I

General introduction

Introduction to myasthenia gravis

Myasthenia gravis (MG) is one of the best characterised neuromuscular or autoimmune disorders. The Greek words myos (muscle) and asthenos (weakness) refer to the presence of pure motor weakness that can affect extraocular, bulbar, axial and limb muscles. Myasthenic weakness is characterised by its fluctuating nature and fatigability. When symptoms are restricted to ophthalmoparesis and ptosis, MG is classified as ocular MG (OMG). Weakness in other muscles is termed generalised MG (GMG). Thomas Willis was probably the first to describe a myasthenic patient in his book "De Anima Brutorum" from 1672. He described a "prudent and honest woman" suffering from fluctuating weakness in the arms and legs, but also from compromised articulation. [1]

> *"For some time she can speak freely and readily enough, but after long, hasty or laborious speaking she is not able to speak a word, but becomes mute as a fish and does not recover till after an hour or two"*

It cannot be proven, nor ruled out, that this was in fact the first patient described with autoantibodies to muscle-specific kinase (MuSK), one of the key elements in neuromuscular signal transmission, as will be highlighted in this thesis. By the end of the 19th century, Wilhelm Erb and Samuel Goldflam provided a detailed description of the disease in which ptosis and ophthalmoparesis frequently occur as first symptoms, followed by weakness of chewing, swallowing and speaking, with a further spread towards neck and limb muscles. The clinical hallmark of MG is the fluctuating character of the weakness, becoming worse upon sustained movement and by the end of the day. [2,3] In 1934, Mary Walker reported a temporary improvement of facial weakness in an MG patient after subcutaneous injections of physostigmine. By then, it was known that physostigmine inhibited the breakdown of the neurotransmitter acetylcholine (ACh). Together with the discovery of the role of ACh in neuromuscular signal transmission by Dale and Feldberg in that same year, the pathology of MG was thought to be a curare-like poisoning of the "motor end-organs or myoneural junctions". [4,5] The first description of histological malformation of the neuromuscular junction (NMJ) in MG patients was made by Coërs and Desmedt in 1959. [6] In 1960, after having studied 440 MG cases, the Scottish neurologist Simpson postulated the still current autoimmune hypothesis of the mechanism of MG, based on observations including thymic hyperplasia and occasional transfer of the disease from a mother to her newborn child during the first weeks of life. He also acknowledged a genetic susceptibility for immunological disorders such as systemic lupus erythematosus in patients and thyroid disease in their relatives. [7] In 1976, the biochemist Lindstrom induced antibodies as a coincidental side-effect of his studies in rabbits injected with the postsynaptic acetylcholinereceptor (AChR) derived

from electric eels. [8] The anti-AChR antibody positive and weak rabbits were recognised as being "myasthenic". The final step was made when Lindstrom *et al*. were able to identify autoantibodies to the AChR as the causative factor for this transmission defect in 87% of 71 MG patients. [9]

Pathogenesis of classical MG with autoantibodies to AChR

The AChR is a cation channel formed by five subunits. In human muscle, two subtypes occur *i.e.* a fetal receptor consisting of two α1 subunits and one β1, γ and δ subunit, and an adult receptor in which the γ subunit is replaced by an ε subunit. The receptors are densely concentrated and located at the crests of the folds of the postsynaptic muscle membrane. A nerve action potential causes ACh to be released from presynaptic vesicles, which then diffuses across the synaptic cleft. Binding of ACh to the AChR depolarizes the postsynaptic endplate membrane generating an endplate potential which then opens Na^+ channels located in the depths of the postsynaptic folds. This causes further depolarisation, finally triggering an action potential that propagates along the muscle fiber leading to muscle contraction. ACh diffuses out of the synaptic cleft or is readily hydrolysed by acetylcholinesterase (AChE) thus ending the depolarisation. The endplate potential generated by ACh release and binding of ACh to the AChR is normally much higher than the threshold needed to generate a muscle action potential. In myasthenic syndromes this safety factor is typically compromised, either by pre- or postsynaptic pathology.

In classical MG with autoantibodies to the AChR (AChR MG), the antibodies affect the neuromuscular transmission in three ways. Firstly, antibodies can directly compete with ACh binding, although titres of such blocking antibodies only weakly correlated with disease severity. [10] More importantly, antibodies are able to crosslink AChRs, thereby increasing AChR turnover through internalisation and lysosomal degradation by muscle fibers. [11]

However, the third and most crucial factor affecting transmission is a reduction in the number of available AChRs and changes in endplate morphology due to complement activation, as was shown by Lennon *et al.* in a rat model using passive and active immunisation. [12] Complement deposition was located at the neuromuscular junction in MG patients correlating with destruction of the folded postsynaptic membrane. [13,14] In 1987, Rodgaard *et al*. published the predominance of antigen-specific IgG subclasses 1 and 3 directed to AChR in MG patients. Those subclasses are well known for their complement activating capacity. [15]

The presence of other autoimmune diseases in patients with AChR MG and their first-degree relatives suggests a role for a genetic predisposition. The human leukocyte antigen (HLA) concerns large number of genes that encode proteins involved in the presentation of antigens to the immune system. An extremely large genetic variability of HLA molecules within

the human population is an important factor determining the flexibility of the acquired immune system. However, several HLA haplotypes are thought to increase the likelihood of developing autoimmune diseases. For example, the HLA A1-B8-DR3 ancestral haplotype is associated with many autoimmune diseases, including Graves' disease, [16] celiac disease, [17] the Lambert-Eaton myasthenic syndrome (LEMS) without small cell lung carcinoma, [18] and type I diabetes mellitus. [19] AChR MG without thymoma and an onset before the age of 40 years is also strongly associated with this haplotype in white Europeans. [20-23] By contrast, late onset MG which occurs more frequently in men has been linked to HLA DR2. [23-25]

Definition of "seronegative" MG

The discovery of antibodies to the AChR in the majority of MG patients in 1976 immediately defined a group of patients diagnosed as MG based on their phenotype in whom no such antibodies could be found. They were defined as "seronegative" MG (SNMG). Between 1976 and 1990, the prevalence of SNMG was reported in at least 12 studies. Overall SNMG was present in 48.1% of patients who had ocular symptoms only (weighted average of n = 412, range 21-83%) and 11.5% of patients with generalised weakness (weighted average of n = 2272, range 6-25%). [9,23,26-35] Although the lower frequency in GMG compared to OMG is consistently found, the large variability probably reflects patient selection and referral bias.
Multiple explanations for the absence of antibodies in the presence of a myasthenic phenotype were proposed including the possibility of seroconversion early in the course of the disease, [36] seroconversion after treatment with immunosuppressive therapy, [29,36,37] or during periods of clinical remission. [38] In children, seroconversion has been described occurring after many years, ranging from 5-10 years, after the onset of (ocular) symptoms. [39]
Adaptations to the assays used to detect AChR antibodies decreased the number of SNMG patients by using human AChR instead of rat or calf derived AChR, [40,41] a mixture of human AChR derived from normal and denervated muscle instead of denervated muscle only, [42] or AChR derived from cell lines. [43] This fine-tuning has continued in recent years through the introduction of cell-based assays using sensitive fluorescence techniques to detect low-affinity antibodies as will be highlighted in the Chapters 3 and 5 of this thesis. [44]
Finally, it became clear that congenital myasthenic syndromes caused by mutations in proteins involved in neuromuscular transmission such as rapsyn and Dok7 could also present as a late onset phenotype. [45,46]
However, strong evidence arose that SNMG was in fact an acquired autoimmune disease caused by autoantibodies directed to unknown antigens. Firstly, clinical symptoms responded to immunosuppressive therapy and plasmapheresis. [27,34,47,48] Secondly, the disease could be transferred from mother to child through the placenta. [49,50] Thirdly, passive transfer using

human serum or IgG from SNMG patients caused a neuromuscular transmission defect and possibly a small loss of AChRs in the mouse diaphragm. [51,52] Moreover, IgG from SNMG patients was shown to bind to an unknown antigen present on a muscle cell line. [53]

Autoantibodies to MuSK in seronegative myasthenia gravis

In 2001, Hoch *et al*. were the first to describe autoantibodies in 17 out of 24 patients with generalised SNMG directed to the extracellular domain of an antigen called muscle-specific kinase (MuSK) using immunofluorescence on transfected cells, immunoprecipitation and ELISA. The autoantibodies also interfered with MuSK function, *i.e.* they prevented the formation of AChR clusters normally induced by nerve-derived agrin. [54] Patients with MG who had autoantibodies to MuSK (MuSK MG) appeared to differ clinically from AChR antibody negative/MuSK antibody negative patients because weakness was more prominent in oculobulbar muscles with frequent respiratory insufficiency. [55] In the MuSK MG group, treatment with oral immunosuppressants seemed less effective. Some MuSK MG patients adversely responded to AChE inhibitors with widespread fasciculations, and muscle atrophy occurred later in the course of the disease. [56] In retrospect, this phenotype had already been identified as a subgroup within a cohort of SNMG patients by Evoli *et al*. in 1996. [57] Studying larger cohorts of AChR Ab- MG patients, the percentage of MuSK MG was adjusted downward leaving a significant group of AChR Ab-/MuSK Ab- patients nonetheless.

The role of MuSK in the neuromuscular junction

Synaptic development of a neuromuscular junction requires specialisation of muscle fibers in the proximity of a motor nerve terminal. This specialisation includes at least two mechanisms, *i.e.* the local transcription of specific proteins by the synaptic nuclei including the AChR subunits, and the redistribution of pre-existent molecules present on the surface of the myofiber. The result is clustering of AChRs on the crests of the postsynaptic membrane folds and the formation of postsynaptic intracellular signalling pathways.
Nerve-derived agrin, a 200 kDa heparin sulphate glycoprotein, proved able to aggregate AChR in cultured chicken myotubes, a process inhibited by polyclonal anti-agrin antibodies. [58] Because phosphotyrosine staining in myofibers is concentrated at the NMJ and tyrosine kinases are often involved in cell-cell interaction, Jennings *et al*. used polymerase chain reaction (PCR) to look for tyrosine kinases in the electric organ and skeletal muscle of the electric ray *Torpedo californica.* They were able to isolate a transmembrane tyrosine kinase with a size of 100 kDa. [59] In 1995, Valenzuela *et al*. cloned similar receptor protein kinases using

cDNA from rat muscle and both normal and denervated human muscles. They introduced the term muscle-specific kinase (MuSK). In this study, MuSK colocalised with AChR at the neuromuscular junction. MuSK expression was upregulated after fusion of myoblasts to form myotubes. In normal adult muscle, MuSK was present only at the neuromuscular junction, but MuSK and AChR transcripts became widely detectable throughout the myofibers after denervation. Activated MuSK dimerizes and triggers phosphorylation of the β subunit of the AChR. [60]

The *MuSK* gene contains 14 exons and maps to chromosome 9q in humans and to chromosome 4 in mice. [61] The extracellular domain of MuSK contains 4 immunoglobulin-like domains and a cystein rich domain. MuSK has a molecular weight of 110 kDa and contains a single transmembrane region followed intracellularly by a juxtamembrane domain containing one of the four tyrosine residues (Tyr553) that is first autophosphorylated upon activation by agrin (Figure 1.1). [62] Next is the catalytic domain containing the tyrosine kinase itself. This kinase contains three tyrosine autophosphorylation sites in an activation loop (Tyr750, 754 and 755). Such a loop is a general characteristic of the receptor tyrosine kinase family that autoregulates catalytic activity by obstructing adenosine triphosphate (ATP) and substrate binding. After phosphorylation of the tyrosine residues, MuSK is switched to a stable active state. [63] Activated MuSK is then rapidly internalised through endocytosis which is also a common property for receptor tyrosine kinases. [64]

Interactions with other proteins

Agrin

In 1996, DeChiara *et al*. reported on a mouse model carrying a *MuSK* gene disruption in which mice were immobile and died at birth. Their skeletal muscles were grossly normal, indicating normal early skeletal muscle development. However, pre- and postsynaptic differentiation was absent, and intramuscular nerves were not able to contact the muscle fibers. [65] Postpartum inhibition of MuSK by RNA interference also induced the disassembly of NMJs that had already been built, indicating also a role for MuSK in NMJ maintenance. [66] Mice lacking agrin showed a remarkably similar phenotype either dying in utero or being stillborn. [67] In 1996, Glass *et al*. reported that agrin was indeed the factor inducing the phosphorylation of MuSK. Even long-lasting phosphorylation of MuSK and AChR, as well as clustering, can be initiated be a single five minute pulse of agrin. [68] In 2006, Stiegler *et al.* showed that the most N-terminal Ig-like domain in MuSK is crucial for this function. [69]

LRP4

No direct binding of agrin to the MuSK ectodomain could be demonstrated. Phosphorylation of MuSK in response to agrin only took place in myotubes, but not in myoblasts or other cell types, transfected with a MuSK expression factor. This suggested the presence of another hypothetical component termed myotube associated specificity component (MASC). [70] Moreover, whereas the intracellular MuSK kinase domain was sufficient for MuSK autophosphorylation and AChR phosphorylation, the ectodomain of MuSK was essential for clustering of the AChRs. [71] In 2006, Weatherbee *et al*. showed that mice with a mutation in the gene encoding the low-density lipoprotein receptor-related protein 4 (LRP4) died at birth and their synapses had both pre- and postsynaptic defects very much like MuSK mutant mice. This included aberrant motor axon growth, axonal branching and a lack of postsynaptic clustering. [72] In 2008, the receptor for neural agrin proved to be LRP4 according to two independent groups. [73,74] LRP4 associates with itself and forms a complex with MuSK in the absence of agrin. LRP4 is expressed specifically in myotubes, but not myoblasts, thereby explaining its function as the above mentioned MASC. The intracellular domain of MuSK becomes tyrosine phosphorylated upon stimulation of LRP4 with agrin. The binding between LRP4 and agrin is enhanced approximately 20 fold by the presence of calcium. [73]

Rapsyn

Similar to MuSK, the intracytoplasmatic protein rapsyn (an acronym for receptor-associated protein of the synapse) was also known to colocalise with AChR at the developing NMJ. In 1995, Gautam *et al*. showed that rapsyn null mice died within hours after birth. There were no detectable AChR clusters anywhere along the muscle fibers which proved that rapsyn was essential for clustering. [75] Recently, the binding between AChR and rapsyn was found to depend on a 20 amino acid motif in the intracellular domain of the AChR β subunit in which one tyrosine amino acid (Tyr390) is phosphorylated through the agrin-MuSK pathway. Because rapsyn has also AChR binding capacity independent of the β loop, it is postulated that more than one rapsyn molecule can bind to phosphorylated AChR. Rapsyn thus acts as a scaffold protein in the NMJ. [76]

Dok7

The cytoplasmatic protein Dok7 (downstream of tyrosine kinase 7) also proved essential for NMJ formation. Okada *et al*. showed that Dok7 null mice died at birth and did not form AChR clusters or NMJs. [77]

The phosphorylated tyrosine in the juxtamembrane domain of MuSK mentioned earlier (Tyr553) becomes a docking site for the phosphotyrosine binding site of Dok7. A tetramer is then created in which a Dok7 dimer binds to two MuSK monomers (Figure 1.1). The juxtapositioning of two MuSK kinase domains facilitates the autophosphorylation of the

MuSK activation loop. Therefore Dok7 is not only a substrate for MuSK, but also activates the catalytic domain. [78] The binding of Dok7 to MuSK is also dependent on the interaction of MuSK and a tumorous imaginal disc protein (Tid1), which function remains to be clarified further. [79]

Acetylcholinesterase (AChE)

An important enzyme in limiting the duration of the presynaptic trigger by the released ACh is acetylcholinesterase (AChE). This enzyme is organised into 3 tetramers of catalytic subunits that are linked to collagen Q (ColQ), a triple helical collagen subunit. ColQ itself is connected to the postsynaptic basal lamina by binding to perlecan, but also to MuSK through its C-terminal domain. [80]

Intracellular interactions

The intracellular pathways leading from MuSK activation to a fully developed NMJ with stable AChR clusters and a sustained MuSK activation are becoming increasingly complex and remain poorly understood. It requires the presences of multiple intracytoplasmatic enzymes like geranylgeranyltransferase I and Rac I essential for the first micro aggregates, Rho GTPase and possibly PAK1 that are involved in the formation of larger clusters and non-receptor tyrosine kinases like Src and Abl that play a role in the early and late response to agrin. Multiple enzymes have been found to contribute to negative feedback pathways and are involved in the disassembly of AChR clusters. As mentioned earlier, activated MuSK is internalised through endocytosis. For this process, the ATPase N-ethylmaleimide sensitive fusion protein (NSF) that binds directly to MuSK is essential. [64] Internalised MuSK is probably degraded upon binding to the ubiquitin tag to direct it to the proteasome. In this respect, a PDZ domain containing RING finger 3 (PDZRN3) ligase has been identified that binds to MuSK and regulates MuSK cell surface levels. [81] MuSK also binds to caveolin 3, a structural protein component of caveolae in muscle cells, but the role of this interaction remains to be elucidated. [82] A delicate balance between these mechanisms probably guides the ongoing process of building and remodelling of NMJs. [83]

Role of MuSK prior to neural innervation

AChR clusters are also formed on immature muscle fibers *prior* to the innervation by motor nerves. In 2001, Lin *et al*. showed that in mice during embryogenesis at day 14.5 AChR clusters were present, but not apposed by a nerve terminal whereas at day 18.5 all remaining clusters were apposed by a nerve terminal. This process, called prepatterning, is restricted to the central part of the muscle fiber. However, no clustering took place in MuSK or rapsyn mutant models indicating that these two proteins are also essential in clustering prior to neural innervation. Agrin was found to be essential in a later stage for stabilisation and growth of the neurally apposed synapses and dispersion of aneural clusters. [84]

Yang *et al*. reported similar results in a mouse model in which motor neurons lose their ability to differentiate through inactivation of the homeodomain transcription factor HB9. AChRs still cluster in these mice, and AChR transcription is increased in the central region of the diaphragm muscle, although in a wider zone than normal. For this clustering, postsynaptic MuSK, but not nerve-derived agrin, proved to be essential. [85] In 2008, Kim and Burden showed that MuSK expression itself is patterned in this central region of the muscle fiber in the absence of neural innervation, and that ectopic MuSK expression induces ectopic AChR clusters promoting axonal outgrowth. [86] Recently, a key protein in this pre-patterning process of developing muscle fibers proved to be the dihydropyridine receptor (DHPR), the L-type calcium channel in muscle. [87] This transmembrane protein acts as a voltage sensor. On depolarization of the sarcolemma, it activates the associated type 1 ryanodine receptor, leading to a release of calcium from the sarcoplasmatic reticulum, which then triggers muscle contraction. Chen *et al*. showed that in knockout mice lacking the β1 subunit of the L-type calcium channel DHPR (Cacnb1-/-), but also in double knockout mice lacking Cacnb1 and HB9, the pre-patterning of AChR clusters was lost. Nonetheless, synapses were established, but myofibers from DHPR deficient mice contained multiple endplates whereas the majority of control myofibers contained only a single endplate.

In summary, MuSK is involved in calcium-dependent aneural prepatterning of AChR clusters and in neural AChR cluster formation in the mature endplate. It is not clear if these aneural clusters are essential for NMJ formation, but they may serve as target for motor axons toward the centre of the muscle fiber. Neural agrin then binds through LRP4 to MuSK, causing autophosphorylation of the juxtamembrane domain and the active loop of the kinase. MuSK is thus switched to an active state which is enhanced by the binding of Dok7, creating a tetramer of two MuSK monomers and one Dok7 dimer. The β subunit of AChR is phosphorylated, enhancing its binding to the rapsyn scaffold protein. Activated MuSK is internalised and its active state is maintained by intracellular kinases, stabilising the NMJ independently of the presence of agrin. The essential role of most of these proteins in human neuromuscular transmission is further substantiated by the description of several congenital myasthenic syndromes involving mutations in MuSK, rapsyn, Dok7 or agrin. [88-91]

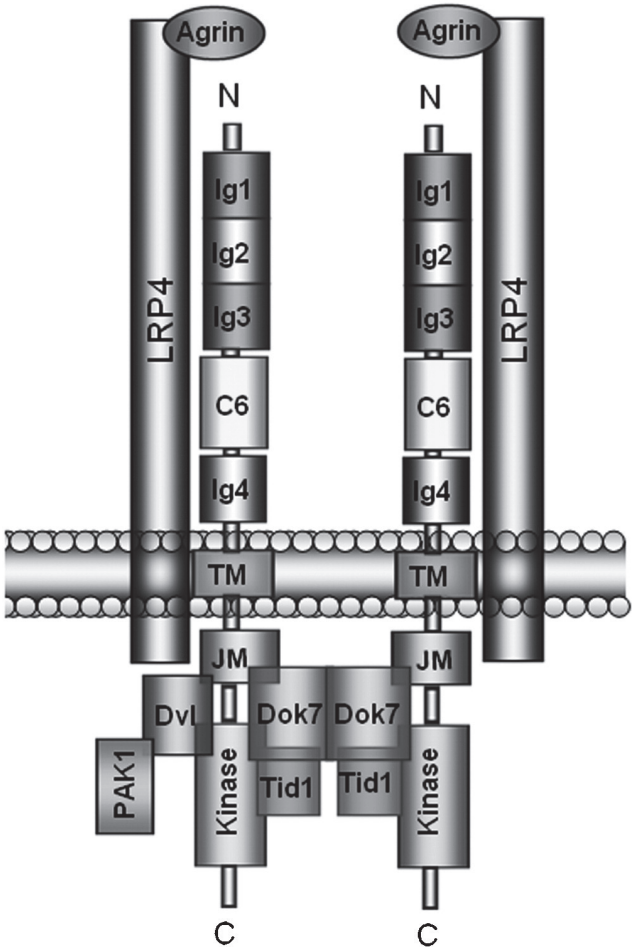

Figure 1.1 The assembled dimeric MuSK signalling complex
The extracellular domain contains 4 immunoglobulin-like domains and a cystein rich domain and interacts with the agrin binding receptor LRP4. Reprinted from the International Journal of Biochemistry and Cell Biology, Volume 43, Ghazanfari *et al*., Muscle specific kinase: organiser of synaptic membrane domains, Page 296, Copyright 2010, with permission from Elsevier Ltd.

Aims of the thesis

The aim of the studies in this thesis was to investigate the pathogenesis and the underlying immunological defect of MuSK MG. To reach this goal a detailed characterisation of the clinical course and features of patients with MuSK MG, and a study of the antibody response to the MuSK protein were first performed.
The discovery of anti-MuSK antibodies in a subset of patients classified until then as having "seronegative" MG (SNMG) raised the question whether these autoantibodies defined a new and distinct clinical disorder different from AChR MG. To investigate this, we first studied the epidemiology of MG subtypes in the Netherlands on a regional level using a well-defined area in the province of Zuid-Holland with a long-lasting and complete MG registry (Chapter 2). This enabled us to calculate population based data on the prevalence and incidence of the different subtypes of generalised MG.
We then undertook a nationwide study of patients with acquired generalised MG in the absence of AChR antibodies. This led to the identification of a relatively large cohort of SNMG and MuSK MG patients. Next, we described the clinical characteristics of MuSK MG patients and compared them to a cohort of AChR MG patients matched for sex and age at onset, and to patients with SNMG (Chapter 3). The immunopathological mechanism of MuSK MG was studied in more detail by investigating the association between MuSK MG and HLA haplotypes, described in Chapter 4. Longitudinal MuSK MG sera were collected to correlate antigen-specific IgG subclasses to clinical disease severity, thus substantiating their role in the immunopathogenesis (Chapter 5). In addition we searched for new antigens in AChR Ab-/MuSK Ab- patients. We hypothesised the existence of antibodies to the postsynaptic ErbB receptor, because ErbB is also a transmembrane protein accessible to an immunoresponse and involved in the development of the NMJ (Chapter 6).
Two case studies highlight the pivotal immunological role of IgG4 autoantibodies to MuSK (Chapter 7) and the electrophysiological characteristics of the neuromuscular transmission defect in MuSK MG (Chapter 8). Finally, overall results are summarised and discussed (Chapter 9).