentie en epitoop. De pathofysiologie van MuSK MG in individuele patiënten hangt waarschijnlijk af van de unieke samenstelling van hun MuSK antistoffen.

Om breder zicht te krijgen op de mogelijke gevolgen van antistoffen in MG buiten de zenuw-spierovergang geeft **hoofdstuk 6** een uitgebreid overzicht van de timing en lokalisatie van genexpressie van MG-gerelateerde genen. Uit deze *in silico* analyse van bestaande expressie databases blijkt dat MuSK en zeven andere genen betrokken bij

(congenitale) myasthenia gravis tot expressie komen op verschillende locaties buiten skeletspieren. Deze genen laten een beperkte co-expressie zien in deze locaties. Dit maakt het onwaarschijnlijk dat deze genen samenwerken met één functioneel doel zoals in skeletspieren. Deze locaties lopen wel risico op niet-spier gerelateerde symptomen van autoantistoffen, of bijwerkingen van nieuwe therapieën gericht op deze eiwitten.

## List of publications

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## Curriculum vitae

Dana Vergoossen was born on the 7th of September 1991 in Haarlem. She graduated from het Alkwin Kollege in Uithoorn in 2009. The next school year she spent attending Kenai Peninsula College in Kenai, Alaska learning about the native Dena'ina language, American politics and fly-fishing. In 2010 she started the BSc in Liberal Arts and Sciences at University College Utrecht, where she was able to combine her love of human biology and psychology. She studied the biology of the brain during her MSc in Neuroscience at the Vrije Universiteit in Amsterdam between 2013 and 2015. During her first internship in the laboratory of Dr. Wilma van de Berg, she investigated amyloid-β pathology in post-mortem human brain tissue from patients with Parkinson's disease and dementia with Lewy bodies. She did her second internship in the lab of Professor Richard Wade-Martins at the University of Oxford studying how the lysosomal enzyme glucocerebrosidase-1 functions under cellular processes implicated in Parkinson's disease.

A week after her master's graduation ceremony in 2016, Dana returned to the University of Oxford to continue her work on the cellular processes in Parkinson's disease, this time in induced pluripotent stem cell derived dopaminergic neurons from patients. In October 2017, the Netherlands called her back and she started her PhD at Leiden University Medical Center under the supervision of Dr. Maartje Huijbers, Professor Silvère van der Maarel and Professor Jan Verschuuren. She studied the cause and consequences of MuSK antibody pathogenicity in myasthenia gravis; the results of which are described in this thesis. Currently, Dana is still studying MuSK myasthenia gravis as a postdoctoral researcher in the lab of Dr. Maartje Huijbers at the Leiden University Medical Center.

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