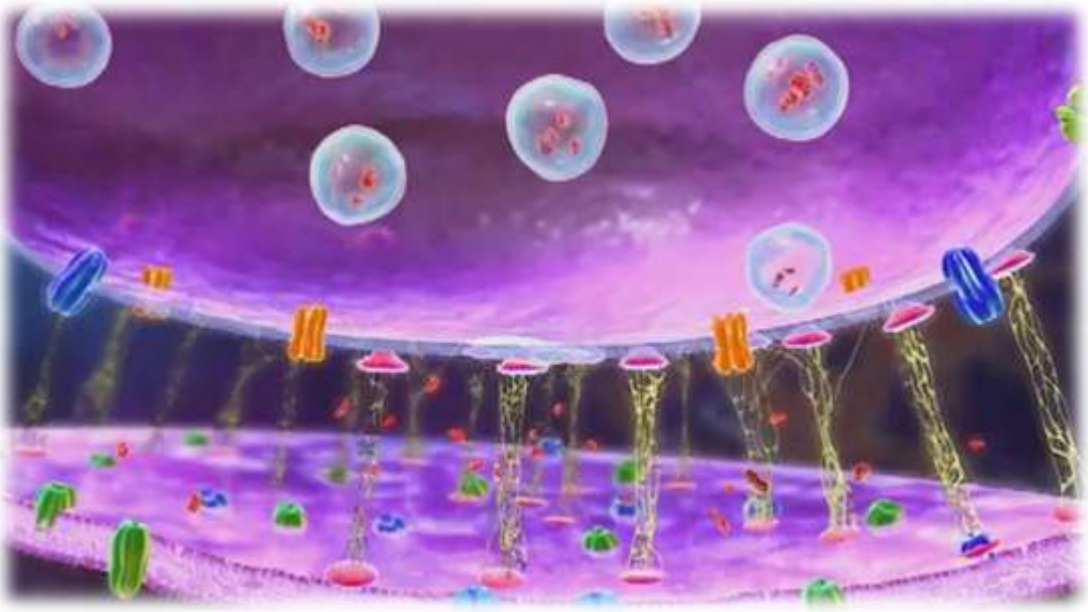
HELLENIC REPUBLIC
National and Kapodistrian University of Athens
EST. 1837



Investigation of antigen derived peptides and nanoparticles as a novel immunotherapy for Myasthenia Gravis

National and Kapodistrian University of Athens
Department of Physiology
Medical School

MSc Molecular Biomedicine

Baltatzidou Vasiliki

Athens 2018

EVALUATION COMMITTEE MEMBERS

Professor of Experimental Physiology George Kollias

Principal Investigator (Β') Zisimopoulou Paraskevi

Senior Researcher (Γ') Lazaridis Konstantinos

Acknowledgements

I would like to express my gratitude to my thesis supervisor Dr. Konstantino Lazaridi for his patient guidance, encouragement and pleasant collaboration throughout the project's duration. Dr. Paraskevi Zisimopoulou and Professor Socrates Tzartos whose valuable advices, support and patience were decisive in the completion of this endeavor. Professor and director Dr. George Kollia for his innovative MSc program that I was fortunate to participate in. Finally, to all my colleagues and family for their unwavering support and continuous encouragement.

Summary

Myasthenia gravis (MG) is an antibody-mediated, T-cell dependent, autoimmune disease, in which autoantibodies are mainly directed against the muscle nicotinic acetylcholine receptor (nAChR) at the neuromuscular junction. Impaired signal transduction due to AChR destruction by the autoantibodies leads to muscle weakness and fatigability. MG is currently treated with non-specific immunomodulatory agents, which may have several side effects. Hence, there is a great need to develop novel, more specific therapies. Such targeted treatments should aim on re-programming the specific autoreactive immune cells and restoring the lost tolerance towards the autoantigens.

Early studies with experimental autoimmune MG (EAMG) animal models showed that mucosal administration of disease-relevant antigens could induce such tolerance, providing the proof of principle for the therapeutic application of the approach. Despite these promising findings, a novel therapy has not yet been established.

We have developed an EAMG model based on immunization of Lewis rats with the human nAChR α1 subunit extracellular domain (hα1 ECD), well suited for testing antigen-specific treatments. We used this model to investigate the therapeutic potency of the ha1-ECD, by different administration routes, doses and time points during disease progression. Our data showed that when given intravenously and in repeated doses, hα1 ECD dramatically ameliorated EAMG symptoms. This was accompanied by reduced loss of nAChRs and lower levels of serum autoantibodies. Although, further investigation of the underlying mechanisms involved is needed, autoantigen-induced tolerization is a promising immunotherapy for MG. Moreover, since MG is a model antibody-mediated autoimmune disease, discovery of novel therapies could be applicable to other similar autoimmune diseases.

Table of Contents

1 Introduction

1.1 Myasthenia gravis

1.1.1 History of Myasthenia gravis

Myasthenia gravis (MG) was first described in 1672 by the anatomist Thomas Willis. Willis observed that the patients were suffering from weakness at their limb muscles, and the intensity of their symptoms fluctuated during the day. More than two centuries later, in 1895 the neurologist and psychiatrist Friedrich Jolly, was the first to show that repetitive stimulation of the nerve that innervates a muscle produces a decreasing muscle contraction in MG patients, which explains their weakness and fatigability. He described two cases with similar clinical symptoms as the ones described by T. Willis, under the name *myasthenia gravis pseudo-paralytica.* The words he chose to describe the disease, *myasthenia and pseudo-paralytica*, originate from the Greek words for muscle and fake paralysis, while *gravis* originates from Latin for heavy or grievous. In 1934, Mary Walker observed that the clinical manifestations of MG were similar to those caused by poisoning from curare, which was used at the time as treatment to severe cases of tetanus. She managed to show that physostigmine, curare's antidote improves the symptoms of MG. Some years later, in 1937 Alfred Blalock, a pioneer heart surgeon, reported signs of improvement in MG patients after thymus removal. The discovery of acetylcholine as a neurotransmitter at the motor axon terminal from Dale and Feldberg, paved the way for better understanding of the disease's pathogenesis [1-3]. Meanwhile, in 1960 MG was categorized as an autoimmune disease and in 1976 Lindstrom demonstrated the role of autoantibodies in MG pathogenesis[4].

1.1.2 Epidemiology

MG is a rare autoimmune disease with prevalence 150-300 per million population and annual incidence of 10 per million person-years [5]. MG prevalence has been rising since the middle of the last century due to improved recognition and diagnosis, medical and intensive care advances and patient longevity [6, 7]. The occurrence of MG is influenced by sex and age and there is a peak in incidence around the ages of 30 and of 50 years old [8]. Women are affected nearly three times more often than men in the younger age group, while the incidence is reversed at the older group with men being affected with higher frequency. Juvenile MG is defined as a disease with onset before the age of 18 years and accounts for roughly 10% of all

cases of MG[9]. Juvenile MG is more commonly reported in East Asia [10], otherwise the incidence follows a relatively equal geographic distribution in both adults and children. In Greece, the incidence of MG has been reported to approximately 70 per million population [11].

1.1.3 Clinical characteristics of Myasthenia gravis

The clinical hallmark of MG consists of fluctuating muscle weakness and fatigability affecting ocular, bulbar, (proximal) limb skeletal muscle groups as well as respiratory muscles. Weakness worsens with repetitive activities and improves with rest. Clinically classification of MG distinguishes the purely ocular myasthenia from generalized myasthenia with mild, moderate and severe manifestation. Ocular myasthenia affects exclusively the outer ocular muscles including the muscle that elevates the upper eyelid and thus presents with ptosis and double vision. These symptoms can be transient, fluctuating or progressive during the day. The ptosis can be unilateral or bilateral, fatigues with up-gaze, and sustained up-gaze for 30 or more seconds will induce it. Only 10-20% of patients show muscle fatigability and weakness restricted to the outer ocular muscles. The majority of the patients proceed to generalized muscle fatigability and weakness within 24 months after the disease onset [12].

Generalized myasthenia is defined as any clinical involvement of muscle groups other than outer ocular muscles independent of its severity [13]. Involvement of the limbs in MG produces predominantly proximal muscle weakness similar to that of other myopathic disorders. However, the arms tend to be more often affected than the legs. Occasionally, distal muscle weakness can be present [14]. Facial muscles are frequently involved and make the patient appear expressionless. Neck extensor and flexor muscles are commonly affected. The weight of the head may overcome the extensors, producing "a dropped head syndrome" [15]. The natural course of MG is general improvement in 57% and remission with complete abatement of symptoms in 13% of the cases after the first 2 years which lasts from 6 months to 4 years. Never the less, severe weakness can be accompanied by high mortality [15, 16]. In only 20% of the patients the disease's condition remains unchanged while mortality from the disease is 5-9%. In patients with ocular and bulbar MG, the weakness can be mild in 2%, and moderate or severe in 11%. In those with generalized MG weakness can

be mild in 2%, moderate in 14% and severe in 15%, the latter associated with dysphagia, depressed cough and reduced vital capacity [6, 17].

1.2 Myasthenia gravis pathophysiology and pathogenesis

1.2.1 Neuromuscular junction

The brain contains billions of nerve cells, or neurons, which receive and integrate signals from the environment. Nervous system activity is made possible by synapses, contacts formed either between neurons or between a neuron and a target cell. Synapses are asymmetric structures in which neurotransmitter molecules are released from the presynaptic membrane and activate receptors on the postsynaptic membrane, thus establishing neuronal communication [18]. The neuromuscular junction (NMJ) involving a motor nerve terminal and the muscle membrane is among the most extensively studied neural synapses, primarily due to its location in the peripheral nervous system, isolated from other synapses, but also because of the availability of a highly abundant source of its close analog within the electric organs of electric fish. As the motor nerve approaches a muscle it branches, innervating many muscle fibers, thus providing a single, unmyelinated nerve terminal to each of the fibers. Its principal role is to transmit the nerve impulses from the nerve terminal to the muscle, leading to muscle contraction.

NMJs efficiently convert the electrical impulses of the motor neuron into action potentials in juxtaposed muscle fiber, a process that depends on the release of large quantities of the neurotransmitter acetylcholine (ACh) molecules by the motor neuron, as well as on the high-density clustering of nicotinic acetylcholine receptors (nAChR) on the muscle membrane.

The mature NMJ (figure 1) consists of three important components that play a pivotal role in signal transduction: (a) the presynaptic nerve terminal where the neurotransmitter acetylcholine (ACh) is synthesized, stored in synaptic vesicles and released, (b) the synaptic basal lamina that occupies the synaptic cleft between the pre- and post-synaptic membrane and (c) the specialized postsynaptic membrane of the muscle containing the necessary neurotransmitter receptors [3, 19-21].

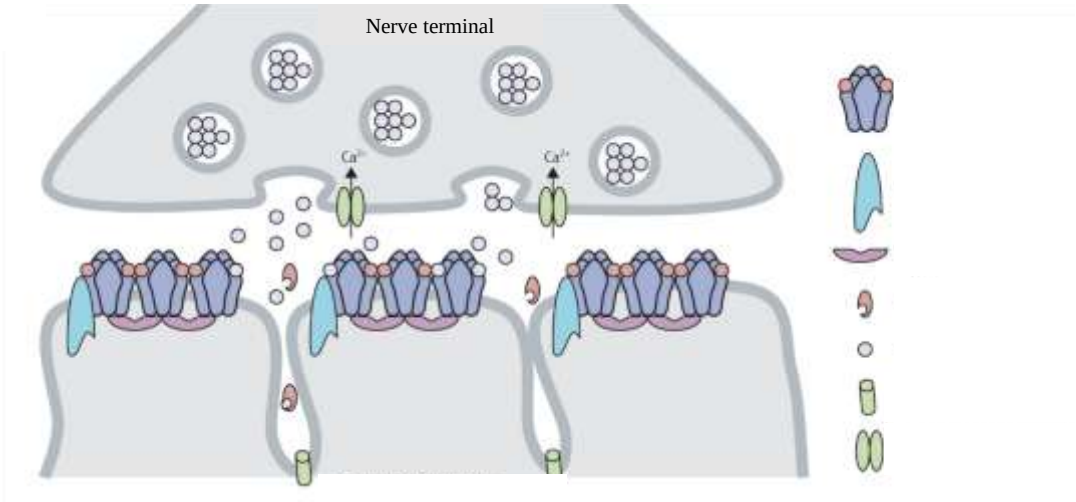


Figure 1. The neuromuscular junction (NMJ) and the molecules involved in synaptic transmission. Components of the NMJ. In the normal NMJ, ACh is released from the nerve terminal following a nerve action potential and interacts with the AChR on the postsynaptic membrane. Voltage-gated Ca2+ channels allow the influx of Ca2+ into the nerve terminal, which facilitates the release of ACh. Voltage-gated Na+ channels on the postsynaptic membrane serve to propagate the muscle action potential on depolarization. Acetylcholinesterase scavenges and hydrolyses unbound ACh. MUSK initiates clustering of the cytoplasmic protein rapsyn and nAChRs and is believed to maintain normal postsynaptic architecture (adapted from Meriggioli and Sanders, Lancet Neurol. 2009).

1.2.2 Presynaptic motor nerve terminal

The presynaptic motor nerve terminal is the NMJ's component where ACh is synthesized from acetyl CoA and choline by the enzymatic action of choline. It is stored in the synaptic vesicles in packets called "quanta" and then released into the synaptic cleft, either spontaneously or as a result of the nerve impulse. When an action potential reaches the nerve terminal, it leads to the opening of voltage-gated calcium channels in the presynaptic release sites (active zones). The mechanism of relaying the calcium signal to synaptic vesicles involves conformational changes in multiple proteins on the synaptic vesicle membrane and the plasma membrane of the nerve terminal[22]. The calcium influx initiates a process called docking of the synaptic vesicles, in which they come into close proximity with the nerve terminal membrane and then undergo priming that allows them to respond to the calcium signal. Three proteins, two on the plasma membrane (syntaxin and synaptic vesicle associated protein 25 or SNAP25) and one on the synaptic vesicle membrane (synaptobrevin) form the docking complex. Subsequently, 10-200 acetylcholine (ACh) containing synaptic vesicles fuse to the plasma membrane, and ACh is released

into the synaptic space through exocytosis [19]. Each synaptic vesicle contains 5.000-20.000 ACh molecules.

1.2.3 Synaptic cleft

Once released from the synaptic vesicles, ACh diffuses rapidly across the synaptic cleft [23], before it acts on its receptor at the postsynaptic membrane. Synaptic clefts are divided into primary and secondary. The primary cleft is the space that separates the presynaptic nerve membrane from the postsynaptic muscle membrane, and it is approximately 70nm wide. It is comprised of the basal lamina, which can bind receptors on adjacent cell membrane surfaces, providing a means of cell adhesion and signaling among NMJ components [20]. The basal lamina constituents include collagen IV, laminin, fibronectin, entactin and perlecan [24]. The secondary clefts are the spaces between the junctional folds of the postsynaptic membrane and they communicate with the primary cleft [3]. Acetylcholinesterase (AChE) which hydrolyses ACh in order to terminate the neuromuscular transmission, is concentrated in the secondary clefts.

1.2.4 Postsynaptic muscle membrane

Once ACh traverses the synaptic cleft it binds to the nAChRs. The motor axon apart from the ACh, secretes agrin, a glycoprotein with a laminin-binding domain that anchors it to the extracellular matrix [20, 21, 23], important for nAChR clustering. Agrin acts by activating a postsynaptic transmembrane kinase, muscle-specific kinase (MuSK). MuSK is a receptor tyrosine kinase, that when activated by agrin, self-phosphorylates and phosphorylates a number of other proteins, like Dok7, a non-catalytic adaptor protein, important for the formation of the NMJ. The agrin/MuSK interaction requires mutual binding to a third transmembrane muscle protein, the low-density lipoprotein receptor-related protein 4 (LRP4) [25]. Through this process the scaffolding protein rapsyn is recruited and the stabilization of the postsynaptic muscle membrane nAChR clusters is achieved [20, 23, 26, 27]. The nAChR is a glycoprotein, forming a cation channel which opens upon ACh binding, resulting in the entry of cations, mainly sodium, into the muscle fiber, leading to the generation of end-plate potential (EPP). When a certain threshold of depolarization is achieved, voltage-gated sodium channels at the bottom of the postsynaptic folds, open, allowing the entry of more sodium ions and generating the muscle action potential and ultimately muscle contraction [28]. ACh in the synaptic cleft is

hydrolyzed by acetylcholinesterase (AChE), and choline is actively transported back into the presynaptic cytosol, where the addition of acetate by ACh transferase resynthesizes ACh for eventual repackaging into vesicles [29].

The surface of a muscle cell membrane opposite to the nerve cell terminal at the NMJ is organized into folds (junctional folds). The normal junctional fold has a slender stalk and a terminal expansion, called "peak". The neurotransmitter ACh's receptors (nAChR) are mostly concentrated in the peaks of these folds (10.000-20.000 AChRs/ μm^2) [30]. At least three cellular mechanisms contribute to the high density of AChRs at the NMJ. Firstly, nAChR might redistribute from primitive clusters to the synaptic area, either by lateral movement, by diffusion in the plasma membrane or by endo- and exocytosis. Secondly, muscle fibers are multinucleated cells, and only the nuclei beneath the post synaptic area are actively transcribing the nAChR genes, contributing to synapse-specific nAChR expression. Thirdly, nAChR turnover is reduced at mature NMJs or when clustered (as shown by the half-life of nAChRs at the NMJ at 8-14 days compared to 17-24 hours for non-clustered or embryonic nAChR) [18]. The high density of nAChRs is pivotal for the initiation of the synaptic action potential in the myofiber.

1.2.5 Pathology of neuromuscular junction in Myasthenia gravis

Myasthenia gravis (MG) is a prototypical, antibody mediated T-cell-dependent autoimmune disease, caused by autoantibodies against neuromuscular junction proteins, leading to impairment of the function of the NMJ and the neuromuscular transmission [31]. This failure of the neuromuscular signal transduction leads to muscle weakness and fatigability, the characteristic symptoms of MG. Antibodies against the nAChR are found in 85% of the patients with generalized muscle weakness and 50% of those with purely ocular involvement [32]. In approximately 6% of the MG patients, autoantibodies against MuSK are present [33], while 9.2% have antibodies against LRP4 [34]. Patients without detectable antibodies against any of the known targets account for 10% of all MG patients [35] and are known as seronegative MG (figure 3).

1.3 Antigenic targets in Myasthenia gravis

1.3.1 The muscle nicotinic acetylcholine receptor

Despite the fact that the role of ACh as neurotransmitter was shown by Sir Henry Dale more than 80 years ago [36], it was only in the 1970s that its receptor was successfully purified with efficient affinity reagents [37, 38]. This discovery paved the way for its further characterization as a single protein. Two factors played a pivotal role in this progress: the presence of very high concentrations of nicotinic AChR (nAChR) in the electric organs of the electric fish *Electrophorus electricus* and *Torpedo californica* as well as the availability of a small polypeptide α-bungarotoxin from the venom of the poisonous snake *Bungarus multicinctus* that binds with very high affinity and selectivity to the nAChR [38]. The nicotinic acetylcholine receptor (nAChR) belongs to the family of Cys-loop ligand ion channels (LGIC), which also includes the serotonin (5-HT_3), glycine and γ- aminobutyric acid A (GABA) receptors [39]. The characteristic feature of this superfamily is a conserved sequence of 13 residues flanked by linked cysteines in the N-terminal domain of each subunit. They are formed by the assembly of five transmembrane subunits, selected from a pool of 17 homologous polypeptides (α1-α10, β1-β4, γ, δ and ε). As the *Torpedo* α subunit contains two adjacent linked cysteine residues, Cys192 and Cys193, which contribute to ligand binding, by convention nAChR subunits with two adjacent cysteine residues at positions analogous to Cys192 and Cys193 in the *Torpedo* α subunit are classified as α subunits [40]. There are many nAChR subtypes, each consisting of a specific combination of subunits, which mediate diverse physiological functions [41]. nAChRs are divided into two groups: (a) the muscle type, found in the vertebrate skeletal muscle, where they mediate the neuromuscular transmission at the NMJ and (b) the neuronal type, found mainly throughout the peripheral nervous system (PNS) and central nervous system (CNS), but also in non-neuronal tissues (keratinocytes, epithelia, macrophages)[41].

All members of the LGIC family share a common topology: each subunit has an N-terminal extracellular domain (ECD), 4 transmembrane domains (M1-M4), an intracellular domain (ICD) between M3 and M4, and a small extracellular tail after M4 [42]. The members of the family also share a conserved loop at their N-terminal ligand binding domain, flunked by two disulfide-bound cysteine residues, called cys-

loop. nAChRs are transmembrane glycoproteins of approximately 290kDa and are composed of five homologous subunits that form an ion channel [43].

The neuronal nAChR subtypes are pentamers and are either homomers (α7, α8, α9 and α10) or heteromers of α and β subunits. In the neuronal nAChRs, the ligand-binding sites lie at the interface between two α-subunits in homomeric receptors or between an α (α2, α3, α4, or α6) and a β (β2 or β4) subunit in heteromeric receptors. The α5 and β3 subunits do not participate in the formation of ligand-binding sites, but contribute to nAChR targeting and localization in neuronal plasma membrane domains [44].

Muscle-type nAChRs. There are five types of muscle-type ACh receptor subunits: α1, β γ, ε and δ. The subunit composition of the muscle nAChR varies depending on the developmental stage. In fetal muscle, the receptor composition is $(\alpha1)_2\beta1\gamma\delta$, whereas in the adult muscle, the composition is $(\alpha1)_2\beta1\varepsilon\delta$ (figure 2) [45]. Muscle-type nAChRs contain two ligand-binding sites for ACh and other cholinergic ligands, which are formed at the interfaces between the α1 and γ / ε subunits and between the α1 and δ subunits, with amino acids from both subunits contributing to the binding site. The ACh binding sites are located at the extracellular domains of nAChR, while 2 molecules of ACh are required to activate the opening of the ion-channel. The α1-ECDs also contain the main immunogenic region (MIR), a region of overlapping epitopes, including amino-acids 67-76 of the α1 subunit, against which a large fraction of autoantibodies against the nAChR is directed in MG [46].

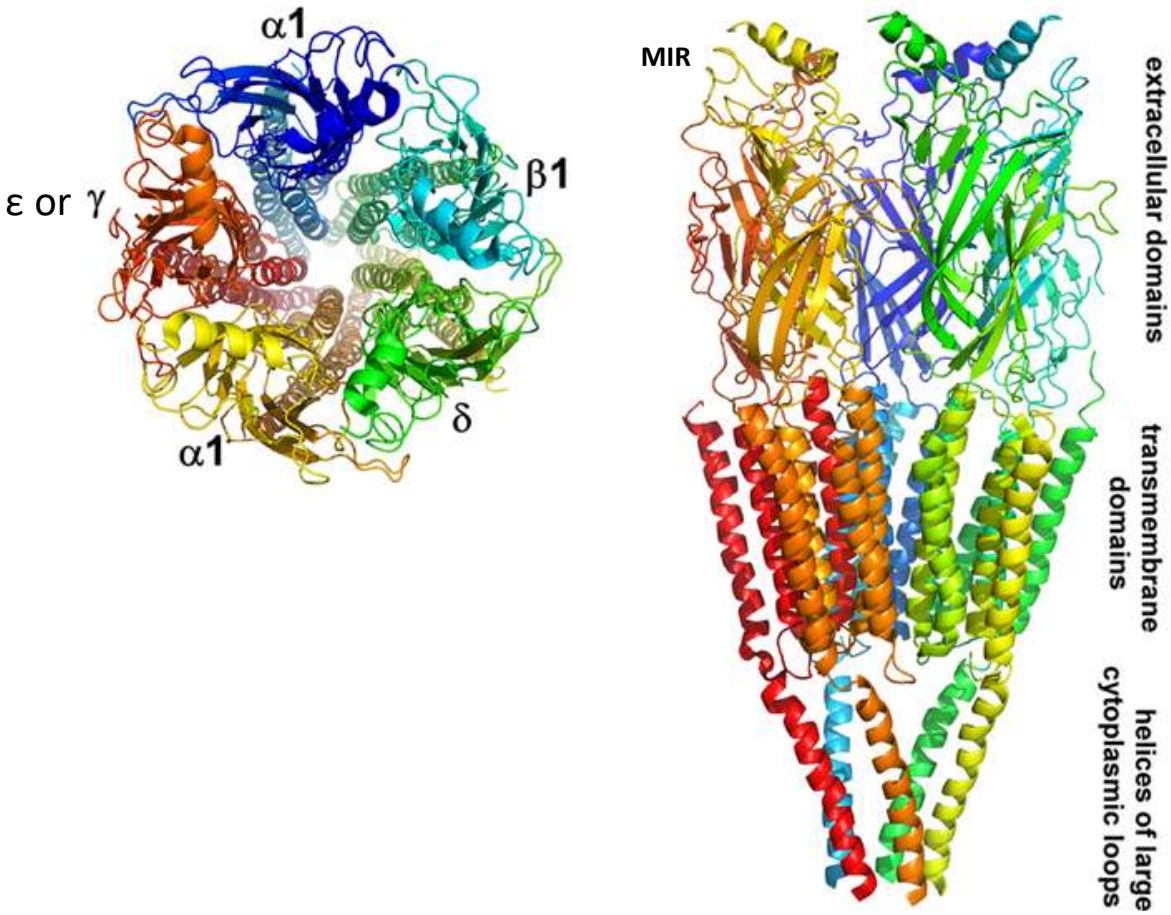


Figure 2. The pentameric structure of the nAChR. Color scheme of the five subunits from N terminus through C terminus of each subunit: α1-blue to cyan; β1-cyan to green; δ-green to yellow; α1-yellow to orange; ε or γ-orange to red (adapted from G. Wells, Frontiers in Bioscience 2008).

1.3.1.1 Anti-nAChR antibodies

Antibodies against the nAChR are found in 85% of all MG patients (nAChR-MG) [5]. In vivo studies have shown that the nAChR antibodies bind to extracellular domains of the receptor [47], thereby impairing signal transduction. nAChR antibodies mostly belong to the IgG1 and IgG3 subclasses, which activate the complement and consequently the formation of the membrane attack complex (MAC), leading to the damage of the postsynaptic membrane. Furthermore, as the antibodies are bivalent, they are capable of cross-linking adjacent nAChRs, thus accelerating their endocytosis and degradation, a process called antigenic modulation. Some nAChR antibodies target the acetylcholine binding site of the receptor, thus potentially directly blocking the signaling pathway (figure 3). However, such antibodies are rare and are probably clinically relevant in few patients [48]. The antibodies against the α1-subunit of the nAChR are more pathogenic than those

against the β-subunit, therefore, the nAChR epitope pattern may influence disease severity [49].

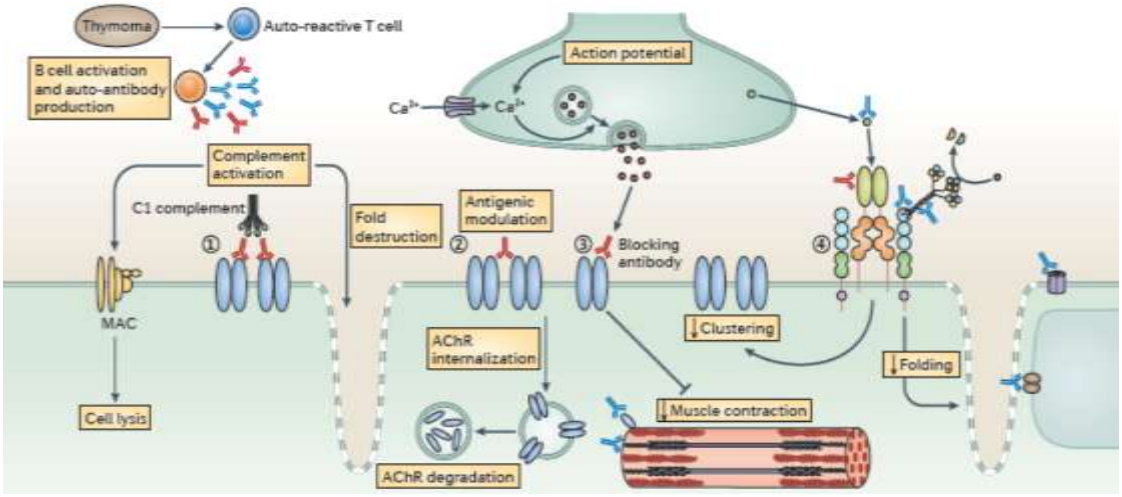


Figure 3. Neuromuscular junction in Myasthenia gravis (MG). Major pathogenic mechanisms of the AChR antibodies in MG include complement activation at the neuromuscular junction, which causes formation of membrane attack complexes (MACs) on the muscle membrane and destruction of the typical folds in the sarcolemma (1); antigenic modulation that results in internalization and degradation of surface AChRs (2); and binding of AChR antibodies at the AChR ligand binding site (3), which could directly block acetylcholine binding and, consequently, channel opening. Anti-MuSK and anti-LRP4 antibodies have been shown to block the intermolecular interactions of MuSK or LRP4 respectively and could thus inhibit the normal mechanisms for maintenance of the organization of the neuromuscular junction (4). Antibodies with known pathogenic involvement in MG are shown in red.

(E. Gillus, G. Skeie, F. Romi, K.Lazaridis, P. Zisimopoulou and S. Tzartos, Nature Reviews 2016).

The majority of antibodies found in nAChR-MG patients target the main immunogenic region (MIR) of the nAChR α1 subunit. The MIR is located at the ECD of the α1 nAChR subunit of the muscle nAChR and is highly immunogenic and myasthenogenic [50]. It is not a single epitope, but a region of closely spaced overlapping epitopes, several of which can be obscured by a single bound monoclonal antibody (mAb) [51]. The MIR loop, (67-76 amino acids of α1 AChR subunit) contains the majority of these epitopes [52]. Amino acids 68 and 71 are particularly important since mutations of either prevent binding of many mAbs to the MIR [53].

Total nAChR antibody concentration does not correlate with symptom severity when patients are compared [54], although fluctuations in nAChR antibody concentration in

an individual patients have been reported to correlate with the severity of muscle weakness and to predict exacerbations [55].

Even though there is a link between the thymus and anti-AChR antibodies, the mechanisms that trigger the production of the autoantibodies are still not clear. For example, some specific drugs such as D-penicillamine, but also IFN-β therapy, can induce MG symptoms. Additionally, viral infections have been suggested as possible triggering factor. Recent data address in part this issue, as they demonstrate that poly(I:C), an agonist of toll-like receptor 3 that mimics a viral infection, can specifically upregulate the expression of α-AChR subunit in human medullary thymic epithelial cells (mTEC) [18, 56], leading to an ectopic autoimmune response.

MG patients with antibodies against the nAChR can be categorized according to their clinical symptoms and disease pathogenesis in: early onset MG, late onset MG or MG patients with thymoma. Thymic abnormalities such as hyperplasia and thymoma are frequently associated with nAChR-MG. Thymoma is mainly present in elderly nAChR-MG patients [57].

In early-onset MG, disease symptoms manifest before the age of 40 and affect mainly women. The ocular muscles are primarily involved and subsequently the patients develop generalized muscle weakness. Usually, early-onset MG patients are diagnosed with hyperplastic thymus, containing germinal centers that may be sites of intense B-cell responses against nAChR [48]. On the other hand, the majority of late-onset MG patients are men older than 60 years old but are presented with normal thymic pathology. Approximately 10% of generalized MG patients have a thymoma and even though their age of onset varies considerably, thymoma is more frequent in the elderlies[58].

1.3.2 Muscle specific kinase

MuSK is a single-pass postsynaptic transmembrane glycoprotein of ~120kDa, containing a total of 5 domains: i) the extracellular domain, which consists of 3 Ig-like domains (Ig1, Ig2 and Ig3) and 1 frizzled-like cysteine-rich domain, ii) the cytoplasmic, iii) the juxtamembrane domain (JM) and iv) the intracellular tyrosine kinase domain. MuSK has a crucial role in signaling between motor neurons and skeletal muscles. MuSK is expressed in skeletal muscles and once activated, stimulates pathways that: (a) cluster and anchor nAChRs and additional muscle proteins that are critical for synaptic transmission, (b) enhance transcription of genes

encoding synaptic proteins in muscle “synaptic nuclei” and (c) promote the production of retrograde signals that stimulate presynaptic differentiation [59]. Its significance is evidenced by the fact that in the absence of MuSK neuromuscular synapses fail to form [60].

MuSK acts in two phases of synapse formation: (1) prepatterning muscle in the prospective synaptic region before innervation and (2) responding to neuronal agrin to form and stabilize synapses. MuSK forms the core of a multi-protein signaling complex. Agrin a heparansulphate proteoglycan released by the nerve terminal of spinal motor neurons binds to LRP4 and phosphorylates MuSK. LRP4 and MuSK interact through their respective extracellular domains. Activated MuSK in concert with Dok-7, which binds to a tyrosine-phosphorylated motif in the JM domain of MuSK [61] and other intracellular proteins stimulates rapsyn to concentrate and anchor the nAChRs at the postsynaptic membrane. Wnt ligands also directly bind to and phosphorylate MuSK to induce nAChR clustering especially at an early stage of the development [62]. The signaling pathway through which MuSK acts to build a nAChR-rich postsynaptic membrane are yet to be fully understood. However, assembly of nAChRs into AChR clusters requires the small GTP-ases Rac and Rho [63]. When neuronal agrin is added to muscle cells, MuSK activation and tyrosine phosphorylation of gernylgernyltransferase I leads to activation of Rac I and formation of tiny nAChR micro-aggregates [64]. Rac activation is followed by the activation of Rho, which is thought to act via PAK I to consolidate nAChR micro-aggregates into large nAChR clusters [65].

The cytoplasmic tyrosine kinase Abl and Src are also activated by MuSK. They play overlapping roles in sustaining MuSK activation and tyrosine phosphorylation of the nAChR β subunit [66]. Tyrosine phosphorylation of the nAChR β subunit, recruits the adaptor protein rapsyn, thereby stabilizing the nAChR cluster [67]. Additionally, MuSK interacts with AChE and collagen Q (ColQ), which are involved in the termination of the signal transduction at the NMJ. Three tetramers of AChE are linked to the triple helical ColQ. AChE/ColQ complex is anchored to the synaptic basal lamina by two mechanisms. Firstly, a pair of heparan sulfate proteoglycan-binding domains in the collagen domain of ColQ bind to heparan sulfate proteoglycans including perlecan at the synaptic cleft [68, 69]. Secondly, the C-

terminal of ColQ binds to the Ig1 and Ig4 regions of MuSK [70]. MuSK thus binds to LRP4, Wnt ligands and ColQ.

Therefore, MuSK plays a pivotal role not only in the formation but also in the maintenance of the NMJ. When MuSK is absent in the developing NMJs, nAChRs are expressed uniformly along the muscle fiber, and motor axons grow without terminating effectively onto muscle fibers to form synapses [60]. MuSK is also essential in maintaining function of the mature NMJ and conditional MuSK inactivation has been shown to lead in synaptic destabilization of the post-developmental NMJ [71].

1.3.2.1 Anti-MuSK antibodies

In 2001, antibodies against MuSK were identified in patients with generalized, nAChR antibody-negative MG [72]. MuSK-MG patients have less often purely ocular weakness at onset, but involvement of bulbar muscles at the start of the disease is more frequent than in nAChR-MG, accompanied with respiratory insufficiency that can be difficult to treat [72].

Antibodies against MuSK can be detected in 1-10% of patients with MG [48]. These antibodies are more prevalent in patients from Mediterranean than in those from Northern Europe, possibly owing to a combination of genetic and environmental factors [54]. The great majority of anti-MuSK-positive patients with MG are female [73]. In contrast to nAChR antibodies, antibodies against MuSK belong mostly to the IgG4 subclass. The human IgG4 subclass is comprised of a group of dynamic antibodies that exchange Fab arms with other antibodies within this subclass in circulation, resulting in a functionally-monovalent antibody carrying two different antigen-binding sites [74]. In the case of MuSK-MG, the result of half-antibody exchange is that patients may have two types of anti-MuSK IgG4 antibodies that contain two different Fab arms, leading to the production of both monovalent and divalent antigen-binding sites for MuSK antigens. Their way of action is thus also different: nAChR antibodies bind to complement and cross link their antigenic target, whilst MuSK antibodies do not activate the complement cascade and a major subclass of these antibodies remain functionally monovalent [75]. Divalent anti-MuSK IgG antibodies can crosslink and activate tyrosine phosphorylation of MuSK in the absence of agrin [76, 77]. In addition, experimental data suggest that these antibodies may also interfere with MuSK function by inducing internalization of MuSK from the

cell surface by accelerating the down-regulation of the cytoplasmic adaptor protein Dok-7, which is indispensable for MuSK function [78]. In contrast, monovalent anti-MuSK antibodies inhibit auto-phosphorylation of MuSK induced by agrin [79]. Monovalent antibodies may also interfere with interactions between MuSK and LRP4-agrin [48]. Instead of affecting nAChR function, MuSK antibodies reduce the postsynaptic density of nAChRs and impair the alignment between the motor nerve terminal and the postsynaptic membrane. Most MuSK antibodies bind to the extracellular N-terminal Ig-like domain of the MuSK [5]. In patients with MuSK-MG, concentrations of anti-MuSK antibodies tend to correlate with disease severity and changes in antibody concentration over time can reflect disease activity [80].

1.3.3 Low-density lipoprotein receptor-related protein 4

Members of the low-density lipoprotein receptor-related protein family are well known for their roles in lipid metabolism, cholesterol homeostasis and Wnt signaling [81]. LRP4 is a member of the LDLR family, contains an very large extracellular N-terminal region that possesses multiple EGF repeats and LDLR repeats, a transmembrane domain and a short C-terminal region [82]. LRP4 is the postsynaptic receptor for the nerve derived agrin, critical for MuSK activation, nAChR clustering, and NMJ formation [62]. More specifically, LRP4 is required for viability and for normal development of the lung, kidney and ectoderm organs. Complete lack of LRP4 leads to paralysis at birth, due to an early block in the development of NMJ [83].

1.3.3.1 Anti-LRP4 antibodies

A subgroup of MG patients, have low affinity antibodies against nAChR [84]. The detection LRP4 antibodies in MG depends on the assay and the examined population. Overall, LRP4 antibodies are detected in 1-5% of all MG patients and in 7-33% of MG patients without nAChR or MuSK antibodies [85]. In animal models, LRP4-immunized mice exhibit muscular weakness, as LRP4 antibodies act directly by disrupting the interaction between LRP4 and agrin and subsequently inhibiting nAChR clustering and mediated neuromuscular transmission [82]. LRP4 antibodies observed in these mice belong mainly to the complement-binding IgG1 subclass[86]. As LRP4 antibodies are primarily reported in MG patients without nAChR or MuSK antibodies, LRP4-MG can be characterized as a distinct disorder [87]. However, LRP4 antibodies have recently been detected in several patients with either nAChR or

MuSK antibodies, in patients with other autoimmune disorders, and in patients with amyotrophic lateral sclerosis. The presence of LRP4 antibodies is associated with milder MG symptoms and LRP4-MG can manifest purely as ocular MG [85].

1.3.4 Autoantibodies directed against skeletal muscle proteins

Antibodies that react with striated muscle antigens may also be found in up to 95% of MG patients with a thymoma and in 50% of late-onset MG patients without thymoma [88]. Striated muscle (strational) antibodies recognize muscle intracellular proteins (titin, myosin, actin and ryanodine receptor) which are not directly accessible to autoantibodies. Strational antibodies are not specific for MG and may occur in patients with other autoimmune diseases and in patients with thymoma without MG [89]. In MG, these antibodies rarely occur in the absence of nAChR antibodies and thus are not generally useful for the diagnosis of MG. Nevertheless, strational antibodies are helpful in the diagnosis of thymomatous MG and can reflect thymic pathology [90].

1.3.4.1 Anti-Titin antibodies

One of the major antigenic targets of strational antibodies is titin, an intracellular protein with a molecular mass of 3000kDa stretching throughout the sarcomere, with a length of more than 1 μm, making it the largest known protein [91]. Ninety percent of the titin mass is contained in a repetitive structure comprising 244 to 297 copies of 2 different 100-residue repeats, the 112 to 165 immunoglobulin superfamily domains and 132 fibronectin-like domains. Titin molecules are arranged in a way that allow augmentation of mechanical stability and tension in the sarcomere [92]. The titin-based tension is a calcium-dependent molecular spring that adapts to the physiological state of the cell [93]. The main immunogenic region of titin is a 30kDa region of the protein, called myasthenia gravis titin-30 (MGT-30) and is located near the A/I-band junction [94, 95]. Titin antibodies can be detected in 20-30% of MG patients with nAChR antibodies, mostly in patients with thymoma-associated disease or late-onset MG [31]. Given its intracellular location, titin antibodies should not interfere with muscle function. However, their presence indicates a more severe form of MG with mild myopathy. Additionally, titin antibodies are a sensitive marker of thymoma in patients with MG whose symptom onset occurs before the age of 50 years [96]. In general, anti-titin antibodies correlate

with disease severity, and may identify patients more likely to be refractory to therapy, including thymectomy [97].

1.3.4.2 Anti-RyR antibodies

The ryanodine receptor (RyR) is a calcium release channel located in the sarcoplasmic reticulum. The name refers to the alkaloid ryanodine that binds selectively to the RyR. There are 2 forms of RyR, skeletal (RyR1) and cardiac (RyR2). The RyR antibodies from MG patients react with both. The RyR is a protein containing 5035 amino acids with a molecular weight of 565 kDa. It is composed of 4 homologous subunits that can build a tetramer with a central channel [98]. The RyR is expressed mainly in striated muscle tissue but is also found in the epithelium and neurons. The longitudinally spreading depolarization along the sarcolemma continues transversally through the T tubules to the terminal cisternae of the sarcoplasmic reticulum, inducing a conformational change in the RyR leading to calcium release which opens the RyR, allowing calcium flow into the sarcoplasm [98]. The RyR epitopes are located on the handle domains of the RyR cytoplasmic assembly, near its junction with the transmembrane assembly [99]. The main immunogenic region is the peptide chain 2 and RyR type 1 fusion protein located to the N-terminus, and in some cases a more centrally located region called peptide chain 25 [100]. Both regions are located near each other in the 3-dimensional conformation of RyR, thus the antibodies against the central region may represent epitope spreading. The RyR antibodies cause allosteric inhibition of RyR function in vitro, inhibiting Ca^{2+} release from the sarcoplasmic reticulum [100, 101]. Antibodies against the RyR are present in 70% of nAChR-MG patients with thymoma and in 14% of patients with late-onset nAChR-MG and their presence of indicates severe MG [95, 102].

1.3.4.3 Anti-k_V1.4 antibodies

Voltage-gated K channel (VGKC) consists of four transmembrane α-subunits that combine as homo- or heterotetramers. Kv1.4 is an α-subunit with a molecular weight of 73kDa located mainly in the brain, peripheral nerves and skeletal and heart muscles. Antibodies against Kv1.4 are detected in 10-20% of patients with MG. In MG, Kv1.4 antibodies seem to cross react with voltage-gated K^+ channels in the heart muscle. In a Japanese cohort Kv1.4 antibodies were associated with severe MG and heart complications [103, 104].

1.3.4.4 Collagen-Q antibodies

Collagen Q is a protein that concentrates and anchors AChE at the NMJ, where it is localized in the extracellular matrix, and thus is accessible to antibodies. Collagen Q is found only at the NMJ. Collagen Q antibodies were recently detected in 3-4% of all MG sera tested and in 1.2-5.5% of the nAChR-, MuSK- and LRP4-antibody negative patients [105]. As mutations of collagen Q might lead to myasthenic syndromes, anti-collagen Q antibodies can be pathogenic. However, collagen Q antibodies have been found in healthy controls [31, 105].

1.3.4.5 Agrin antibodies

Antibodies against agrin can be detected in a minority of patients with MG, either with or without antibodies against nAChR, MuSK or Lrp4. Agrin antibodies are present only in patients with MG, suggesting that they are specific for the disease. Due to its pivotal role in the formation, maintenance of the NMJ and in the signal transmission between the motor neuron and the muscle, interference with agrin's function leads to insufficient neuromuscular transmission. Still, there is not established a direct pathogenic effect of agrin antibodies, but such antibodies inhibit MuSK phosphorylation and nAChR clustering in vitro [106, 107].

1.3.5 The role of the thymus

The thymus is the primary lymphoid organ for T lymphocyte production and establishment of central tolerance. Precursor T-cells migrate from the bone marrow to the thymus throughout life and the early T cell precursors localize in the thymic cortex. Maturating cells undergo cell division and T-cell receptor (TCR)-chain rearrangement and develop into double -positive $CD4^+CD8^+$ thymocytes. Immature T-cells undergo either positive or negative selection and finally $CD4^+$ or $CD8^+$ single-positive T cells located in the thymic medulla leave the thymus to enter the peripheral circulation [108]. Autoreactive T cells are eliminated during interactions between the developing thymocytes and thymic stromal cells that include epithelial cells, mesenchymal cells, dendritic cells and a few myoid cells [109]. This process is called negative selection. Self-tolerant T cells then continue their differentiation and are exported to the periphery. A key factor in thymic central tolerance is the autoimmune regulator (AIRE), which controls the expression of tissue specific antigens presented mainly by the medullary thymic epithelial cells (mTEC). Examples, of such self-antigens are insulin and nAChR, which are typically expressed in pancreas and the

muscle respectively and they are both expressed in mTEC [110]. When the interaction between developing thymocytes and tissue-specific antigens is strong, these cells are considered autoreactive and are eliminated [111]. The effectiveness of tolerance induced in the thymus is highly dependent on the level of expression of the self-antigen in the thymus [112]. Moreover, the thymus is the source of regulatory T cells (Tregs) that contribute to the immune regulation in physiological conditions and are often dysregulated in autoimmune diseases. Regarding MG, there seems to be a strong association between thymic pathology and MG disease manifestation. Most patients have follicular hyperplasia or a thymoma and there is a correlation between the degree of follicular hyperplasia and the level of anti-nAChR antibodies [113], which decreases after thymectomy [114]. The hyperplastic thymus includes all the components of the anti-nAChR response: loss of nAChRs, B-cells producing anti-nAChR antibodies and anti-nAChR autoreactive T cells [115]. The current theory of the immunopathogenesis of thymoma-related autoimmunity is that potentially autoreactive T cells are positively selected and released to the periphery where they are activated to provide help for auto-antibody-producing B cells by mechanisms that are still under investigation. Negative selection and regulation of potentially autoreactive T cells might be impaired in thymoma due to a deficiency in the expression of AIRE and the selective loss of Tregs [116].

1.4 Lambert-Eaton myasthenic syndrome

Lambert-Eaton myasthenic syndrome (LEMS), is an autoimmune disorder affecting presynaptic P/Q-type voltage-gated calcium channels (VGCC) of the neuromuscular junction, causing impaired ACh release thus leading to muscle weakness. The P/Q-type VGCC is the basis for the action potential-induced Ca2+ influx, which triggers fusion of the ACh-containing synaptic vesicles with the nerve terminal plasma membrane resulting to the release of ACh into the synaptic cleft [21]. Small-cell lung cancer and autonomic symptoms are common in LEMS [117]. Serum antibodies to P/Q-type BGCC, which are present on small-cell lung cancer cells and at motor nerve terminals, were detected by radioimmunoassay in 1995 [118]. Even though, the antigenic trigger for the production of anti-VGCC antibodies in patients without cancer is unknown, there has been found an association with the HLA haplotype B8 Vr2 DQ2 [119].

1.5 Immunogenetics in MG

The biological and clinical heterogeneity of autoimmune MG seems to correlate with genetic markers, most notably the HLA genes [120]. There has been found an association between HLA-DR3 and B8 alleles with early onset MG with thymic hyperplasia [121]. Late-onset MG is less strongly associated with HLA-DR2 and B7 [122]. HLA-DR3 and DR7 seem to have opposite effects on MG phenotype, DR3 has a positive association with early-onset MG and a negative association with late-onset MG , while DR7 has the opposite effects [121]. No clear genetic links have been found for thymomatous MG, but thymoma patients with particular genetic profiles have a higher risk of developing MG [123]. Recently, an association with DR14-DQ5 has been reported in patients with anti-MuSK antibodies [124]. Several non-HLA genes (*PTPN22, FCGR2, CHRNA1, CTLA-4*) have also been found to be associated with MG, and some of them are also associated with other autoimmune diseases, representing a non-specific susceptibility to autoimmunity [122]. Exception to this group of susceptibility genes, is the *CHRNA1* gene, which encodes the α-subunit of the nAChR and might provide pathogenic clues specific for MG [6]. The *PTPN22* gene encodes a member of the tyrosine phosphatase subfamily and interferes with signaling in T cells, leading to the inhibition of T cell activation. The *CTLA-4* molecule is highly polymorphic and plays an immunoregulatory role in limiting the excessive activation of T cells [125]. In addition to genetic analyses, numerous studies in progress investigate the impact of epigenetic modifications on selected genes that may play a role in autoimmune diseases.

Overall, due to the disease's heterogeneity with differences in clinical presentation, age of onset, autoantibody profile and the presence or absence of thymic pathology there have been identified several MG clinical subtypes.

Broadly MG is classified as follows [15, 126]:

1) Early onset MG: age at onset <50 years. Thymic hyperplasia, usually females, high levels of anti-nAChR antibodies[127]
2) Late onset MG: age onset >50 years. Thymic atrophy, mainly males
3) Thymoma-associated MG (10-15%)
4) MG with anti-MuSK antibodies

5) Ocular MG: symptoms only affecting extraocular muscles
6) MG with no detectable nAChR and MuSK antibodies.

1.6 Diagnosis of Myasthenia gravis

In patients with a characteristic history it may be relatively easy to make a diagnosis on clinical grounds alone. However, it is important to confirm diagnosis of myasthenia gravis before committing patients to long term treatment [3]. Several tests and diagnostic method have been developed in order to identify, as accurately as possible, the presence or absence of the disease.

Edrophonium chloride (Tensilon) test. Edrophonium is a short-acting AChE inhibitor that works within a few seconds (30 seconds) and prolongs the duration of action of ACh at the NMJ. Its effect lasts for a few minutes (approximately 5 minutes). Edrophonium is administered intravenously and the patient is observed for objective improvement in muscle strength particularly the eyelid ptosis or extraocular muscle movement. Only unequivocal improvement in strength of a sentinel muscle should be accepted as a positive result. Patients must be connected to cardiac and blood pressure monitors prior to injection due to possible risks of arrhythmia and hypotension. Atropine should be available if an adverse event like severe bradycardia occurs. Side effects from Edrophonium include increased salivation and sweating, nausea, stomach cramping and muscle fasciculation. Hypotension and brachycardia are infrequent and generally resolve with rest in the supine position. Tensilon test has a sensitivity of 71.5%-95% for the diagnosis of MG [128, 129].

Ice pack test. This test can be applied when ptosis is present. The application of an ice pack to lids of the affected eyes improves ptosis due to myasthenia gravis in 80% of the cases, but it does not when there are other etiologies [130]. This response is explained on the basis that cooling presumably slows down the kinetics of nAChRs. It is performed by placing an ice pack over the eye for 2-5 minutes and assessing for improvement in ptosis [131].

Anti-nAChR antibody test. A radioimmunoprecipitation assay based on a mixture of solubilized embryonic and adult nAChRs is the most rigorously validated test for nAChR antibodies and is the most reliable among the validated nAChR antibody tests. These testing kits are commercially available. Positive results have a near 100% specificity for MG in symptomatic individuals [48, 54, 132]. Such high specificity is very important for a first-line screening test that is used also in patients

with vague muscular symptoms or fatigue as the primary symptom. Even though, ELISA and immunofluorescence assays have been developed as non-radioactive alternatives with good sensitivity and specificity, they are inferior to radioimmunoprecipitation [5, 133]. Cell-based assays, where nAChR molecules are clustered on the membranes of cultured test cells, offer better sensitivity, and enable detection of low-affinity antibodies as they can detect antibodies in 4-66% of patients with generalized MG in whom antibodies cannot be detected with the radioimmunoassay [134]. The antibodies detected with the different methods, all belong to the same IgG subclasses, suggesting that the etiology and pathogenesis is the same in the total of patients with nAChR antibodies.

Anti-MuSK antibody detection. As mentioned previously, in patients with MuSK-MG, concentrations of anti-MuSK antibody tend to correlate with disease severity and changes in antibody titers over time can reflect disease activity [80]. Immunoprecipitation with radioimmunoassays is the standard test for MuSK antibody detection, and ELISA tests are also available, although neither are as sensitive as cell-based assays [75, 135]. The specificity of the cell-based assay is estimated to be 97-98%. MuSK-MG and nAChR-MG are distinct disease entities and rarely occur in the same patient. However, a few single-patient reports have demonstrated the existence of both in the same patient, particularly when the most sensitive antibody detection techniques were used [136].

Anti-LRP4 antibody detection. Even though the anti-LRP4 antibodies have been recently identified, LRP4 antibody testing has lately become commercially available. Anti-LRP4 antibodies are found in approximately 9.2% (range 2-50%) of patients with MG who are negative for both anti-nAChR and anti-MuSK antibodies [34]. Cell based assays using the human LRP4 that have been developed for the specific detection of anti-LRP4 antibodies [85], have also identified double positive MG patients (nAChR/LRP4-MG and MuSK/LRP4- MG) representing more severe cases than the average single-positive MG patient. RIPA can also be used for the detection of LRP4 antibodies. LRP4 antibodies are predominantly of the IgG1 and IgG2 subtypes. The prevalence is higher in women than in men, with an average disease onset at ages 33.4 for females and 41.9 for males.

Electrophysiological tests. The two principal electrophysiological tests for the diagnosis of MG are the repetitive nerve stimulation study and the single fiber

electromyography. Repetitive nerve stimulation tests neuromuscular transmission. It is performed by stimulating the nerve supramaximally at 2-3 Hz. A 10% decrease between the first and the fifth evoked muscle action potential is diagnostic for MG. In the case that no decrease is observed, exercise can be used to induce exhaustion of the muscles and document potential induction. The test is abnormal in approximately 75% of patients with generalized MG and 50% of patients with ocular MG [137].

Single-fiber electromyography (SFEMG) is the most sensitive diagnostic test for MG. It is done by using a special needle electrode that allows identification of action potentials from individual muscle fibers. It allows the simultaneous recording of the action potentials of the two muscle fibers innervated by the same motor axon. The variability in time of the second action potential relative to the first is called "jitter". SFEMG reveal abnormal jitter in 95-99% of patients with MG if appropriate muscles are examined [15, 137]. Even though it is a highly sensitive testing method, increased jitter is not specific for primary NMJ disease. It may be abnormal in motor neuron disease, polymyositis, peripheral neuropathy, Lambert-Eaton myasthenic syndrome and other neuromuscular disorder. However, it is specific for a disorder of neuromuscular transmission when no other abnormalities are seen in standard needle electromyography examination [129].

1.7 Current treatments for Myasthenia gravis

Therapeutic options in Myasthenia gravis patients include cholinesterase inhibitors, thymectomy, immunosuppressive agents and short-term immunomodulation with plasma-exchange and intravenous immunoglobulin [138].

1.7.1 Symptomatic therapy

Acetylcholinesterase inhibitor (AChEI) pyridostigmine bromide represents the first-choice of treatment in all types of autoimmune MG [54]. Neostigmine, another acetylcholinesterase inhibitor with a shorter half-life, can also be used. Ambenonium chloride is another acetylcholinesterase inhibitor, though for most patients, it is less effective than pyridostigmine or neostigmine. These drugs inhibit acetylcholine degradation, thereby increasing the availability of acetylcholine in the synapse. All MG subgroups besides MuSK-MG usually respond well to this treatment [54].

In MuSK-MG, the response to acetylcholinesterase inhibition is often insufficient, reflecting differences between MG subgroups due to the antibody-induced pathology in the postsynaptic muscle membrane. Relevant studies have reported a good response in only 50% of the patients with MuSK-MG, while 10% did not respond at all [136]. The beneficial effect of pyridostigmine is specific to MG. The dose must be adjusted to achieve the optimum in regard to therapeutic effect but also to the minimum of the adverse effects. Another approach to symptomatic treatment consists of drugs that increase the amount of acetylcholine via se presynaptic mechanism. For example, ephedrine and 3,4-diaminopyridine. They are less effective compared to AChEIs but can mildly reduce MG symptoms [48, 139].

1.7.2 Immunosuppressive therapy

Nearly all patients with late-onset MG, thymoma MG, and MuSK-MG require immunosuppressive therapy to suppress antibody production and autoantibody-induced detrimental effects at the neuromuscular junction. Early-onset MG can sometimes be ameliorated by symptomatic therapy alone, but the majority of the patients with early-onset MG may need, temporarily pharmacological immunosuppression [5].

Steroids. First-line immunosuppressive treatment in autoimmune MG consists of steroids like prednisone/prednisolone or their combination with azathrioprine (AZA) [140]. These drugs have broad action on the immune system and have been shown to be beneficial in all MG subgroups. Current treatment recommendations

suggest that prednisolone alone should be given only as short-term treatment (< 1year). Long-term prednisolone therapy can be considered for the treatment of ocular MG, but for the majority of the other MG patient groups combination of immunosuppressive treatments is preferred in order to obtain maximum effect with minimal side effects [102, 141]. If immunosuppressive therapy induces pharmacological remission or a marked improvement, it should be maintained in the long-term, but the dose should be adjusted in order to avoid adverse effects[5]. Side effects of chronic corticosteroids are osteoporosis, cataract, diabetes mellitus, hypertension, gastrointestinal irritation, glaucoma, weight gain and skin disorders [142].

Azathrioprine (AZA) is a purine analog and prodrug which is converted in the liver into its active metabolites 6-mercaptopurine and thioinosinic acid. These metabolites act in an immunosuppressive, anti-proliferative, and cytotoxic way as they reduce the purine synthesis and are incorporated into replicating DNA, halting replication. This leads to the increased apoptosis if T and B cells [13]. AZA is widely used in combination with corticosteroids, as it is more beneficial and leads to faster improvement compared to AZA alone as well as less side effects than steroid monotherapy [140].

Cyclosporin A (CsA) acts as a calcineurin inhibitor reducing lymphocyte activation, differentiation and function via binding of cytosolic cyclophilin in lymphocytes [13]. This in turn inhibits the upregulation of IL-2 gene expression by helper T cells [142]. CsA has been successfully administered as monotherapy in patients with generalized MG and it is often given along with corticosteroids treatment. Clinical effects after its administration are evident after 4-6 weeks [13]. CsA's side effects include arterial hypertension, myelosuppression, opportunistic infection, nephrotoxicity, headache as well as tremor which need careful clinical monitoring [143].

Cyclophosphamide is an alkylating agent inhibiting DNA replication. Acts mainly on B lymphocytes and it has been shown to be effective in treatment-resistant MG cases [144], but its use is limited by the risk of adverse side effects, including late development of malignancies or infertility, to patients intolerant or non-responsive to other treatment modalities [102].

Methotrexate (MTX) reduces the activity of dihydrofolate reductase leading among others to impaired DNA, RNA, protein synthesis and reduced proliferation of lymphocytes [13]. Potential side effects are leucopenia, thrombopenia, anemia, infections, hepatotoxicity, stomatitis, gastrointestinal symptoms, arthralgia, osteoporosis, pulmonary disorders, nephrotoxicity and elevated risk of malignancies. MTX is teratogenic and is not recommended during pregnancy and lactation [13].

Rituximab is a monoclonal chimeric antibody that binds to the B cell surface antigen CD20 and depletes many types of lymphatic B cells. Treatment with rituximab in MG is recommended in moderate and severe MG patients as well in MuSK-Ab-positive-MG patients, which are not responsive to first-line immunosuppressive treatments [54, 145]. A possible explanation for the particularly high efficacy of rituximab in MuSK-MG patients is that anti-MuSK IgG4 antibodies are produced by $CD20^+$ short-lived plasma cells whereas the predominant IgG1 and 3 response in nAChR-MG patients is induced by $CD20^-$ long-lived cells [146]. Potential side effects are infections, precipitating other autoimmune diseases and rarely JC-virus-associated progressive multifocal leukoencephalopathy [5]. Apart from rituximab, the humanized monoclonal antibody eculizumab which inhibits the terminal complement complex by blocking enzymatic activation of complement 5, has shown positive effects by ameliorating the symptoms of generalized nAChR-MG [13].

Intravenous immunoglobulins. The immunomodulating effect of intravenous immunoglobulins (IVIG) is mediated via their large quantity of Fab- or Fc-fragments of polyclonal healthy donor immunoglobulins [13]. Among potential mechanisms are Fab fragment-mediated activities such as suppression or neutralization of autoantibodies, cytokines, activated complement components and restoration of idiotypic-anti-idiotypic networks, blockade of leukocyte adhesion-molecule binding, targeting of specific immune cell-surface receptors as well as modulation of dendritic cells. Moreover, potential Fc-dependent activities of IgG include saturation of FcRn (protective receptor in many tissues that attenuates the catabolism of IgG), blockade of inflammation activating crystallizable fragment portion of IgG Fc receptors (FcγR), upregulation of the inhibitory anti-inflammatory receptor FcγRIIb on effector macrophages and immunomodulation by sialylated IgG [147]. The treatment effect usually appears within 2-5 days and lasts no longer than 2-3 months due to recurring

autoantibody synthesis [148, 149]. Nonspecific side effects of IVIG may occur after a few hours of infusion including facial rash, angina pectoris, nausea, shivering, back pain, fever, sweating, headache and hypotension [150]. More severe side effects develop in about 1-2.5% of the IVIG-treated patients and include allergic reactions, infections, pulmonary edema, acute renal failure, venous thrombosis, stroke, myocardial infraction, hemolysis, and aseptic meningitis [13].

Plasma exchange (PE) & Immunoadsorption (IA). In plasma exchange, plasma is separated from the corpuscular blood components and substituted by an exchange fluid. The way of action of this treatment is based on the non-specific elimination of autoantibodies and other pathogenic humoral factors like immune complexes, toxins and cytokines [13]. In contrast, IA is a more selective technique for removing IgG antibodies by binding to a specific matrix (protein A or tryptophan) [151]. Plasmapheresis is generally used as a treatment of myasthenic crisis [152]. In most cases, 6-8 treatment cycles are needed in order to reach stabilization and induce remission [13]. The therapeutic effect of remission typically persists for some weeks [143].

Thymectomy. Thymectomy is the surgical removal of the thymus gland which has been shown to play a role in the development of MG. Thymic abnormalities in MG, such as hyperplasia and thymoma, strongly suggest a thymus-mediated immune response in MG [153]. Active germinal centers and an ongoing export of disease-inducing T-lymphocytes will be effectively stopped by thymectomy [154]. Thymectomy is mandatory in patients with thymoma and is recommended as an option for non-thymomatous patients with generalized MG and specifically for those with nAChR-antibodies and of age younger than 60 years old [142]. The advantage of thymectomy is the potential of long-term benefit reducing or, in some cases, stopping chronic medical therapies. Despite great progress in research, MG treatment still has limitations, such unspecific immunosuppression and multiple adverse side effects. Ongoing research is contributing in bridging this gap. To this end, animal models of the disease are invaluable in understanding the disease pathophysiology, designing increasingly specific drugs and testing them pre-clinically.

1.8 Experimental Autoimmune Myasthenia Gravis

The first report on an experimental model of MG (Experimental Autoimmune MG, EAMG) was published more than 30 years ago, showing that rabbits immunized with nAChR, isolated from the *Electrophorus electricus* electrical organ, developed MG-like symptoms [155]. Since then EAMG has been induced in a wide variety of experimental animals: rabbits [156], rats (Lewis strain is preferred, but Brown-Norway or Fisher might also be used) [157], guinea pigs [158], mice [159] and rhesus monkeys [160]. It is usually induced by active immunization with *Torpedo californica electroplax* nAChR (T-AChR) in Complete Freund's Adjuvant (CFA) as well as by passive transfer of MG sera or anti-nAChR antibodies [156, 161]. The most detailed models and studies have been performed in rats (65%) and mice (35%). The incidence of clinical EAMG in rats is higher (about 90%) than mice (70-85%), therefore indirectly indicating the superiority of the rat EAMG model [161]. The susceptibility to EAMG is influenced by sex, age and genetic background of the different rat and mice strains [162]. EAMG shares several immunopathological features with the human MG, such as the presence of anti-nAChR antibodies in the serum, complement activation and deposits at the NMJ, major histocompatibility complex class II-restricted presentation of nAChR epitopes and involvement of T helper cells (Th) in the production autoantibodies, as well as several clinical features, such as muscle weakness and fatigability, reduced response after repetitive nerve stimulation, increased curare sensitivity and temporary improvement of muscle strength following treatment with anticholinesterase drugs [163].

EAMG is commonly induced in 6-8 weeks old Lewis rats with a single administration of TAChR in CFA. Clinical signs of the disease are due to anti-TAChR antibodies cross-reacting with the self (rat) nAChR on the NMJ. EAMG induced by TAChR is characterized by two distinct phases: (1) a transient acute phase with a mild muscular weakness, beginning approximately 8-10 days post immunization (p.i.) and recovering after 3-4 days and (2) a severe, progressive chronic phase starting approximately 25-30 days p.i., ending often in death [158].

The immunization with TAChR results in the generation of anti-rat nAChR antibodies and consequently, to the activation of the complement cascade which leads

to the degradation of the muscle end-plate and loss of nAChRs and ultimately to the impairment of the neuromuscular signal transduction. In more detail, IgM directed to TAChR are readily detectable in rat serum during the acute phase of EAMG, while more than half of the antibody repertoire belong to IgG subtype, a finding that suggests that a switch from IgM to IgG production occurs very early in the process. During the chronic phase, 35 days p.i. all detectable anti-TAChR antibodies belong to the IgG class. These late-IgGs, contain both T-AChR and R-AChR antibodies and cross-react with the R-AChR with high affinity [156]. As EAMG progresses into its chronic phase, the titer of cross-reacting antibodies continues to increase and the concentration of muscle nAChRs is greatly reduced. The development of the chronic phase is associated with dramatic increase in titer of serum antibody against muscle nAChR, decrease of post-synaptic membrane area and simplification of its folded structure. The impairment of neuromuscular transmission could result from decreased muscle nAChR content as the amount of receptor extracted from the muscles of myasthenic animals is significantly reduced (approximately 30% compared to normal muscles). Moreover, in chronic EAMG a large portion of the remaining nAChR is bound by antibodies and their function is partially or completely impaired [164].

The course of EAMG and disease severity, is evaluated by monitoring the loss of body weight and muscular strength of the immunized animals. Myasthenic symptoms, assessed prior or after exercise (repetitive paw grips in the cage grid for 30 seconds), include tremor, hunched posture, muscle weakness and fatigue. Grading of the symptoms (clinical scoring) is as follows [165, 166]:

0. no clinical signs observed
1. no clinical signs observed before exercise, appearance of weakness after exercise due to fatigue
2. clinical signs present before exercise such as hunched posture, weak grip or head down
3. no ability to grip, hind limb paralysis, respiratory distress, immobility
4. moribund

Since EAMG is used not only to decipher the human MG pathogenesis but also to discover novel antigen specific therapies, immunization with TAChR might lead to undermining any positive results, as they would be evaluated on a model induced by non-human sequences. Therefore, we standardized and characterized an EAMG

animal model induced with the human nAChR ECD [165]. Lewis rats were immunized with each of the human nAChR subunit ECDs (α1, β1, γ, δ or ε) which resulted in the typical EAMG symptoms, reduction in the muscle nAChR content and pathological electromyographic findings, albeit with different potencies. Immunization with the α1 ECD resulted in higher rates of symptom development, content with the pivotal role of the MIR containing α1 subunit in MG pathogenicity. The use of the human α1 subunit ECD lead to the development of typical, chronic EAMG symptoms 6 to 7 weeks post immunization, in the majority of immunized rats (~95%). Although, there was absence of the transient symptoms that appear in rats about one week after immunization with TAChR and the appearance of symptoms was delayed about 2 weeks, the symptoms persist for several weeks allowing monitoring of symptomatic rats over an extended period. The course of the clinical condition of the animals immunized with hα1 ECD in terms of their body weight and clinical scores is presented in figure 4. Apart from assessing the rats' clinical status, measurement of their serum antibody titers is pivotal in better understanding EAMG's progress. We have set two indicating blood sampling dates throughout the experiment for all our data to be comparable, 45 days and 100 days after the immunization, which represent the symptoms manifestations period and the experiment's termination respectively.

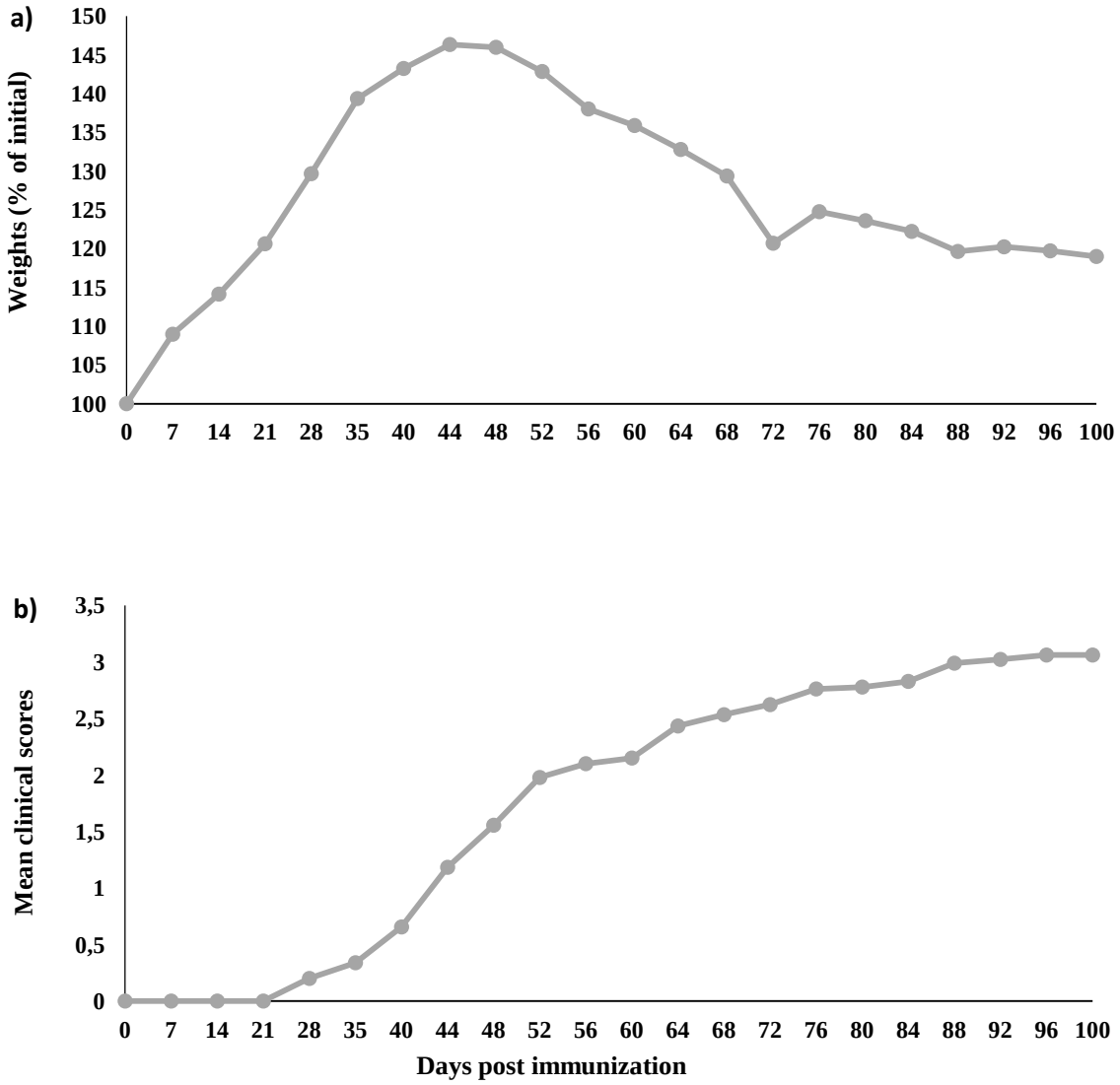


Figure 4. Lewis rats body weights, expressed in percentage (%) of the initial and their mean clinical scores as measured after the immunization with hα1 ECD (Day 0).

Thus, we developed a very useful and well characterized tool, which relies on human nAChR sequences, for the study of novel antigen-specific therapies. As such, this model has been already successfully used for the preclinical evaluation of antigen-specific immunoadsorption, using sepharose-immobilized human nAChR ECDs for the treatment of MG [167] . The currently established approaches for the improvement of patients who are refractory to other treatments or need an immediate effect, include plasmapheresis and IgG-immunoadsorption. However, both these treatments result in the non-specific removal of all other plasma components and immunoglobulins, respectively. Therefore, the selective removal of the pathogenic autoantibodies is an attractive alternative. Repeated immunoadsorption sessions in

EAMG rats resulted in a marked decrease of their serum antibody titers and the subsequent improvement of their clinical conditions. The encouraging results from this study suggest that, antigen-specific immunoadsorption could be an effective alternative therapeutic approach for MG.

1.9 Towards an antigen specific therapy for MG

The current MG treatments are not specific, increasing the possibility to elicit adverse side effects. Thus, several attempts have been made for the discovery of novel antigen-specific therapies. The premise for such a therapy to be successful would be to inhibit the immune response against the target antigens specifically, without interfering with the general function of the immune system [168].

Different approaches have been attempted to re-establish immune tolerance in EAMG animal models. Mucosal administration, prior or after immunization with *Torpedo* nAChR (TAChR), of nAChR or its fragments have produced encouraging preliminary results. In more detail, nasal administration of *Torpedo* AChR [169], or the human acetylcholine α-subunit [170] as well as use of targeted fusion proteins with nAChR fragments [171] have been shown to ameliorate or even prevent the clinical manifestations of the disease. Improvement of the animals' clinical condition was accompanied from reduction of anti-nAChR serum antibodies and improved muscle nAChR content. Furthermore, oral administration of immunodominant TAChR T-cell epitopes [168], or the whole TAChR [172] or rat nAChR (r-nAChR) [173] showed similar positive effects with the nasal administration.

Despite these promising results, no such treatment has been established yet, due to problems encountered with the reproducibility of the animal models and the strenuous procedures needed to acquire the necessary amounts of the treatment antigen (nAChR from *Torpedo californica*).

Based on these results and having in our disposal a highly reproducible animal model, along with easily obtained large quantities of the disease-relevant antigen, we focused our attention to the treatment of EAMG. **In particular, the aim** of this study was to evaluate the therapeutic potency of the human nAChR α1 ECD (hα1 ECD) in the aforementioned human nAChR-induced animal model. Therefore, we set to investigate the therapeutic potency of different routes of administration, amount of antigen administered (dose) and frequency of administration of the hα1 ECD. In more detail:

1. We tested the effect of two different routes of administration, the intranasal or the intravenous, in hα1 ECD immunized Lewis rats.
2. We investigated the efficiency of the hα1 ECD when administered in daily repetitive doses or a single dose
3. We tested the effect of starting the treatment at different time points post immunization.
4. Finally, we examined the therapeutic potency of different doses of hα1 ECD.

In order to evaluate the efficacy of hα1 ECD as a therapeutic agent, we monitored the rats' clinical score, body weight, nAChR antibody titers and muscle nAChR content.

2 Materials

2.1 Laboratory equipment

The necessary instruments and devices for the realization of this study are presented in the following list:

- Ultra-pure water production system, MilliQ Direct 8, Millipore
- Incubation chamber for yeast cultures, CDR ICN100DG
- Incubator for yeast cultures in agitation GALLENKAMP, MRC
- Spectrophotometer Nanodrop 2000C, Thermo Scientific
- Water baths Memmert and Digiterm 3000542
- Sterilizing oven REYPA Steam Sterilizer
- Centrifuge KUBOTA 7780 (heads AG-580CA and AG-5006 and plastic centrifuge tubes and glasses suitable for centrifugation at high turns)
- Microcentrifuge Eppendorf 5410
- Cold microcentrifuge Eppendorf 5415 R
- Table centrifuge Juan CR422
- Solution microfiltration system Millipore
- Solution ultrafiltration system PALL Corp. Ultrasette 10K (includes membranes that block molecules with a molecular mass >10kDa)
- Peristaltic pump Millipore/Masterflex
- Electronic precision scale for measuring small quantities, Mettler, model AESO
- Electronic scale KERN
- Magnetic stirrers HEIDOLPH
- Mixers, Vortex-GENIE 2
- Spyramix
- Pheometer, HANNA
- Radioactivity washer with 12 filter holders, Millipore
- Γ-counter, 1470 Wizard, Perkin Elmer
- Anesthesia machine, Parkland Scientific
- Tissue homogenizer, 230V Grainger

2.2 Consumables

- Petri dishes for solid cultures of yeast, GreinerBio-One
- Conical flasks 500mL, 1L, 2L ISOLAB
- Plastic centrifuge tubes 250mL, 500mL, Corning
- Plastic centrifuge tubes 250mL, 500mL KUBOTA
- Disposable polypropylene test tubes 15 and 50mL, Greiner
- Disposable plastic tubes of 1.5mL GreinerBio-One
- Plastic tips, and plastic pipettes 1, 2, 5, 10 and 25 mL, Costar
- Plastic tips for repeating pipettes, Eppendorf
- Disposable plastic cells, SARSTEDT
- Filters with pore diameter 0.22 and 0.45 μm, Millex, Millipore
- Filters with pore diameter 0.22μm, NALGENE
- Filtering paper 3MM, Whatmann
- Chromatography columns, BIORAD
- Syringes 2, 5, 10 mL, Becton, Dickinson and Company
- Needles 25G, 30G and 22G Becton, Dickinson and Company
- Insulin syringes 1mL, 27G Becton, Dickinson and Company
- Surgical scalpel blades, Swann-Morton

2.3 Reagents

The reagents used were of analytical grade clarity from Sigma, Applichem.

- Agarose, Sigma
- Bacto-peptone, Bacto-tryptone, Bacto-yeast extract, Becton, Dickinson and Company
- Yeast Nitrogen Base (YNB), Sigma
- Imidazole, Sigma
- Glycerol, Applichem
- Ni2+-NTA agarose beads, Qiagen
- $Na^{125}I$ for α-bungarotoxin (α-btx) and ha1 ECD radiolabeling
- Albumin from bovine serum (BSA), Applichem
- Iodoacetamide, Applichem
- Hepes, Applichem
- EDTA, Applichem

- Phenylmethylsulfonyl fluoride (PMSF), Sigma
- Sodium Chloride, Applichem
- Potassium di-hydrogen, Applichem
- Di-Potassium hydrogen, Applichem
- Triton X-100, Applichem
- Monoclonal antibody mAb198 against the hα1 nAChR subunit, derived from hybrid mouse strain S194/5.XX0.BU.1
- rabbit anti-rat IgG, DakoCytomation
- Complete Fraud's Adjuvant, Becton, Dickinson and Company
- Isoflurane, IsoFluo, ESTEVE

2.4 Media Recipes

The culture media for Pichia pastoris cultures were used after liquid sterilization (120^{o}C, 20 minutes, 2 Atm) or after filtration with a sterile filter with pore diameter 0.22μm and were comprised of:

YPD +/- zeocin	1% (w / v) yeast extract, 2% (w / v) peptone, 2% (w / v) dextrose
YPS-agar **+/- zeocin**	YPD containing 1.5% (w / v) agar
RDB-agar	1M sorbitol, 2% (w / v) dextrose, 1.342% (w / v) YNB, 4x10-5 (w / v) biotin, 0.005% (w / v) for each amino acids L-glutamic acid, L-lysine, L-methionine, L-leucine, L-isoleucine, 2% (w / v) agar
BMGY	1% (w / v) yeast extract, 2% (w / v) peptone, 100 Mm phosphate buffer, pH 7.0, 1.34 (w / v) YNB, 4x10-5 (w / v) biotin, 1% (v / v) glycerol
BMMY	1% (w / v) yeast extract, 2% (w / v) peptone, 100 Mm phosphate buffer, pH 7.0, 1.34 (w / v) YNB, 4x10-5 (w / v) biotin, 0.5% (v / v) methanol

2.5 Buffer solutions

PBS 10X	60 mM phosphate buffer pH 7.4, 137 mM NaCl, 2.7 mM KCl
Radioimmunoassay Wash Solution	PBS 1X + 0.5% Tritom x-100
Homogenization Buffer A	0.05M TrisCl (1M stock) pH=7.5,0.1M NaCl, 0.001M EDTA, 0.01M NaN3, 0.01M iodoacetamide, 0.001M PMSF
Homogenization Buffer B	Buffer A + 1% Triton x-100
Phosphate Buffer (PB)	1M K_2HPO_4, 1M KH_2PO_4
Protein isolation dialysis buffer	300mM NaCL, 50mM PB pH=8.0, 2% Glycerol

3 Methods

3.1 Liquid and solid cultures of yeast *Pichia pastoris* cells

P. pastoris is a single-cell methylotropic yeast which is used for heterologous expression of proteins from higher eukaryotic organisms. As a eukaryotic organism, yeast has the advantages of higher eykaryotic expression systems and therefore can produce soluble, properly folded recombinant proteins that have undergone the necessary post-translational modifications (glycosylation, phosphorylation, disulfide bond formation). Compared to other eykaryotic systems used for heterologous expression *P. pastoris* is faster, easier and less costly, and usually gives higher levels of heterologous protein expression.

P. pastoris has a potent promoter, which induces protein expression after the addition of methanol to the medium, allowing controlled expression of heterologous proteins. *P. pastoris* can grow with methanol as the sole source of carbon. The first step, to methanol's catabolism is its oxidation to formaldehyde and peroxide hydrogen, a reaction catalyzed by the enzyme alcoholic oxidase. There are two alcohol oxidase genes with high homology, the *AOXI* and *AOX2*, with *AOXI* being responsible for most of the alcohol oxidase's activity in the cell. Because *P. pastoris* secretes very few of its own proteins, isolation of heterologously expressed secreted proteins is easy.

For the current thesis project we used transformed yeast strains, containing plasmids with our protein of interest, hα1 ECD. These hα1 ECD constructs have their Cys-loop exchanged with the Cys-loop from the acetylcholine binding protein (AChBP) from the snail *Lymnaea stagnalis.* AChBP is a soluble protein, composed of five identical subunits, which share an overall 20-24% identity with the ECDs of the nAChR. This mutation results in high expression yields [174]. Moreover, the hα1 ECD construct contains an N-terminal Flag tag [175] as well as a 6xHis tag at the C-terminus [174] used for its purification with antibody or metal affinity chromatography respectively.

3.1.1 Protein expression in P. pastoris

First, *P. pastoris* cells from stock stored in -80^{o}C were streaked on RDB medium, lacking histidine, and were incubated in 30^{o}C. Colonies are usually developed after 48-72h.

Individual colonies of transformed *P. pastoris* cells containing the hα1 ECD protein gene were transferred to 200ml (x2) of BMGY medium and were incubated for 16-20h, overnight (o/n), at 30^{o}C under stirring at 200rpm. After the o/n incubation when the culture had reached $O.D._{600nm}$= 1.0, we transferred 50 ml of yeast culture from the BMGY flasks to each of 8 flasks of BMMY medium (500ml each) and were incubated for 3 days at 22^{o}C under stirring at 200rpm. In the BMMY medium glycerol had been replaced from 0.5% (v / v) methanol in order to induce protein expression. As the *P. pastoris* cells require good ventilation for growth, the 500ml of BBMY were placed in flasks with volume capacity of 2L. At day 4, as induced protein expression had been completed, the BMMY liquid culture's supernatant containing the secreted protein was collected, by centrifugation at 7.000 rpm, for 15 min at 4^{o}C, using a KUBOTA centrifuge.

3.1.2 Protein isolation from *P. pastoris* supernatant

The process for hα1 ECD's isolation consists of two steps:

a) Microfiltration

The yeast culture supernatant was transferred to a metal sterilized cauldron, which was hermetically sealed. The vessel was connected to a nitrogen gas bottle, under pressure, through a hose which was attached at a specific point on the top of the can. Opposite to the hose's connection site, a metal microfiltration device (Millipore), was connected via a rubber on the top of the can. In the microfiltration device were placed in a horizontal arrangement from top to bottom, Whatman 3M filter paper, gauze, filter with pore diameter 0.45μm, gauze and filter with pore diameter 0.2μm. At the bottom of the microfiltration device was attached a rubber strap, which ended up in conical flask, with volume capacity 10L. In this large flask the filtered supernatant was collected, after the passage of pressurized nitrogen gas ~1Atm, in the metal can.

b) Ultrafiltration and dialysis

After the process of microfiltration the supernatant was condensed through a Millipore tangential flow device, which contained a membrane that excluded molecules with molecular weight >10kDa, while smaller molecules through the

membrane with the solution. The flow of the protein solution to be condensed abuts in this specific membrane. Prior to use, the device was washed with 2L of ddH_2O for 20 minutes without recycling in order to remove the 0.3M NaOH solution in which the device was required to be stored. Thereafter, the flow of the protein solution was applied through the ultrafiltration device by recycling with a peristaltic pump of adjustable volumetric flow. At the end of the condensation process, the protein solution was changed by adding 1L of 50mM PB buffer, 300mM NaCL and 2% Glycerol pH 8.0 for each liter of initial supernatant. The solution was then again condensed. By maintaining a constant volumetric outlet flow rate of 35ml/min, 1L of protein volume was condensed to a final volume of 100ml over 25 minutes.

3.1.3 Protein purification by metal affinity chromatography with Ni^{2+}-NTA agarose column

In an alkaline environment, proteins containing six consecutive histidine residues (6xHis-tag) bind with high affinity to the nickel beads column, because in an alkaline environment the histidine residues are negatively charged, forming a ring surrounding the nickel cations. A specific volume of a nickel suspension solution containing 50% (v / v) Ni^{2+}-NTA agarose beads was centrifuged at 1000rpm, for 5 minutes at 4^{o}C, in a Juan centrifuge, and the supernatant was carefully removed. The Ni^{2+}-NTA beads were equilibrated with 50mM PB buffer, 300mM NaCl pH 8.0 solution buffer with gentle agitation. The supernatant was removed, each time, by centrifugation at 1000 rpm, for 5 minutes at 4^{o}C. After the dialysis, the condensed *P. pastoris* supernatant of 150 ml volume, containing the soluble hα1 ECD protein, was mixed with the equilibrated Ni^{2+}-NTA agarose beads. The hα1 ECD bound to the agarose beads upon o/n incubation with gentle agitation at 4^{o}C with gentle agitation. Afterwards, the Ni^{2+}-NTA with the bound hα1 ECD were placed in a glass chromatography column.

The packed Ni^{2+}-NTA and hα1 ECD were washed with 10 column volumes of buffer solution consisting of 50mM PB, 300mM NaCl and 10mM Imidazole, pH 8.0. Gradual elution is performed first with 50mM PB, 300mM NaCl and 20mM Imidazole pH 8.0. The purified hα1 ECD is eluted with 50mM PB, 300mM NaCl and 150mM Imidazole pH 8.0 in one or more fractions.

3.1.4 Protein quantification with Bradford colorimetric method

Coomassie brilliant blue dye binds to protein molecules and more specifically to lysine (K) residues to produce a colored product. The color intensity depends on the concentration of the protein in the solution. In order to be able to quantitate a protein in a solution, a standard calibration curve is made with BSA at various concentrations. For the standard curve, solutions of 100μl were prepared, with 0.125, 0.250, .0500 or 1.5μg / μl BSA concentrations in 50mM Hepes, 300mM NaCl pH 8.0. In 20μl of the pre-mentioned dilutions we added 1ml of Biorad protein assay solution, which was diluted five times with ddH_2O and we measured the solutions' absorbance at 595nm. The same procedure was followed for the unknown sample. From the standard reference curve, which was designed based on the absorbance of the BSA solutions, the protein concentration in the unknown sample was calculated in μg/μl.

3.2 Chemical modification

Chemical agents are commonly used for the denaturation of proteins. When pH and duration of the reaction are controlled, the modification is normally very specific for cysteinyl residues. In solution, the reaction can be carried out under denaturing conditions, in either 6M guanidine/HCl or 8M urea, to optimize accessibility of cysteine side chains that may be protected from reagent access by the structure of the native protein. B-mercaptoethanol (2-ME) is used to reduce disulfide bonds that form between thiol groups of cysteine residues. Breaking of the disulfide bonds, both the tertiary structure and the quaternary structure of the proteins can be disrupted. Chemical modification of hα1 ECD for conformational studies was performed by reduction and carboxymethylation, with 0.1M of 2-ME in 6M guanidine HCl/0.2M Tris buffer, pH8.8.

3.3 Animals and EAMG induction

6-7 week old female Lewis rats were obtained from the animal breeding unit of the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute. They were maintained in the large rodent unit of the Department. All experiments described were conducted according to the regulations and guidelines for animal care.

Before immunization the rats were anaesthetized with 3% isoflurane supplemented with oxygen. A 250μl portion of emulsion containing a 1:1 ratio of antigen/CFA was administered to each rat. Owing to loss of some of the viscous emulsion on the walls of the eppendorf tubes and in the hub of the syringe, excess emulsion was prepared. The rats were immunized with 80μg hα1 ECD, diluted to the appropriate PBS volume, for a total volume of 125μl. The 125μl of CFA were supplemented with 2mg/ml inactivated Mycobacterium turbeculosis H37RA. The antigen/CFA mixture was emulsified with 20 repeated syringe extrusions, using 2ml syringes with 22G needles.

To proceed to the animals' immunization, we slowly loaded the antigen/CFA emulsified mixture into a 2 ml syringe with a 25G needle, as it is very viscous and easy to form bubbles when transferred. The rats were immunized once in the hind footpads (75μl to each footpad) and at the base of the tail (100μl in two doses).

3.3.1 Treatment protocols

The therapeutic capacity of hα1 ECD was first evaluated by intranasal administration at different timepoints and at different doses after the immunization. 5μg hα1 ECD diluted in 20μl PBS 1X, 10μl in each nostril, were administered 7 (N=10), 28 (N=8) or 42 (N=8) days after the immunization. The treatment was repeated once for 12 consecutive days. 5μg (N=7) or 100μg (N=6) were administered intranasally 7 days after the immunization, one dose/day for 12 consecutive days. For the intranasal administration the animals were not anaesthetized but immobilized with hand grip.

For the studies regarding the effect of hα1 ECD's conformation on its therapeutic capacity we administered intranasally 5μg hα1 ECD (N=7) in its native conformation and 5μg hα1 ECD denatured (N=7).

Afterwards we proceeded to assess the therapeutic potency of hα1 ECD when administered intravenously. The rats were anaesthetized with 3% isoflurane supplemented with oxygen and hα1 ECD was administered intravenously through the tail vein with a 1ml insulin syringe with a 27G needle. 5μg (N=6), 25μg (N=6) or 100μg (N=6) of hα1 ECD were administered intravenously diluted in 200μl PBS 1X. The treatment was initiated 7 days after the immunization and lasted for 12 consecutive days (one dose/day) or only once (N=7).

The control animals in both treatment protocol, intranasal and intravenous, received only PBS 1X as treatment and were subjected to the same procedures and conditions.

3.3.2 Animal monitoring and EAMG scoring

The rats were monitored once a week for the first 4 weeks after immunization and daily thereafter, until the termination of the experiment. Each monitoring visit consisted of weighing of the rats, followed by observation on a flat bench for signs of muscle weakness. EAMG was scored as follows: 0-no symptoms, 1-no symptoms observed at rest but only after exercise (repetitive grasping of a rack for 30s), 2-symptoms present before exercise, 3-very severe symptoms, moribund, 4-death.

3.4 Detection of anti-hα1 ECD antibodies with radioimmunoassay (RIPA).

The radioimmunoassay (RIPA) is a sensitive technique for the detection of antibodies against a specific antigen in serum samples. It is the routine technique for the detection of autoantibodies against nAChR and MuSK in Myasthenia gravis. RIPA's principal of function is based on the antibodies' property to specifically bind to their antigen, forming complexes of high molecular weight. The first step is the radio-labeling of the protein-antigen of interest, which is carried out by other members of the laboratory. The second step is the incubation of the radiolabeled antigen with the rat serum sample. If the sample contains antibodies against the protein-antigen, a complex will be formed. Afterwards, incubation with antibodies from other species that bind to the first antibodies results in heavier complexes that are readily precipitated by centrifugation. In the samples in which antibodies against the protein-antigen are present, radioactivity emitting from the labeled protein-antigen will be trapped in the precipitate. The last step involves washing with a suitable detergent-containing solution to remove unbound radioactivity and measure the radioactivity of the sample. The greater the amount of antibodies against the antigen, the more the radioactive counts per minute measured.

In more detail, the RIPA experimental procedure followed for this master thesis was:

Initially, 50000cpm of labeled antigen (hα1 ECD) were incubated with 2μl rat serum in a final volume of 50μl (with the appropriate volume of PBS-BSA 0.2%) for 2 hours

at 4°C. We also added in the reaction 2μl of normal rat serum (NRS) in order to block possible non-specific binding. Subsequently, 10μl of rabbit anti-rat IgG were added and the mixture was incubated for 2 hours at 4°C, so as to create a precipitate. Next, 1ml of washing solution was added and centrifugation at 2500rpm for 15 minutes at 4°C, followed. Thereafter, the precipitated pellet was resuspended and a second wash with subsequent centrifugation followed. The supernatant was removed and the radioactivity bound to the precipitate was measured in γ-counter. The radioactivity of the precipitate was proportional to the amount of anti-hα1 ECD antibodies bound to the labeled hα1 ECD.

3.5 RIPA for the detection of anti-r-nAChR antibodies

The first attempt for the detection of antibodies against the muscle nAChR was based on immunoprecipitation of the antibodies, after they were incubated with solubilized nAChR from human muscle. Solubilized nAChR was labeled with radioactive bungarotoxin (^{125}I-α-bungarotoxin), a snake toxin that binds with very high affinity to nAChR. Therefore, for this master thesis project we utilized this alternative RIPA method to detect the anti-r-nAChR antibodies. In more detail, 20μl of nAChR isolated from muscles of healthy Lewis rats were incubated with 50000cpm of ^{125}I-α-btx for 1 hour at 4°C. After the incubation 2μl of rat serum sample combined with 2μl of NRS were added to the reaction, to a total volume of 50μl (with appropriate volume of PBS-BSA 0.2% w/ v) and the mix was incubated for 2 hours at 4°C. 10μl of rabbit anti-rat IgG, secondary antibody were added and the samples were incubated for 2 hours at 4°C. Ultimately, we proceeded to the removal of excess, unbound radioactivity, following the steps described in section 3.5 and measurement in γ-counter.

3.6 Rat muscle nAChR isolation and quantitation

3.6.1 Muscle homogenization

In order to fully assess the clinical status of the animals with EAMG and the effect of the treatment, was essential to quantitate their muscles nAChR content. At the end of each experiment, the animals were sacrificed by CO_2 and their tibialis anterior muscles were dissected and homogenized in order to quantitate their nAChR content. More specifically, first muscles were isolated and processed to remove their

connective membranes and tendons. Throughout the experimental procedure we needed to work on ice. The samples were weighed in order to calculate the appropriate amount of lysis buffer (Buffer A, § 2.5) required. 5ml/g of Buffer A were added and each sample was homogenized in Graiger tissue homogenizer. The muscles were initially crushed with repeated, gentle moves for 10-15 minutes (depending on muscle size) until they were completely dissolved. The solution was then centrifuged at 17.000g for 20 minutes at 4^{o}C. The supernatant was discarded while the precipitate was resuspended in Buffer B (Buffer A+ 1% Triton X-100), until it was fully homogeneous. The new solution containing the membranes of the dissolved muscle cells was incubated for 3 hours at 4^{o}C, in circular stirrer. Buffer B's detergent resulted to muscle membranes disruption and solubilization and the subsequent release of their proteins, including nAChRs. Centrifugation at 17.000g for 20 minutes at 4^{o}C followed. The supernatant containing the nAChRs, was collected with 1ml syringe and a 25G needle. If the samples were not immediately further analyzed, we added 10% glycerol and were stored in -80^{o}C.

3.6.2 nAChR content quantitation

For the quantitation of the homogenized muscles' nAChR content we incubated 50μl of muscle extract with 50.000cpm of ^{125}I-α-btx for 1 hour at 4^{o}C, to a final volume 90μl (with the appropriate PBS-BSA 0.2% w/v volume). For negative controls we used 50μl of the same muscle extracts incubated with both ^{125}I-α-btx and non-radiolabeled α-btx (cold), in 1/200 analogy. Next, we added 10μl of the monoclonal antibody mAb198 (diluted 1/100 in PBS-BSA 0.2% w/v), which was specific for nAChR's MIR, and incubated for 2 hours or overnight at 4^{o}C. Addition of 10μl of secondary antibody, rabbit anti-rat IgG followed and the samples were incubated for 2 hours at 4^{o}C. Addition of 1ml of washing solution (PBS 1X -0.5% Triton X-100) and centrifugation at 2500rpm for 15 minutes at 4^{o}C, followed. Thereafter, the precipitated pellet was resuspended and a second wash with subsequent centrifugation followed. The supernatant was removed and the radioactivity bound to the precipitate was measured in γ-counter. The results were presented as α-btx binding sites per g muscle.

3.6.3 Estimation of hα1 ECD's half life

The amount of time an intravenously administered molecule remains in the blood circulation is very important for the evaluation of its therapeutic capacity as well for the determination of the dosage amount and frequency. Therefore, to fine-tune hα1 ECD's delivery conditions (dose and frequency), but also to maximize its positive effects we sought to determine its half-life when administered intravenously. For that purpose, we injected radiolabeled hα1 ECD and measured the radioactivity's levels through time in the blood circulation of rats. In more detail, in 11 female Lewis rats immunized with hα1 ECD, we administered intravenously, using 1ml insulin syringes with 27G needles, 100μg hα1 ECD supplemented with 15μl ^{125}I-hα1 ECD corresponding to 10^^{6} cpm. The animals were anaesthetized with 3% isoflurane supplemented with oxygen. After the injection they were kept in concrete lead boxes to reduce the radioactivity dispersion. Blood samples were obtain at 5, 15, 30, 60, 120, 180 and 360 minutes after the injection, through the tail artery with 1ml insulin syringes. Prior to radioactivity the volume of the samples was measured for the cpm/μl ratio. Radioactivity was measured in 1470 Wizard γ-counter. After the experiment the animals were sacrificed by CO_2.

3.7 Statistical analysis

The clinical scores and antibody titers were analyzed using the Spearman's rank correlation coefficient. Muscle nAChR contents of the different treatment groups were compared using analysis of variance (ANOVA) with Tukey hic test (GraphPad Prism 6).

4 Results

In MG, the ECD of the nAChR α1 subunit contains the MIR and is considered the most immunogenic, when comparing the pathogenic capacity of all the nAChR subunits [165]. Therefore, the development of an antigen specific therapy based on the α1 subunit ECD should be the optimal approach. The main goal of this project was to investigate the therapeutic potency of the hα1 ECD for MG using immunized Lewis rats as an EAMG model.

All animals were monitored for changes in their clinical status. The primary parameter for the evaluation of the progression of EAMG and assessment of the effect of the treatment is the EAMG score of each animal as follows: (0) no clinical symptoms, (1) signs of weakness present only after exercise, (2) signs of weakness prior to exercise, (3) no ability to grip, hind limb paralysis, immobility and (4) moribund. The animals' clinical scoring was done once every week for the first 35 days and every day thereafter and included repetitive paw grips in the cage grip test for 30 seconds and observation on a flat bench. Similarly, their body weight was measured once a week for the first 35 days after the immunization and every day onwards, as symptoms start manifesting from this day and keep aggravating in the normal course of EAMG progression. Affected animals start losing weight, since chewing becomes more difficult, thus measuring their body weight constitutes an important parameter, but it should be kept under consideration that other factors may also contribute to body weight changes.

Additionally, to more comprehensively assess the therapeutic potency of hα1 ECD, we also measured the serum autoantibody titers. Immunization with the hα1 ECD in CFA, which results in an immunological response and subsequent epitope spreading, is responsible for the generation of the pathogenic rat-nAChR antibodies (r-nAChR) [165]. In our rat EAMG model the r-nAChR antibody titers correlate with symptom severity [165], therefore, their quantitation would be an indication of a

positive effect observed by the administration of the hα1 ECD. To determine the r-nAChR antibody titers we performed RIPA with rat serum samples using as antigen muscle nAChR isolated from denervated muscles of healthy Lewis rats. As we cannot directly label radioactively the whole nAChR we used I^{125} labeled α-bungarotoxin, which binds with high affinity to the α-subunit of nAChR [176]. Through this method, we were able to measure the r-nAChR antibodies and their titers. For the measurement of the hα1 ECD antibody titers we performed RIPA with directly I^{125} radiolabeled hα1-ECD.

The destruction of available nAChR molecules by the pathogenic autoantibodies results in impairment of the signal transduction and thus to the manifestation of muscle weakness and fatigability. Therefore, another important parameter that is useful to assess the effect of hα1 ECD treatment is the quantitation of the rats' muscle nAChR content. To this end, after euthanasia the tibialis anterior muscles were dissected and their nAChR content was quantified with RIPA using I^{125} labeled α-bungarotoxin.

4.1 Evaluation of the treatment initiation time point

Based on previous studies, showing that nasal administration of the disease-relevant antigen can ameliorate or prevent the manifestation of disease symptoms [170], we administered 5μg of hα1 ECD nasally, to evaluate its effect in our EAMG model. Initially, we investigated the most appropriate time point for the initiation of the treatment. As EAMG symptoms manifest in the period between 35 and 50 days post immunization (p.i.), we started the treatment 7 days p.i. (D7, early time point), 28 days p.i. (D28, late time point, prior to symptoms) and 42 days p.i. (D42, late time point, around symptom onset). Control animals were immunized but received an equal volume of PBS only. All the animals underwent the same procedures with regard to handling for treatment administration and monitoring. For each of the experimental groups 10, 8 and 8 animals received hα1 ECD, and 10, 6 and 6 animals received PBS (in the D7, D28 and D42 groups respectively). The treatments consisted of 12 daily doses.

The clinical status of the treated animals was assessed primarily by recording their clinical scores (figure 1). The animals that did not receive hα1 ECD and instead received only PBS as treatment showed the typical EAMG symptom manifestations starting at the expected onset period (approximately 35-40 days p.i.), which kept worsening until the experiment's termination (100 days p.i.) without signs of remission. The rats that started the treatment at D7 showed slightly delayed development of EAMG symptoms, around 47 days p.i. Additionally, they had milder disease manifestations in comparison to the other treatment and control groups as indicated from the lower mean clinical scores, not exceeding a score of 2 throughout the observation period. On the other hand, the clinical status of the D28, as well as at the D42 groups, followed a similar deteriorating pattern with the controls, although they did not appear to reach the same levels of severity.

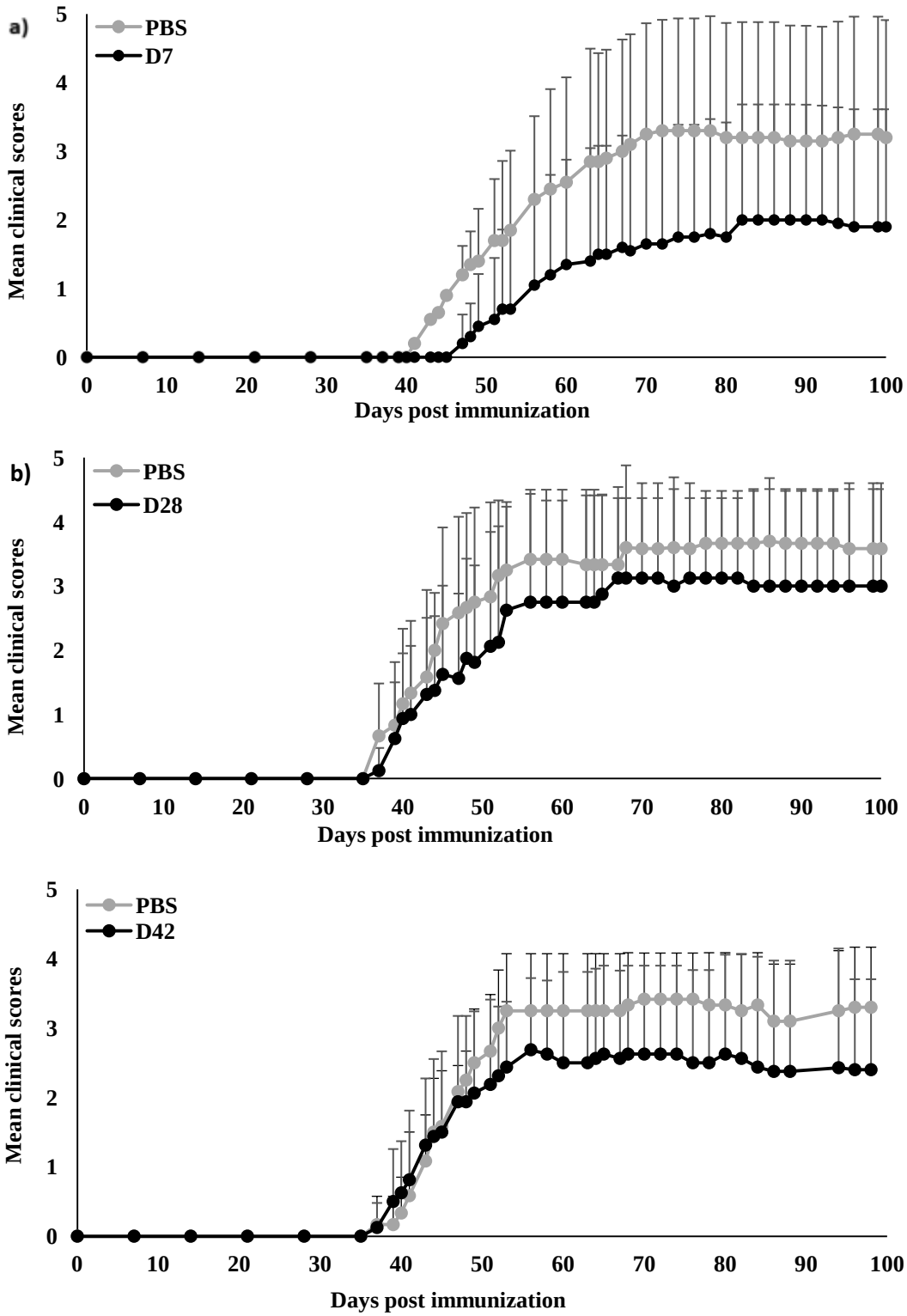


Figure 1. Clinical scores of rats treated nasally with 5µg ha1 ECD starting at different times after immunization: a) Animals starting treatment on D7, b) animals starting on D28 and c) animals starting on D42. The grey lines represent the average scores of animals treated with PBS (N=10, 6 and 6 for a, b and c respectively), the black lines represent the average scores of the hα1 ECD treatment group in each scenario, (N=10, 8 and 8 respectively).

Another parameter we measured to further assess the effect of the treatment was the rats' weights, expressed as percentage (%) of the initial for each animal. The weights' progress throughout the monitoring period for the animals of each treatment group is presented in figure 2. The animals treated only with PBS followed a declining course throughout the experiment, losing a significant proportion of their body weights after the onset of symptom manifestation. In contrast, the animals whose treatment was initiated at D7 (figure 2a), even though they had weight loss, it was limited compared to the controls, an effect that lasted until the experiment's termination. The treatment seems to slightly ameliorate the disease's manifestations as the continuous weight loss observed in the PBS treated animals is not present. Here also, the observed weight loss manifests at a later time–point than in controls. On the other hand, our results from the administration of the treatment at the intermediate time-point D28, or late time point D42 (figure2b & 2c) show a similar weight fluctuation as the controls. Subsequently, we measured the serum antibody titers as to better evaluate the treatment's effect when administered at the alternate time-points on the humoral response against the EAMG-related protein (hα1 ECD antibodies) and the endogenous muscle r-nAChRs (r-nAChR antibodies).

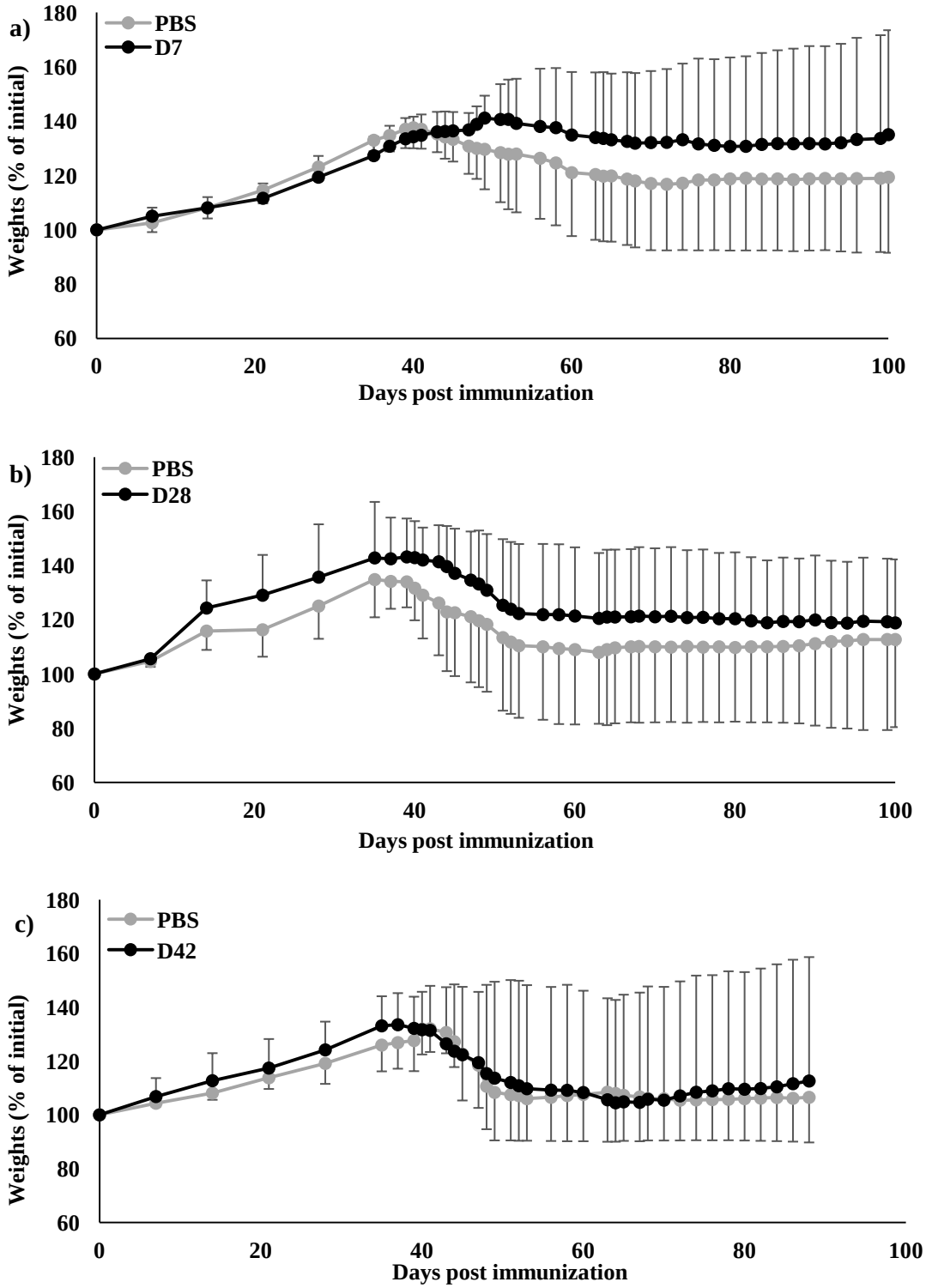
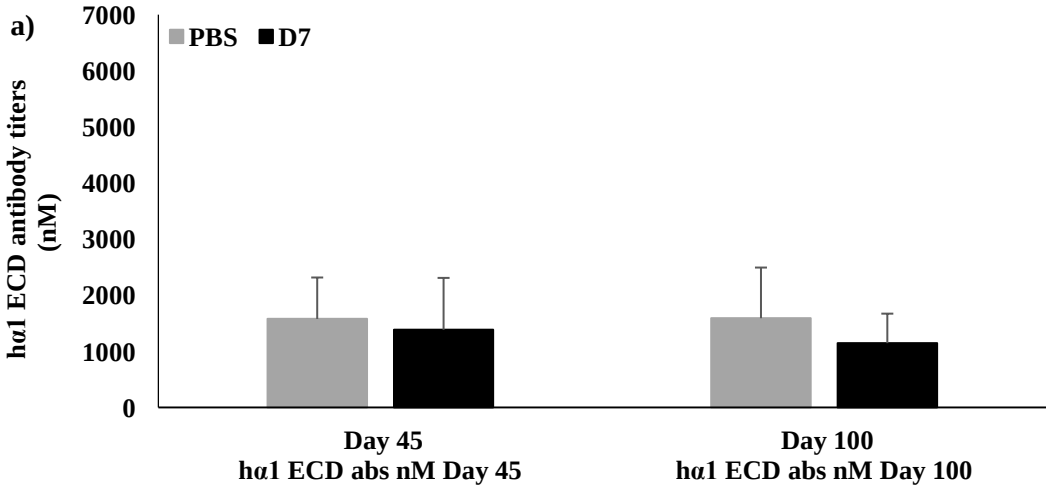


Figure 2. Body weights of nasally treated rats with 5µg ha1 ECD. The average body weights for each group of rats is represented as a percentage (%) of the initial weights at different times after immunization (Day 0): a) animals starting treatment on D7, b) animals starting treatment on D28 and c) animals starting treatment on D42. The grey lines represent the animals that were treated with PBS (N=10, 6 and 6 for a, b and c respectively). Black lines represent the hα1 ECD treatment groups (N=10, 8 and 8 respectively).

In order to measure the serum hα1 ECD antibody titers, we used serum samples collected at two different time points, 45 and 100 days after immunization (figure 3). The measurements taken by the three different conditions when compared to the controls of each group, do not show any effect of the treatment on the hα1-ECD antibody levels. The apparent differences between the control and treatment groups are not statistically significant. Therefore, when taking into consideration these data we can conclude that the treatment does not seem to affect the titers of the antibodies against hα1 ECD.



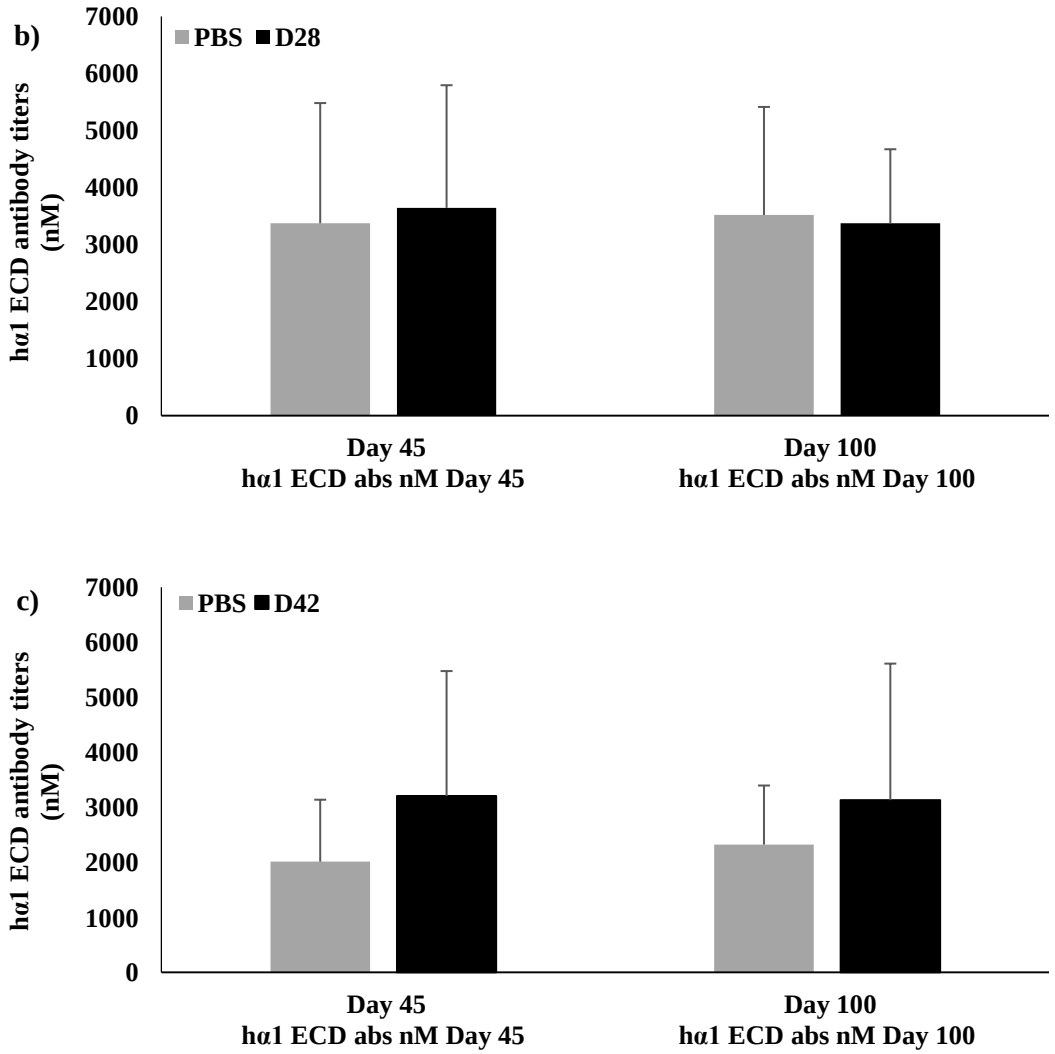
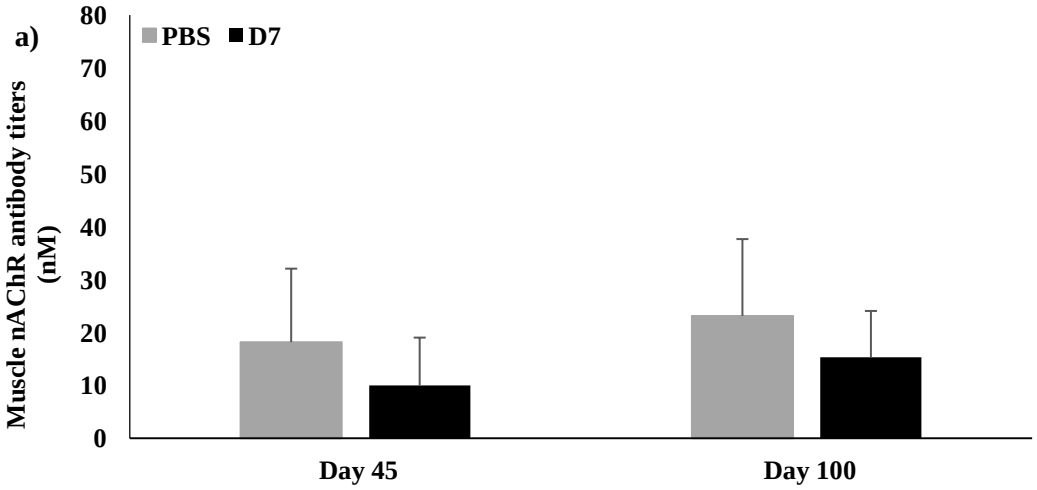
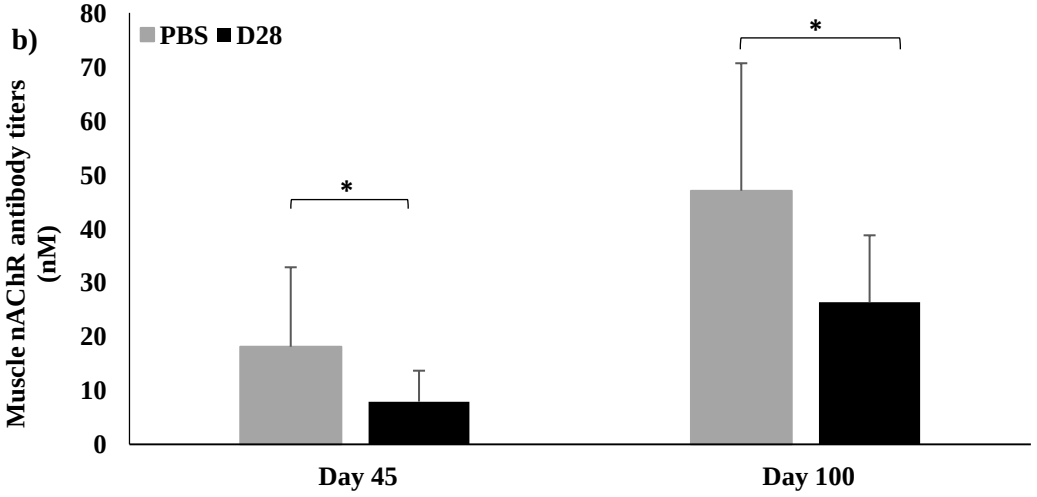


Figure 3. Titers for the ha1 ECD antibodies. The mean hα1 ECD antibody titers of each group was measured in serum samples taken at days 45 and 100 p.i.: a) Treatment initiation 7 days p.i., b) treatment initiation 28 days p.i. and c) treatment initiation 42 days p.i. Grey bars represent the average titers of PBS treated animals (N= 10, 6 and 6 respectively), while black bars represent the average titers of the ha1 ECD treated animals (N= 10, 8 and 8 respectively).

To further assess the treatment's efficacy, we measured the titers of the antibodies against the r-nAChR, since these are the pathogenic molecules that lead to the symptoms manifestations. As previously, we measured the r-nAChR antibody titers in serum samples isolated at 45 and 100 days p.i. (figure 4). The results from the quantitation of the mean r-nAChR antibody titers show a reduction trend in all the experimental treatment groups when compared to the r-nAChR antibody levels corresponding to the PBS treated animals. The small reduction of r-nAChR antibodies of the D7 treatment animals (figure 4a), even though not statistically significant, is consistent with their improved clinical scores and ameliorated weight loss. On the

other hand, the r-nAChR antibody levels from the animals with treatment initiation at D28 (figure 4b) show a statistically significant reduction. In this group, the reduced r-nAChR levels coincide with improved clinical status. The animals treated at the later time-point of EAMG progression, at D42, did not present with a change in their antibody levels, since the small decrease in the treated animals was again not significant. Overall, our data regarding the levels of the pathogenic antibodies against the muscle r-nAChR suggest that nasally administered hα1, has a possible positive effect.





c)

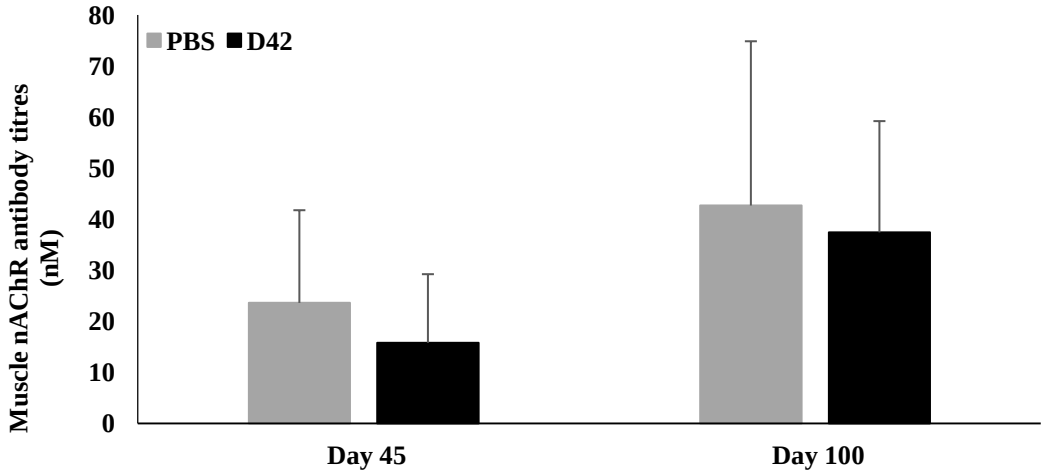
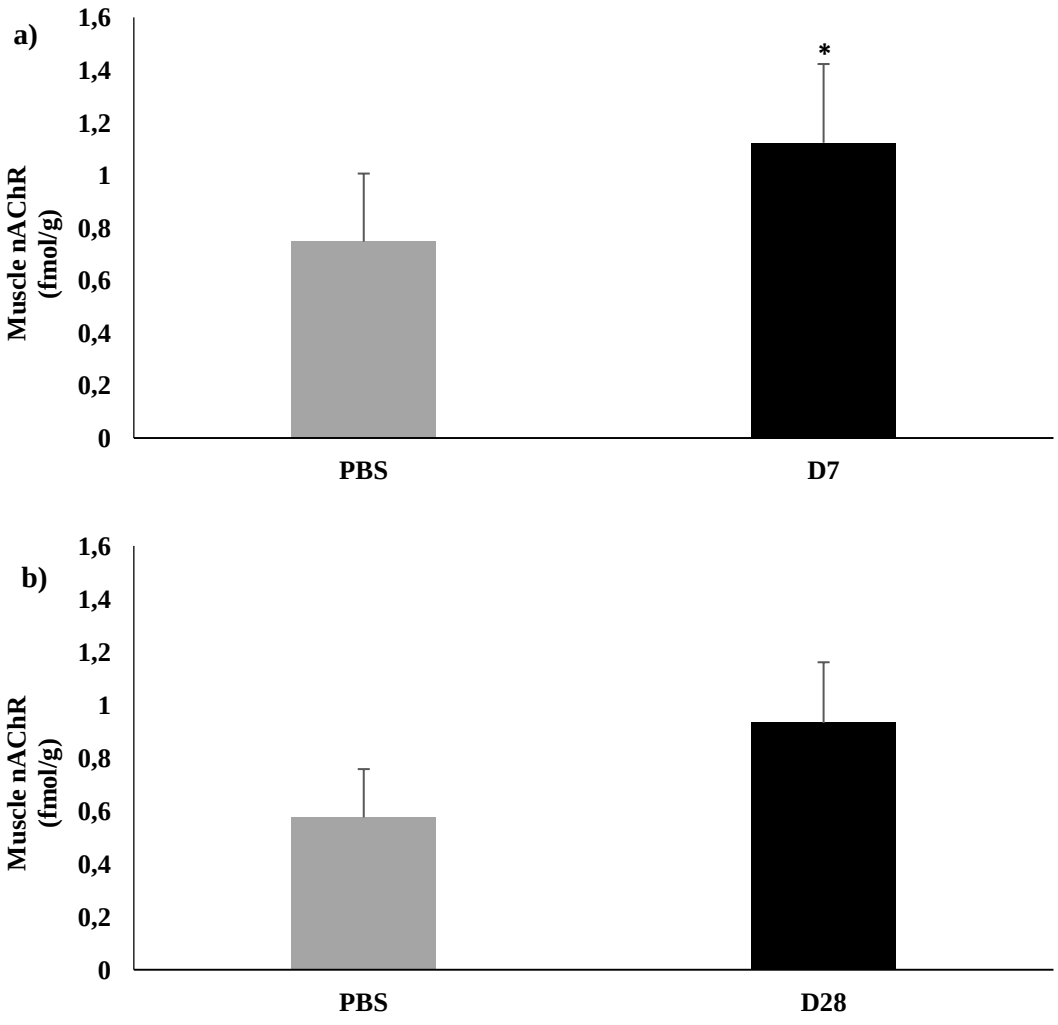


Figure 4. R-nAChR antibody titers. The mean r-nAChR antibody titers of each treatment group in serum samples taken 45 and 100 days p.i. a) Treatment initiation 7 days p.i., b) treatment initiation 28 days p.i. and c) treatment initiation 42 days p.i. Grey bars represent the PBS treated animals (N=10, 6 and 6 respectively), black bars the animals from D7, D28 & D42 treatment initiation points (N=10, 8 and 8 respectively). *$p<0.05$

The muscles isolated from the groups that received intranasal treatment at the different time points, D7, D28, or D42 (figure 5 a, b and c respectively) had increased nAChR muscle content compared to controls, suggesting that the treatment might have resulted in reduction of the damage at the post-synaptic membrane. The highest concentration in muscle nAChR content was observed in the muscles isolated from the rats that received the treatment at D7 (figure 5a). The detected increase was statistically significant ($p<0.05$) and therefore it provides another indication of the positive effect of the treatment when it is initiated at the earliest time point. The increased r-nAChR content found in the muscles of rats treated at the later time-points (D28 and D42), although not statistically significant, could indicate a possible positive effect of the treatment, but with no capacity at this point to reverse the progress of the disease.

Overall, the data gathered from this series of experiments suggest a positive effect of the hα1 ECD on the EAMG primed animals. Administration at the early (D7) and intermediate (D28) time-points, resulted in improved clinical status accompanied from lower levels of the r-nAChR antibodies and increased muscle nAChR content. Even though, the response to the treatment was limited when it was administered at the later time-point of D42, a trend of reduced r-nAChR antibody titers and increased muscle nAChR contents was still evident. Taking into consideration our results in

their entirety and the slightly overall greater improvement of the animals whose treatment started at the earlier time-point (D7), we set D7 as the treatment initiation date for our further experimental procedures.



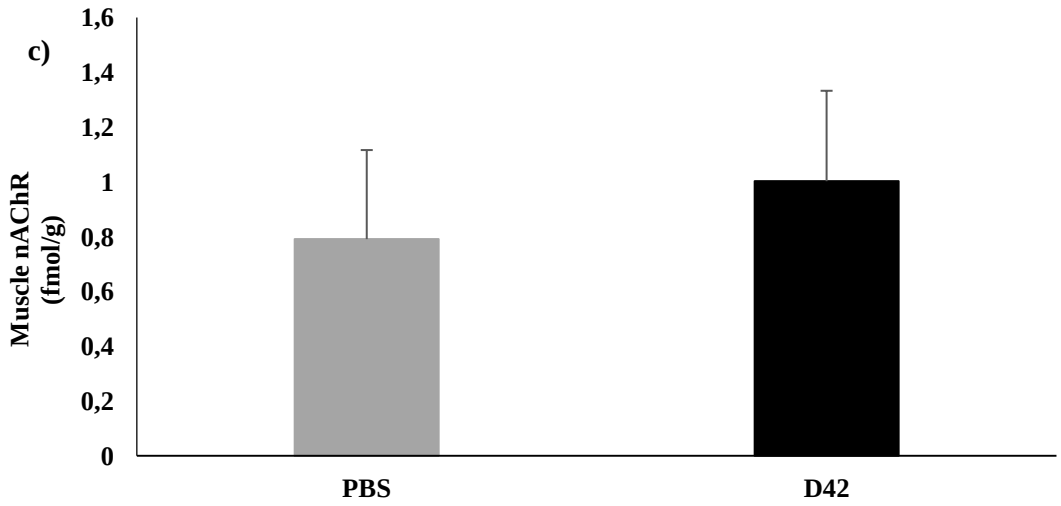


Figure 5. Muscle nAChR content of the treatment groups. Quantitation of the content of nAChR of the Tibialis anterior muscle, expressed in fmol/g tissue. a) Treatment initiation 7 days p.i., b) treatment initiation 28 days p.i. and c) treatment initiation 42 days p.i. Grey bars represent the average of PBS treated animals (N=10, 6 and 6 respectively), black bars represent the average of animals treated with hα1 ECD (N=10, 8 and 8 respectively). *p<0.05.

4.2 High dose intranasal

Our initial results showed a positive effect of hα1 ECD when administered intranasally at the earliest time point of 7 days p.i., but because this effect was marginal, we decided to investigate whether it would be improved by increasing the administered dose. Therefore, we administered 100 μg of hα1 ECD nasally (N=6), starting the treatment 7 days after the immunization and repeating for 12 consecutive days. Again, the controls, received only PBS as treatment (N=6).

As expected, the PBS treated animals followed the typical course of EAMG, as their muscle weakness was evident from the first days of the symptoms manifestations period and kept aggravating until the experiment's termination (figure 6 grey line), with mean clinical score 3.5. The administration of 100μg of hα1 ECD lead only to a mild amelioration of EAMG symptoms. Animals showed muscle weakness prior to exercise but without exceeding mean scores of 2.7 ± 1.02 (figure 6, black line). Also, the animals from the hα1 ECD treatment group exhibited a marginally later onset of symptoms. Nevertheless, we did not observe any statistically significant differences. The body weights of the control and treated animals are presented in figure 7. The animals that were treated with PBS (grey line) lost a significant amount of their body weight after the symptoms manifested and followed a declining course until the end of the experiment. The animals that received treatment

also lost weight during the symptomatic period presenting the same pattern as the controls.

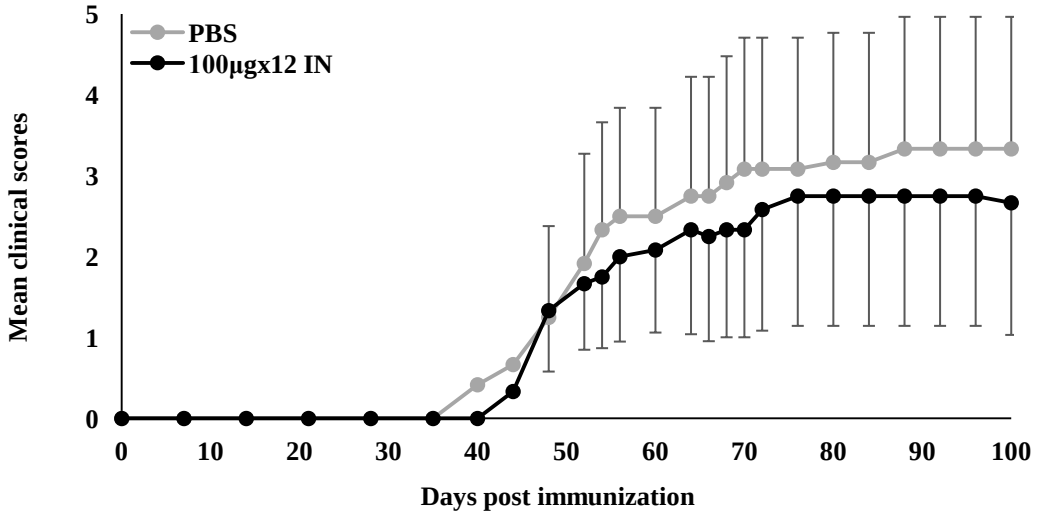


Figure 6. Clinical scores of rats treated nasally with 100µg ha1 ECD, measurements at different times after immunization (Day 0). Grey: PBS animals (N=6), black: animals treated with 100µg hα1 ECD (N=6).

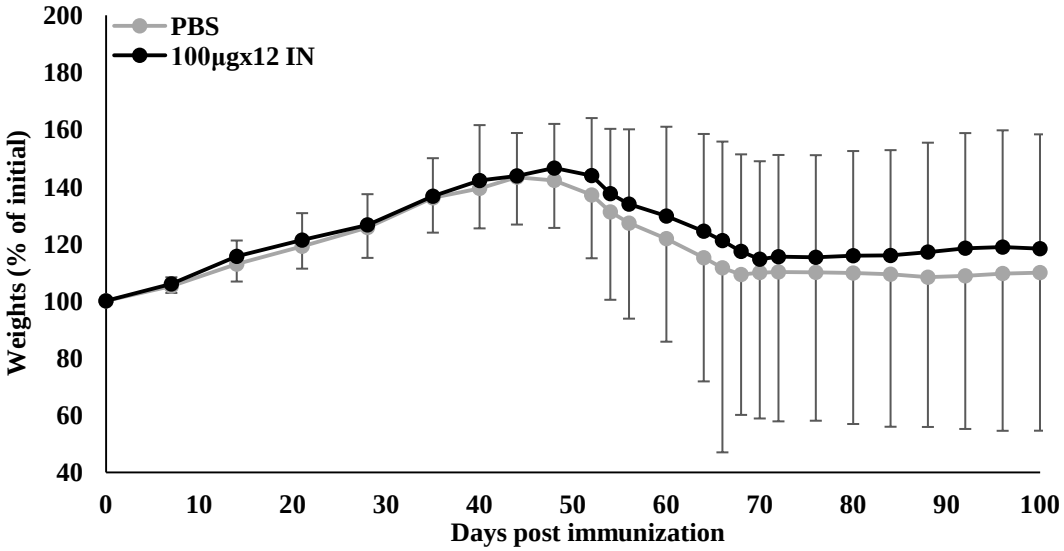


Figure 7. Body weights of nasally treated rats with 100µg ha1 ECD, 7 days p.i. The average body weights for each group of rats is represented in percentages (%) of the initial weights and is shown at different times after immunization. Grey: mean weights of animals treated only with PBS (N=6), black: animals treated with 100µg hα1 ECD (N=6).

Next, we investigated whether the increased dose of hα1 ECD affected the serum titers of hα1 ECD antibodies, in the EAMG afflicted animals (figure 8). Interestingly, the group that received hα1 ECD presented with an increase trend of the hα1 ECD antibodies, though with no statistical significance compared to the controls. Subsequently we measured the serum titers of the pathogenic r-nAChR antibodies as shown in figure 9. We did not detect any significant changes in the antibody titers occurring when administering the higher dose of hα1 ECD (figure 9). Furthermore, in this case our results do not show consistency in the reduction trend as observed in the previous series of experiments.

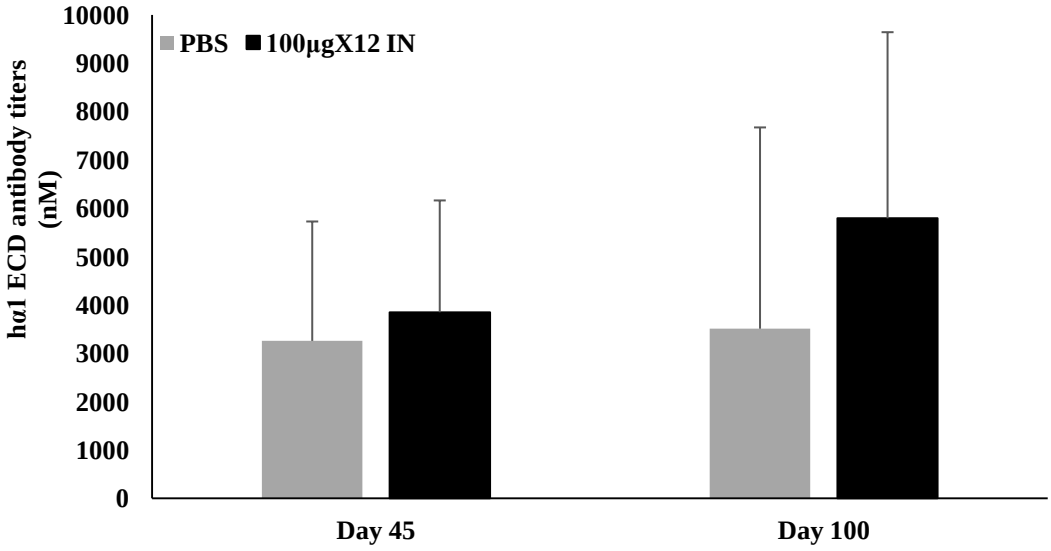


Figure 8. Ha1 ECD antibody titers. The mean hα1 ECD antibody titers of the different groups as measured in serum samples taken at day 45 and day 100 p.i. Black: animals that received 100µg hα1 ECD (N=6), grey: PBS animals (N=6).

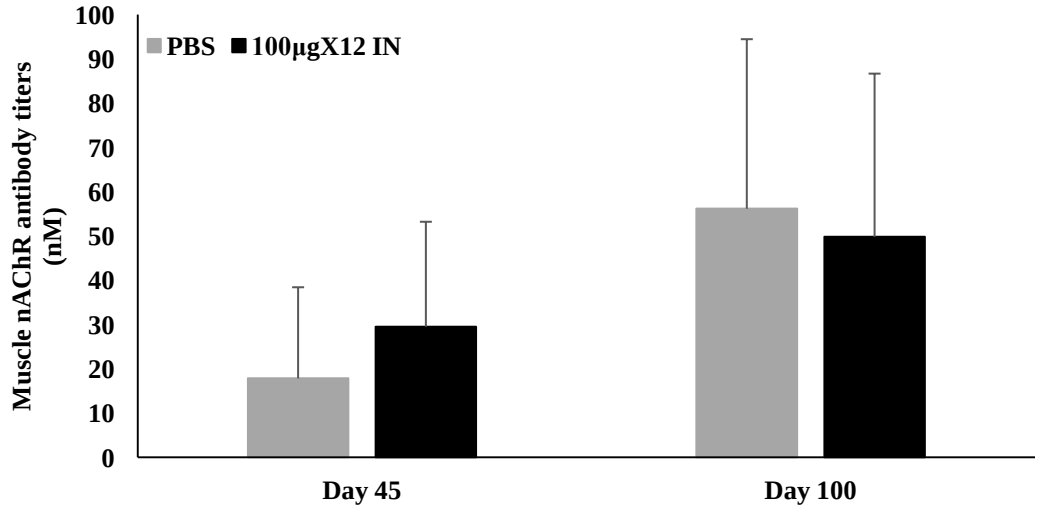


Figure 9. R-nAChR antibody titers. The mean r-nAChR antibody titers of each therapy group as measured in serum samples taken 45 and 100 days p.i.: Black: animals that received 100µg hα1 ECD (N=6), grey: PBS animals (N=6).

To complete our investigation of the effect of the higher dose of hα1 ECD we measured the nAChR muscle content of the treated animals and the results (figure 10).

In accordance with our findings so far, our data did not indicate any change of the muscle nAChR content of the treated animals. Overall, these data indicate that the higher dose of hα1 ECD did not result in an improvement of hα1 ECD's therapeutic potency.

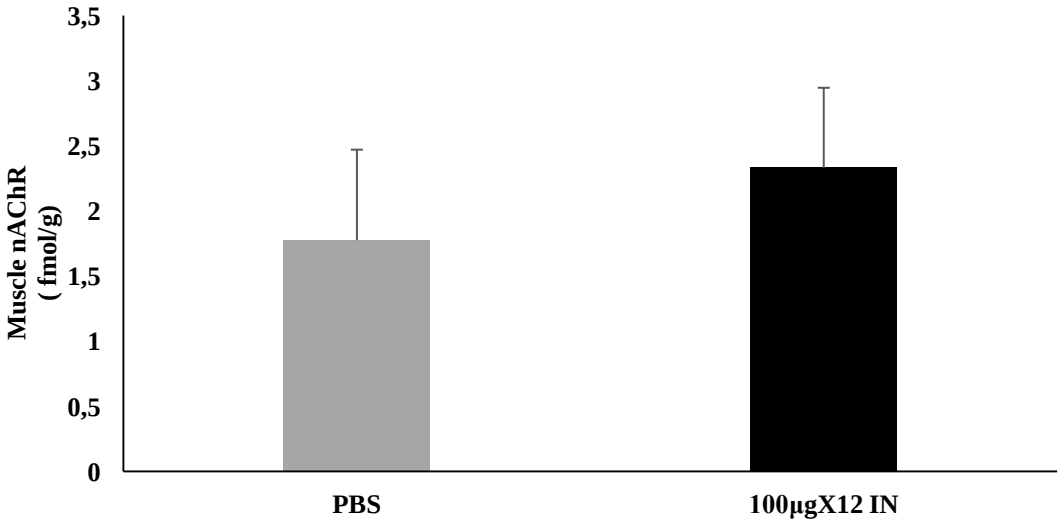


Figure 10. Muscle nAChR content of the treatment groups. Quantitation of the mean content of nAChR of the treatment group, expressed in fmol/g tissue. Black: treatment group that received intranasally 100µg hα1 ECD (N=6) and grey: PBS animals (N=6).

4.3 Antigen's conformation role in efficacy

Previous studies for the induction of mucosal tolerance in EAMG animal models have shown that the autoantigen's conformation can affect its capacity to induce tolerance [177]. Therefore, we wanted to investigate the significance of hα1 ECD's conformation in the induction of nasal tolerance in our model. In order to address whether changes in hα1 ECD's conformation affect the positive effect we had seen in our previous results, we retained the amount of dose (5µg hα1 ECD), frequency (12 daily doses) and treatment initiation time (D7) and we administered the hα1 ECD either in its native conformation or denatured.

In this experiment 5 animals were used as controls, receiving only PBS as treatment, 7 animals received intranasally 5µg hα1 ECD in its native conformation and 7 animals 5µg denatured hα1 ECD. Firstly, we assessed their clinical status by measuring their clinical scores (figure 11) and their weights (figure 12). In regard to the clinical scores, both treatments, native and denatured hα1 ECD, ameliorated EAMG clinical manifestations, with the animals exhibiting symptoms with maximum

average clinical scores 2.8 and 2.2, respectively. The animals that were treated only with PBS had the typical EAMG clinical image: symptoms manifestations started at the expected period, severe disease manifestations with maximum mean clinical score 3.7 (figure 11) and substantial loss of bodyweight (figure 12). On the other hand, the animals from both treatment groups had decreased weight loss in comparison to controls, while there was not substantial difference observed between the two treatments (figure 12).

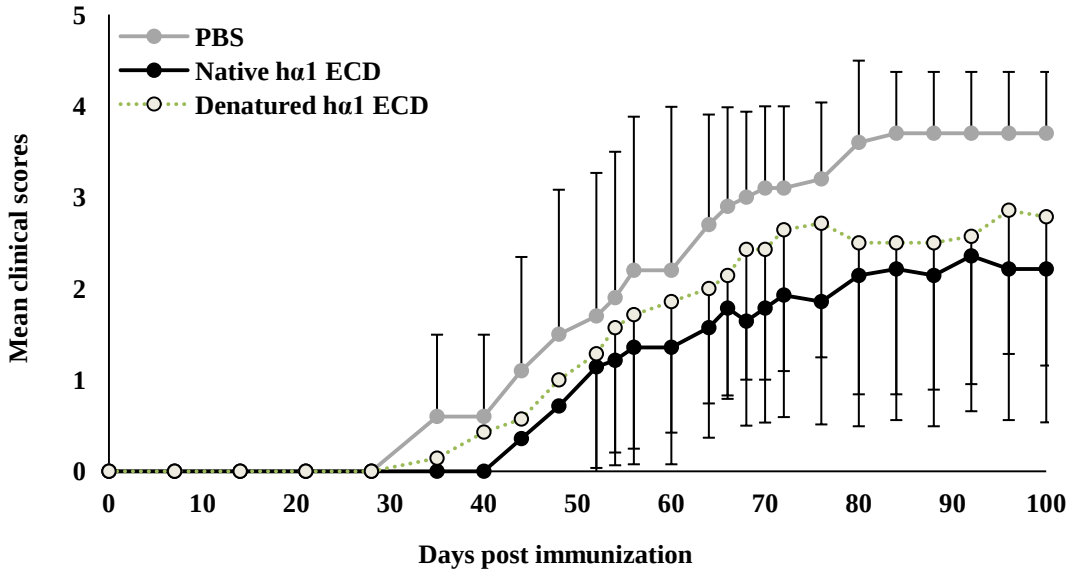


Figure 11. Clinical scores of rats treated nasally with 5μg of native hα1 ECD or 5μg denatured ha1 ECD. Grey: animals treated only with PBS (N=5), black: animals treated with 5μg native hα1 ECD (N=7) and grey dotted line: animals treated with 5μg denatured hα1 ECD (N=7).

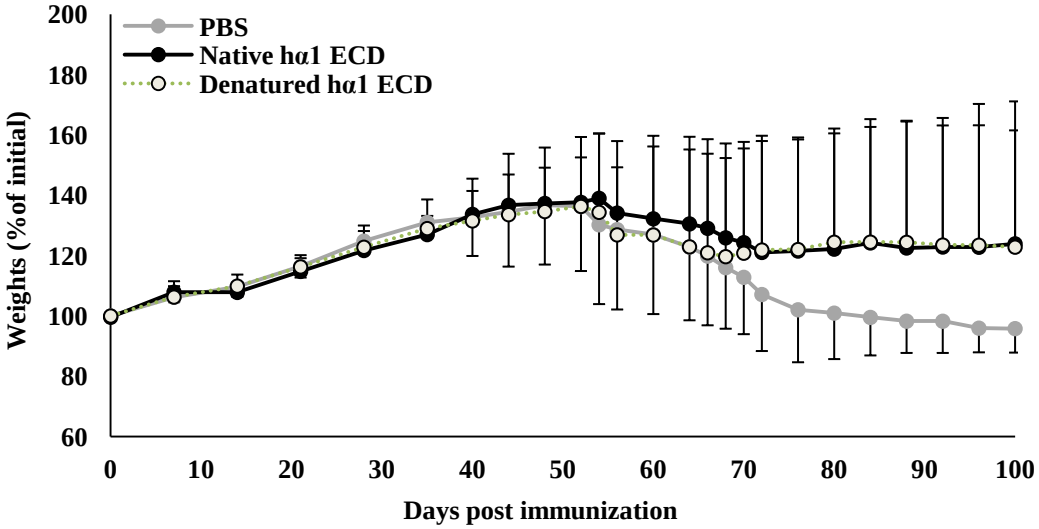


Figure 12. Body weights of nasally treated rats with 5µg native ha1 ECD or 5µg denatured ha1 ECD, 7 days p.i. The average body weights for each group of rats is represented in percentages (%) of the initial weights and is shown at different times after immunization. Grey: animals treated only with PBS (N=5), black: animals treated with 5µg hα1 ECD in native conformation (N=7) and grey dotted line: animals treated with 5µg hα1 ECD denatured (N=7).

When measuring the serum antibody titers from blood samples of the 45th and 100th day after the immunization (figure 13) we did not observe an effect of either of the treatments on the hα1 ECD antibodies, neither when comparing them with the controls nor between them. Next, we measured the serum r-nAChR antibody levels (figure 14) and found that neither of the treatments caused detectable effects on the serum r-nAChR titers of the samples taken 45 days p.i. On the other hand, the serum titers of r-nAChR antibodies from the samples taken 100 days p.i. were decreased in comparison to the ones representing the PBS treated group, supporting our previous findings showing a reduction tendency of the r-nAChR antibody levels in the treated animals. However, the observed difference in the r-nAChR titers between the native or denaturated hα1 ECD and the controls, was not statistically significant.

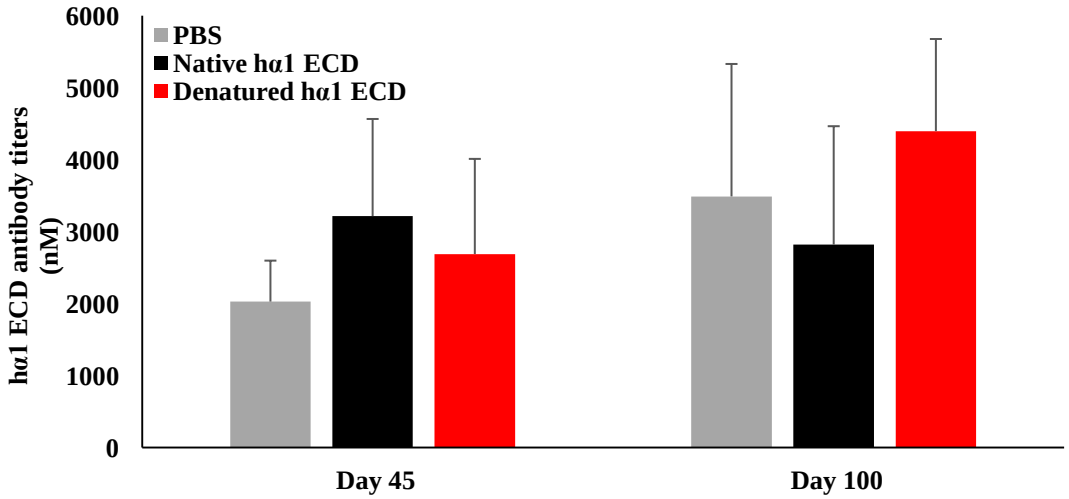


Figure 13. Serum ha1 ECD antibody titers. The mean hα1 ECD antibody titers of the different groups, as measured in serum samples taken at 45 and 100 days p.i. Grey bars: animals treated with only PBS (N=5), black: animals treated with native hα1 ECD (N=7) and red: animals treated with denatured hα1 ECD (N=7).

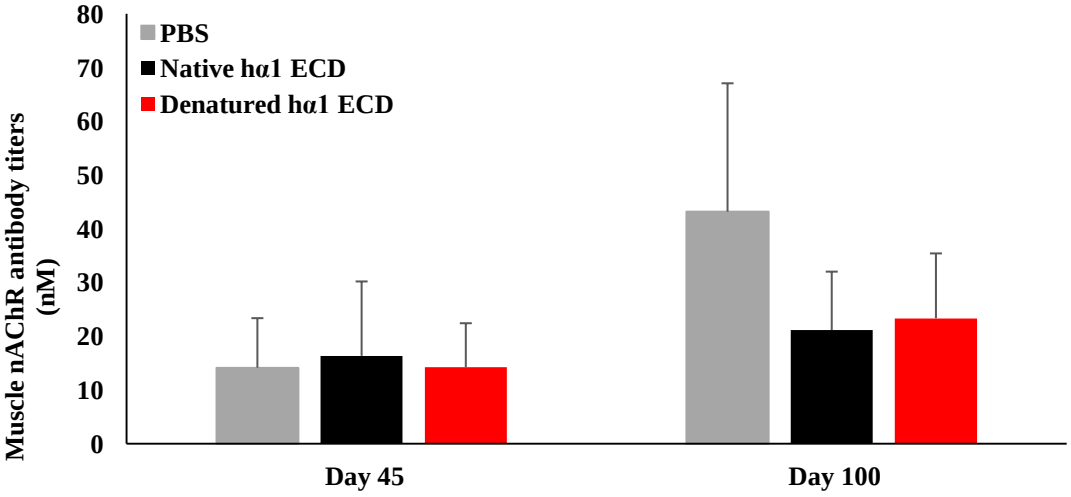


Figure 14. Serum r-nAChR antibody titers. The mean r-nAChR antibody titers of each therapy group as measured in serum samples taken 45 and 100 days p.i. Grey bars: PBS treated animals (N=5), black bars: native hα1 ECD treated animals (N=7) and red bars: denatured hα1 ECD treated animals (N=7).

In order to fully evaluate the effect of the applied therapy in each occasion, we subsequently measured the nAChR content of the muscles of the treated animals (figure 15). The muscles isolated from animals that received only PBS as treatment had decreased muscle nAChR concentration (1.25 ± 0.5fmol/g). In contrast, the muscles that were derived from animals that received 5μg of native or denatured hα1 ECD had higher muscle nAChR concentrations compared to controls (native hα1 ECD: 1.9 ± 1.06 fmol/g & denatured hα1 ECD: 1.44 ± 1.05 fmol/g). Our data show an apparent increase in the overall muscle nAChR concentration, in the animals that received the native hα1 ECD, when compared to the nAChR amount corresponding to denatured hα1 ECD treated animals. However, the difference was not significant and therefore, we can assume that there is no substantial difference in the efficacy of the two treatments. Overall, these data corroborate the previously observed positive effect of the intranasal administration of hα1 ECD and at the same time suggest that, in our case, the tolerogen's spatial conformation does not affect its therapeutic potency.

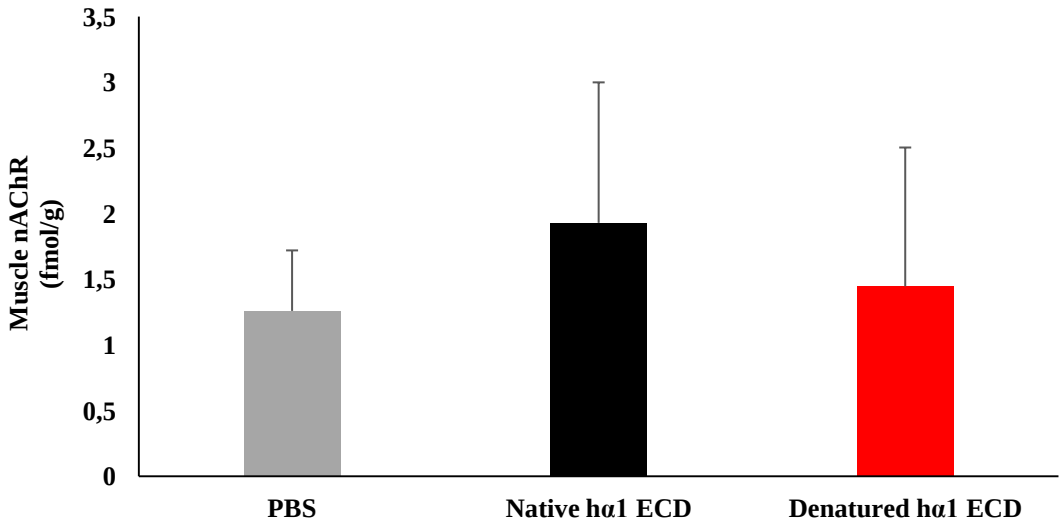


Figure 15. Muscle nAChR content of the treatment groups. Quantitation of the muscle nAChR content of the different groups, expressed in fmol/g tissue. Grey bar: PBS treated animals (N=5), black: native hα1 ECD treated animals (N=7) and red: denatured hα1 ECD treated animals (N=7).

4.4 Intravenous administration

The results extracted from the previous experiments, even though encouraging and indicative of a positive effect of the intranasal administration of hα1 ECD, did not provide clear conclusions regarding the extend of its therapeutic potency. Taking into consideration the observed trend in ameliorating the EAMG manifestations, as well as the successful outcomes of studies with intravenous administration of disease-relevant peptides in animal models of relevant autoimmune diseases like Experimental Autoimmune Encephalomyelitis (EAE), the animal model for Multiple Sclerosis (MS), were they have successfully induced tolerance by administering intravenously the disease specific peptide myelin basic protein (MBP) [178], we investigated whether the intravenous route of administration would show a similar effect in our EAMG model. Nasal administration of peptides and proteins consists a promising drug delivery route due to the large mucosal surface for dose absorption, the rapid drug absorption through the highly vascularized mucosa, the ease of administration and the fact that it is non-invasive. Furthermore, in our therapeutic protocol nasal administration of hα1 ECD showed a positive effect by ameliorating EAMG symptoms manifestations in the treated Lewis rats. Nevertheless, the data we have

gathered so far did not provide robust results in regard to hα1 ECD's efficacy as treatment, when administered nasally.

We administered intravenously hα1 ECD in different doses, initiating the treatment 7 days p.i. and repeating for 12 consecutive days. To this end, 6 Lewis rats received only PBS as treatment, 6 received 5μg hα1 ECD, 6 received 25μg hα1 ECD and 6 received 100μg hα1 ECD per dose. The treatment was administered intravenously at the animals' lateral tail veins. Their monitoring and sample gathering was done at the appointed time points as previously described.

Initially we evaluated the rats' clinical status by measuring their clinical scores as presented in figure 16. The animals that received only PBS as treatment, were severely affected by EAMG, reaching mean clinical score of 3.3 On the contrary, the animals from all treatment groups had milder disease manifestations. The hα1 ECD's effect on the clinical status of the treated animals showed a dose dependent effect, where increasing the dose resulted in greater amelioration of symptoms manifestation. Furthermore, the animals that received any of the treatments had delayed disease onset in comparison to controls. In more detail, administration of 5μg hα1 ECD resulted in animals with EAMG scores not exceeding an average of 2.5. Animals that were treated with the intermediate dose of 25μg presented with symptoms mostly after they were subjected to exercise, with a maximum mean clinical scores of 1. Ultimately, the animals that received 100μg of hα1 ECD were the least affected from EAMG, showing signs of mild muscle weakness after exercise, with the average clinical score not exceeding 0.5. The improvement in the manifestation of symptoms observed in the clinical image of the group treated with the intermediate (25μg) and the highest dose (100μg) was statistically significant compared to controls ($p<0.05$ & $p<0.01$ respectively). In regard to their weights, all treated animals responded well to the intravenously administered doses and showed a positive effect of hα1 ECD, albeit with different intensity in each case (figure 17). Animals treated only with PBS lost a significant amount of their body weight after the symptoms manifested, following the typical EAMG progression course. The animals that received 5μg hα1 ECD, also lost weight, but the reduction was lower than that observed in the controls. The other two doses of 25μg and 100μg of hα1 ECD had a similar effect on the animals' body weight, since there was minimal weight loss that is usually present in the EAMG affected animals.

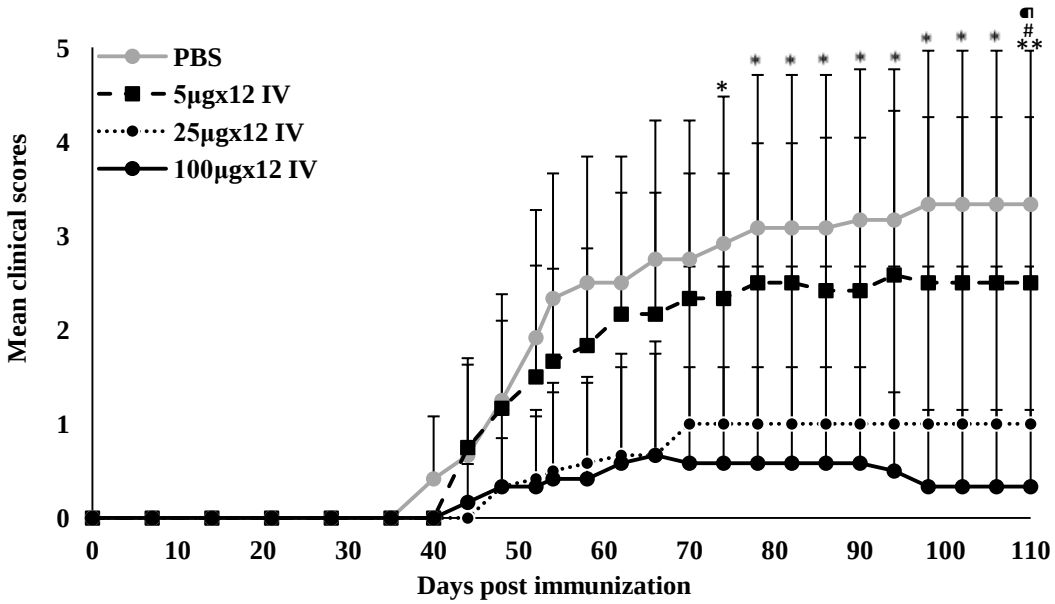


Figure 16. Clinical scores of rats treated intravenously with 5µg, 25µg, 100µg hα1 ECD or PBS. Grey: animals treated only with PBS (N=6), dashed squares: animals treated with 5µg hα1 ECD (N=6), black dotted: 25µg hα1 ECD (N=6) and black: 100µg hα1 ECD (N=6). Administration of 25µg and 100µg lead to statistically significant lower clinical scores compared to controls and to the group treated with 5µg hα1 ECD. Statistical significance: 100µg compared to controls * = $p<0.05$ & ** = $p<0.01$; 25µg compared to controls # = $p<0.05$; 100µg compared to 5µg hα1 ECD ¶ = $p<0.05$.

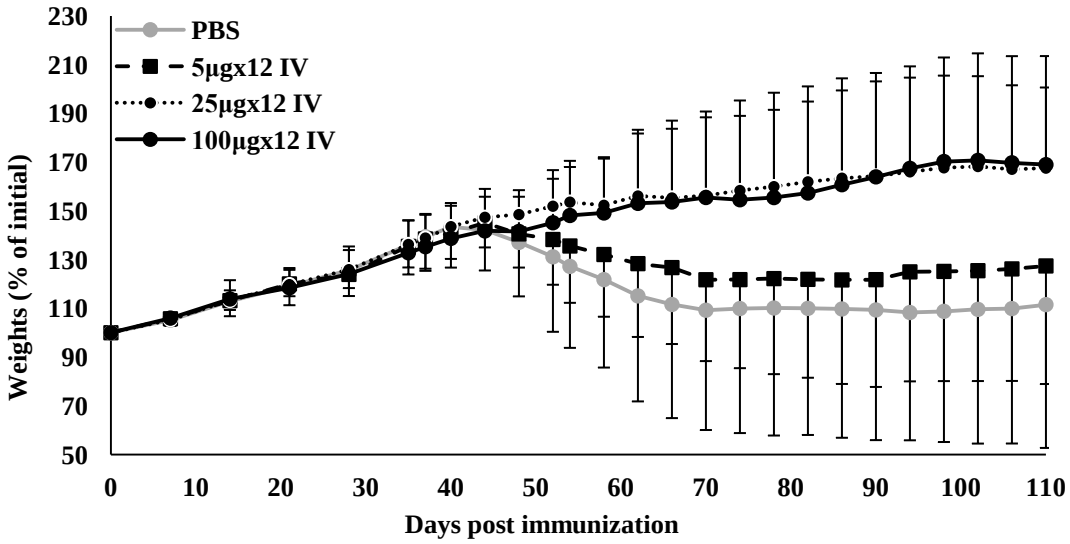


Figure 17. Body weights of intravenously treated rats with 5µg, 25µg, 100µg hα1 ECD or only PBS. The average body weights for each group of rats is represented as percentages (%) of the initial weights and is shown at different times after immunization. Grey: animals treated only with PBS (N=6), dashed squares: animals treated with 5µg hα1 ECD (N=6), black dotted: 25µg hα1 ECD (N=6) and black: 100µg hα1 ECD (N=6).

Subsequently, we went on to elucidate the effect of the different intravenous hα1 ECD doses on the serum antibody titers (figure 18). The serum samples tested were isolated from the treated animals in the 45th and 100th day after the immunization. Even though, the low (5µg hα1 ECD) and the intermediate (25µg hα1 ECD) doses did not affect the hα1 ECD serum antibody titers, sera isolated from animals that received the high dose (100 µg hα1 ECD) resulted in a lower mean of hα1 ECD serum antibody titers. Even though the reduction of hα1 ECD levels compared to untreated animals was not statistically significant, for the first time we observed a sustained effect upon the hα1 ECD titers, which was evident until end of the experiment.

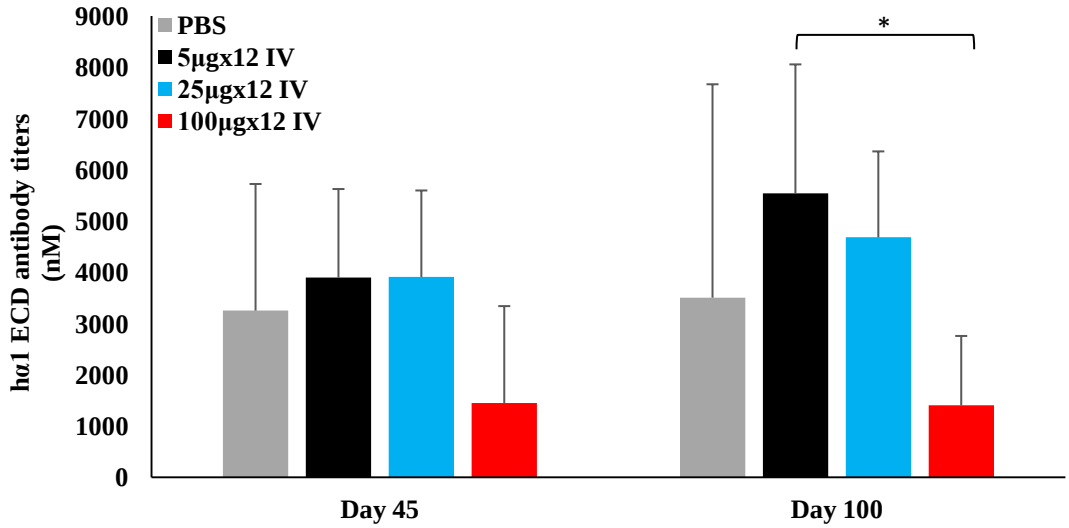


Figure 18. Serum hα1 ECD antibody titers. The mean hα1 ECD antibody titers of the treatment groups, as measured in serum samples taken at day 45 and day 100 p.i. Grey: PBS treated animals (N=6), black: 5μg hα1 ECD treated animals (N=6), blue: 25μg hα1 ECD animals (N=6) and red: 100μg hα1 ECD treated animals (N=6). (*$p<0.05$).

In addition to the hα1 ECD antibodies, as previously, we measured the r-nAChR serum antibody titers (figure 19). Our results show that the r-nAChR antibody concentrations in the sera of treated animals were decreased in comparison to controls in a dose dependent manner, an effect that was sustained throughout the experiment. The dose of 100μg hα1 ECD had the greatest effect as the titers of r-nAChR antibodies were seven to ten times lower than the controls (Day 100 r-nAChR titers 4.7 ± 5 nM compared to 56.15 ± 38.3nM in PBS). Interestingly, the differences in the r-nAChR antibody concentrations between the PBS and 100μg treated animals was not statistically significant. The majority of the animals (66%) showed no clinical manifestations of the disease, in accordance with the reduced amount of r-nAChR antibodies detected. Lastly, we quantified the nAChR content of the muscles isolated from the treated animals (figure 20). The animals that received only PBS as treatment had the lowest nAChR muscle content due to the destruction of the nAChR molecules from the autoantibodies. In contrast, the concentrations of nAChRs in the muscles

isolated from the animals that received 5 μg, 25 μg or 100 μg hα1 ECD were increased in comparison to controls. Furthermore, we observed again a dose dependent mode of action, as an increase in hα1 ECD dose resulted in greater concentration in muscle nAChRs. The greatest effect was observed in the group that received 100μg hα1 ECD, as the muscle nAChR concentrations reached those from healthy controls (3.4 fmol/g ± 0.6) [165, 167].

Our data suggest a positive effect of the intravenous administration of hα1 ECD on the immunized animals, as evidenced by reduced weight loss, lower clinical scores and r-nAChR serum antibody titers, as well as increased nAChR muscle content. The hα1 ECD administration showed a dose-dependent effect and even though all administered doses had a positive effect, the highest one (100 μg) lead to the greatest amelioration of symptom manifestations.

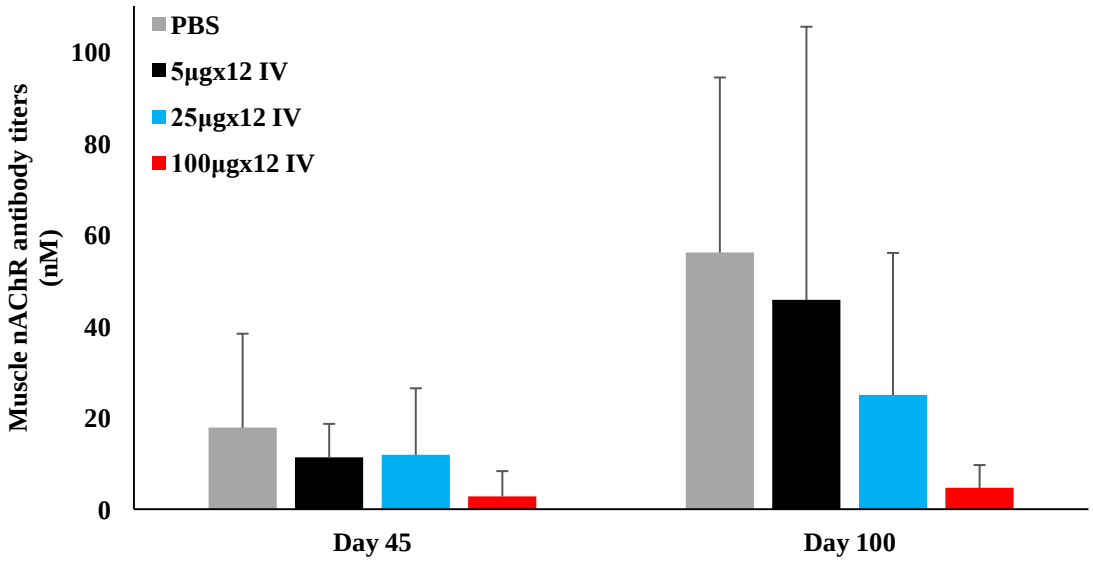


Figure 19. Serum r-nAChR antibody titers. The mean r-nAChR antibody titers of the treatment groups, as measured in serum samples taken at day 45 and day 100 p.i. Grey: PBS treated animals (N=6), black: 5μg hα1 ECD treated animals (N=6), blue: 25μg hα1 ECD animals (N=6) and red: 100μg hα1 ECD treated animals (N=6).

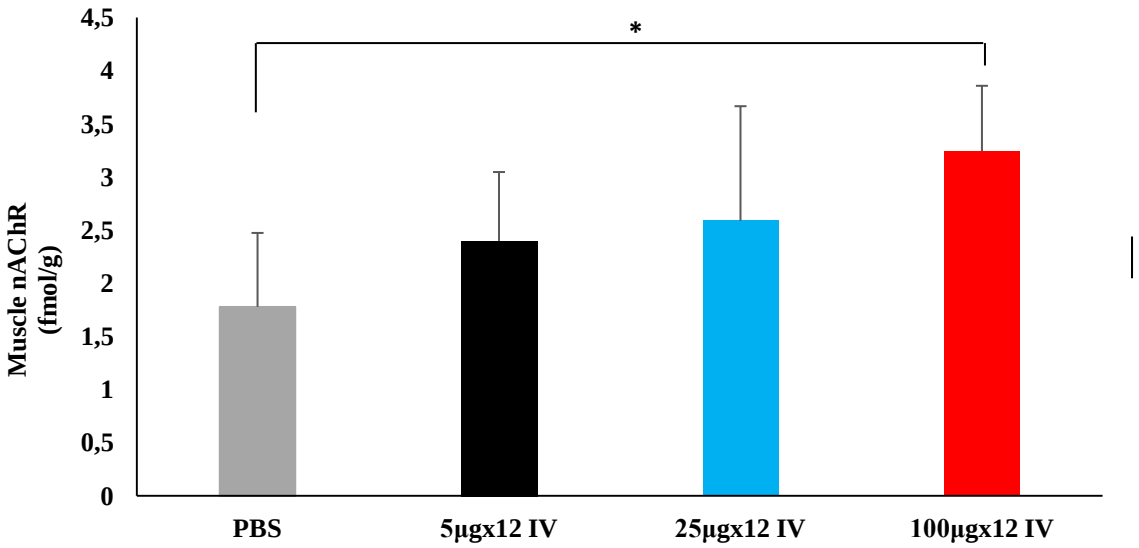


Figure 20. Muscle nAChR content of the treatment groups. Quantitation of the mean content of nAChR of the treatment group, expressed in fmol/g tissue. Grey: PBS treated animals (N=6), black: 5µg hα1 ECD treated animals (N=6), blue: 25µg hα1 ECD animals (N=6) and red: 100µg hα1 ECD treated animals (N=6). (*p<0.05).

4.5 Hα1 ECD serum half life

The intravenous administration of hα1 ECD for 12 consecutive days showed a robust effect on the clinical parameters measured for EAMG evaluation. However, a large dose of 100 µg hα1 ECD was required for a significant positive outcome. Therefore, in order to better understand the dosage amount and the frequency of administration required, we proceeded to determine the hα1 ECD's half-life. For this reason, we administered intravenously 100µg hα1 ECD in combination with radiolabeled ^{125}I-hα1 ECD in immunized Lewis rats (N=11), 7 days p.i. so as to mimic the treatment conditions and measured the radioactivity in blood samples obtained at different time-points. The results are presented as the average percentage of the total hα1 ECD that is serum-detected at each time point (figure 21).

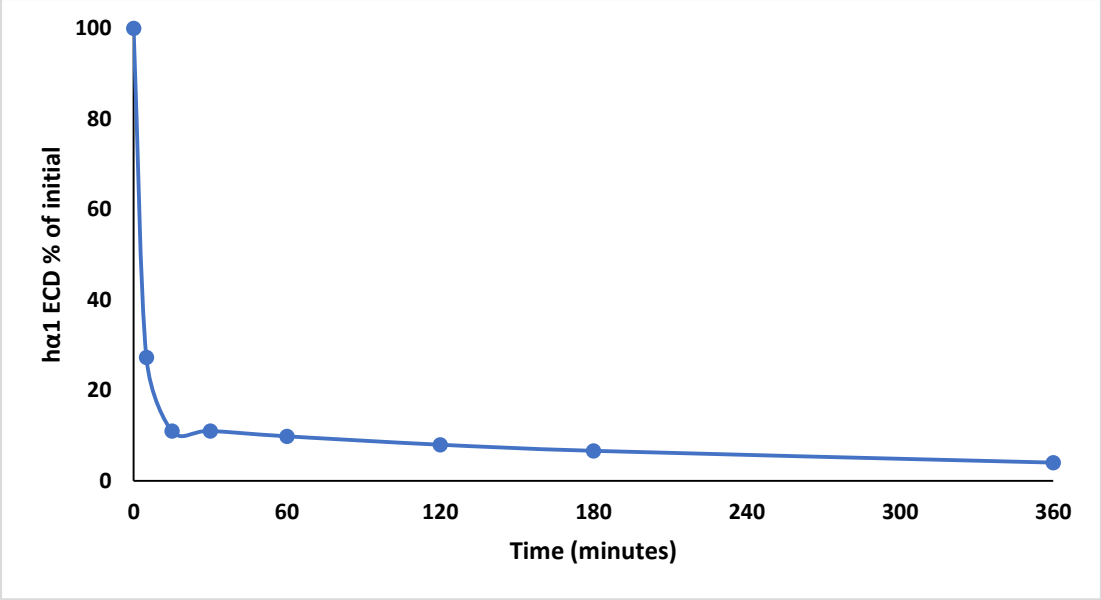


Figure 21. Hα1 ECD serum half-life. Mean radioactivity measurements for each sampling time-point, expressed in percentage of initial (%). The radioactivity measurements correspond to the hα1 ECD content at each time-point and therefore, to the amount available in the blood circulation of the Lewis rats.

We observed a significant reduction in serum hα1 ECD immediately after the intravenous administration (5 min post injection) and a continuous decline thereafter, albeit at a lower rate. Overall, these data indicate that the concentration of hα1 ECD is reduced within a very short period of time in circulation (within minutes after injection). The short hα1 ECD's half-life may explain the need of repeated, high doses in order to exert its therapeutic capacity.

In summary, our data indicate a positive effect of hα1 ECD on the EAMG animals, when administered either intranasally or intravenously. Greater amelioration was observed when the tolerogen was administered intravenously at an earlier time-point (D7), at the highest dose (100μg) for 12 consecutive days.

5 Discussion

MG is a prototypical antibody mediated, T-cell dependent autoimmune disease caused by antibodies targeting components of the neuromuscular junction. This process leads to impaired signal transduction and, consequently, to muscle weakness and fatigability [5]. The exact cause that leads to the initiation of the autoimmune response to nAChR is still unknown [56]. Evidence suggests that a complex interaction between multiple genotypes of low penetrance and several, largely unidentified, environmental factors contribute to the pathogenesis of MG [179]. Various therapeutic strategies have been used over the years to alleviate MG symptoms. These strategies aim at improving the transmission of the nerve impulse to muscle or at lowering the immune system activity with corticosteroids and immunosuppressant drugs. Besides the long-term management of the disease, the severe myasthenic crises require particular and immediate treatments, including intravenous immunoglobulin (IVIg) infusions, plasmapheresis and non-specific

intensive care unit management in case of respiratory involvement. Despite of all the alternative therapeutic approaches for the disease's management, MG remains a chronic disease and symptoms tend to persist in many patients [180]. Furthermore, the current MG treatments are not specific, increasing the possibility to elicit side effects. Many attempts have been made for the discovery of novel antigen-specific therapies, which are based on the manipulation of the immune system, in order to inhibit the immune response specifically against the target antigens, without disrupting the general function of the immune system.

So far, there have been several studies on autoimmune diseases, which have demonstrated that mucosal administration of disease relevant antigens can induce immune tolerance. Some attempts aiming to induce tolerance in EAMG animal models have been made, showing encouraging results, nevertheless without advancing to clinical trials for a candidate antigen-specific therapy. Since this gap in the development of a relative treatment is yet to be filled and as we have in our disposal improved methods and tools, we aimed to investigate the potency of hα1 ECD as an antigen-specific therapy in our EAMG animal model.

Initially we based our experimental design on past studies, which reportedly have achieved tolerance in EAMG, when the nAChR or its fragments were administered nasally. In more detail, it has been demonstrated that nasal administration of 5μg of recombinant fragments of the hα1 ECD (Hα1-210) in female Lewis rats, prior to or after immunization with nAChR isolated from the electric organ of *Torpedo californica* (TAChR), and for 12 consecutive days, results in amelioration of EAMG symptoms in the treated animals (lower mean clinical scores, decreased serum antibody titers and increased muscle nAChR content) [170]. The same team has also attempted to delineate the underlying mechanisms of the therapeutic effect observed when recombinant hα1 ECD fragments (Hα1-205) were administered nasally. Lewis rats were again immunized with TAChR and received 1μg of Hα1-205, 8 days after disease induction and for 12 consecutive days. Their observations suggest, that the mechanism responsible for the induction of nasal tolerance in their study, is based on active suppression involving a shift from Th1 (IL-2, IL-12, IFN-γ) to a Th2 (IL-10)/Th3 (TGF-β)-regulated nAChR-specific response through the downregulation of co-stimulatory factors [181]. Taking into consideration their encouraging results from the positive response to the intranasal treatment they investigated the therapeutic

potency of Hα1-205 when administered orally. They employed prevention and therapeutic protocols and the rats received 600μg per dose, twice a week. The data from this series of experiments were similar to their previous observations, as active suppression seems to be implicated also in the induction of oral tolerance. Additionally, they report a switch in nAChR specific IgG antibody isotypes from IgG2 to IgG1 [182]. Furthermore, in a more recent study, C57BL6/N mice immunized with TAChR, received nasally 5μg of a fusion protein (mCTA1-T146), carrying a dominant epitope corresponding to the amino acid sequence 146-162, from the α1 subunit of the TAChR. They have previously demonstrated that this fusion protein acts as immunomodulator that can carry different disease-relevant peptides and is an effective tolerogenic vector for the suppression of auto-aggressive $CD4^+$ T cells. This construct is an inactivated mutant of the CTA1 subunit of cholera toxin, which in its native form exerts strong ADP-ribosylating effects [183]. Moreover, it contains a dimer of a fragment of *Staphylococcus aureus* protein A, which targets classical dendritic cells [184]. The treatment with the fusion protein was administered either preventively (at an early phase of disease-induction) or therapeutically (during the chronic phase of the disease), in 15 or 10 daily intranasal doses. The data indicate a positive effect of the treatment at the early as well as at the later time-point of administration, on the EAMG manifestations, reflected in suppressed nAChR-specific antibody levels and preserved muscle nAChR content [171]. However, the majority of these studies have been performed on animals that were immunized with the TAChR. As EAMG is an animal model for a human disease, the efficiency of antigen specific therapies evaluated on animal models that are induced by non-human sequences could be underestimated. Taking into consideration the promising observations extracted from the relevant studies and having in our disposal a highly reproducible EAMG animal model, induced by the human α1 ECD, we investigated the therapeutic potency of hα1 ECD.

Initially, we wanted to determine the most appropriate treatment initiation date that would lead to the optimal effect of hα1 ECD. For this purpose, we chose 3 different time-points to initiate the treatment, as to assess its potency at multiple stages of the disease. We administered hα1 ECD intranasally to female Lewis rats 7, 28 or 42 days after the immunization repeating for 12 consecutive days. At day 7 after the immunization, the immune response is at its initial stages. In our EAMG model

the symptoms manifest between 35 and 50 days after the immunization. Therefore, at day 28 there is inflammation and underlying destruction of the NMJ, but usually without the manifestation of symptoms. Therefore, this intermediate time-point, is a good indicator for the efficacy of hα1 ECD. Furthermore, we have observed that in our model, the health of the affected animals deteriorates fast after the symptoms have emerged and many of them need to be sacrificed. So, by administering the treatment at 28 days p.i., we can have a reliable number of animals to be able to extract conclusions for the treatment's effect. Finally, at the late time point of 42 days after the immunization, the symptoms are evident in the majority of the affected animals, as the damage at the NMJ from the targeting autoantibodies has progressed significantly. So far, there are no published studies at this late disease stage, possibly due to difficulties with treatment of animals at this late stage of the disease.

Our data support that nasal administration of hα1 ECD at the earliest time point (D7), after the induction of EAMG has a more positive effect. The clinical status of the treated animals was consistent when taking into consideration the rats' body weights and clinical scores, showing a protective effect when hα1 ECD was administered at D7. The observed improvement was accompanied by lower serum nAChR antibody titers that might explain the marginally increased nAChR content found in these animals' muscles. However, our findings did not show an extensive effect on the treated animals when compared to controls at the molecular level, contrary to indications from previous studies [170, 171]. The animals receiving the treatment at the intermediate (D28) and late time-point (D42), followed a similar deteriorating pattern with the controls, although overall, they did not reach the same levels of disease severity. This is likely due to the different stages of NMJ damage during the progress of the disease, between the alternate treatment initiation time-points. Even though, there were indications of a possible ameliorating effect, the destruction caused by the autoantibodies probably could not be reversed at the later time-points. As the data gathered from this series of experiments suggest that administration of hα1 ECD at D7 was more efficient, we decided to set it as treatment initiation day for our subsequent experiments and follow the same administration frequency of 12 consecutive daily doses.

Despite these encouraging preliminary results, we did not detect robust alternations to the progress of disease in the treated animals. Thus, we decided to

investigate whether a higher dose would result to greater amelioration of the symptoms. However, increase of the intranasally administered amount of hα1 ECD did not lead to a more robust effect on the treated animals. The data from these experiments suggest a positive effect of the treatment (lower mean clinical scores, increased nAChR content), albeit with no significant difference between the two doses of hα1 ECD (5μg & 100μg hα1 ECD).

These results are not in line with the previous studies in which higher dose of nasally administered nAChR lead to greater symptoms amelioration and even more so in a dose-dependent manner [185]. In these studies, the doses administered are even higher (300μg or 600μg) than those we administered and also contain the entire molecule of TAChR. This may lead to alternate cell populations' involvement and antigen presentation pathways. Furthermore, differences in the source from which the antigen of immunization and treatment is acquired (TAChR vs. hα1 ECD expressed in yeast) as well as the type of antigen (whole molecule vs fragment), might be responsible for such discrepancies in the results.

Another important aspect we investigated was whether the antigen's conformation affects its capacity to modulate the immune response, when given intranasally. Fuchs and colleagues have previously investigated the effect of the human nAChR α-subunit fragment's conformation on its therapeutic potency when orally administered in its native form or denatured, in EAMG Lewis rats [177]. Their data showed lack of therapeutic efficiency of the fragment with native conformation, and even exacerbation of the disease, while the denatured fragment lead to EAMG suppression. They hypothesize that the different humoral responses elicited from the two fragments, results from the repertoire of T and B cell epitopes they are bearing. The native fragment, may be recognized by autoreactive B cells already existing, while the denatured fragment probably bears significantly less, or no B cell epitopes. Their findings highlight the importance of the nature of the tolerogen, as it may determine the outcome of the treatment, its safety and whether it will result in improvement or exacerbation of the disease.

Our data so far do not indicate a negative effect of the hα1 ECD when administered in its native conformation, as recorded by previous studies [177]. Nonetheless, we investigated the efficacy of the denatured hα1 ECD and compared the potency of the two proteins with different conformations. In our EAMG model we

did not observe significant differences between the groups that received the two treatments. Both native and denatured hα1 ECD, elicited similar effects on the treated animals, that resulted in improved clinical status compared to controls, with lower mean clinical scores and reduced weight loss. The molecular findings showed lower mean r-nAChR serum antibody titers and increased muscle nAChR content, consistent with the amelioration of EAMG symptoms manifestations, however the differences observed when compared to controls were not statistically significant in this aspect. The fact that our findings do not agree with previous studies on the impact of the antigen's spatial conformation on its therapeutic potency, could be due to differences in the EAMG animal models used as well as the origin of the antigens administered for immunization and treatment. Nevertheless, further investigation is required to fully assess the role of the antigen's spatial conformation in its therapeutic capacity.

Until now there are no published successful attempts of intravenous administration of nAChR or its domains as treatment for EAMG. Of note, a relevant series of experiments was performed by Spack and colleagues, who managed to ameliorate EAMG symptoms manifestations by administering intravenously solubilized MHC Class II and TAChR α1 subunit complexes [186]. One of their control groups received only the nAChR α1 subunit in 3 doses of 5μg each, on the days 1, 4 and 7 after immunization with TAChR, but it did not have any positive effect on the treated animals. On the other hand, in models of other autoimmune diseases like Experimental Autoimmune Encephalomyelitis (EAE), there have been studies that report successful suppression of the disease when administering disease relevant antigens [187-189].

Taking into consideration all the above, we investigated the efficiency of hα1 ECD when administered repeatedly intravenously. Our results were very encouraging since not only were EAMG symptoms ameliorated in all treatment groups but the effect of hα1 ECD appeared to be dose-dependent. Moreover, the highest dose (100μg) had a robust effect, as their clinical status and the molecular findings, suggest robust suppression of the disease manifestations. The nAChR content found in the muscles isolated from the animals treated with the highest dose, was at the levels of the content in the muscles of healthy controls, probably owing to the almost diminished r-nAChR antibody serum titers. All the animals survived until the

experiment's termination, the majority of which (66%) did not present any EAMG symptoms, whereas the remaining showed only mild disease manifestations.

Our findings suggest an almost curative effect of hα1 ECD, when administered in the highest dose examined so far in our experiments. At this point it is important to denote, that the positive therapeutic effect we observed in this series of experiments, is probably not the result of the binding of serum r-nAChR antibodies to the injected hα1 ECD. If that were the case, we would not observe the lasting therapeutic effect of hα1 ECD's throughout the experiment, which lasts for several weeks after the treatment's termination.

Data from multiple studies support that low doses of a tolerogen favor active suppression, while high antigen doses favor clonal deletion and clonal anergy [177, 190-192]. In our case, the differences observed in the efficacy and dose responsiveness of the alternate routes of hα1 ECD administration, intranasal and intravenous, suggest that the mechanisms involved in the induction of immune tolerance differ considerably. We can also assume that in our model the prevalent mechanism in the nasally induced tolerance, is not as effective as the one in the intravenous administration.

In addition, we wanted to investigate whether the observed positive effect was due to the amount of the tolerogen or the frequency of the doses. The animals treated only once exhibited milder symptoms manifestations, but signs of progressed EAMG were evident. The profound difference observed in the effect of hα1 ECD, when administered in high doses but in different dose frequencies, could be due to the time needed for the injected protein to be eliminated from the rats' bodies.

In regard to the hα1 ECD's bioavailability, the intravenous administration is advantageous as it ensures that the administered dose in its entirety will reach the systemic circulation. Assessment of the pharmacokinetic properties of a potential drug is necessary in order to extrapolate the doses from animals to humans and increase clinical trial safety. Pharmacokinetics is one of four different methods, including dose by factor, similar drug, pharmacokinetically guided and comparative approaches that are used to determine the new drug's initial dose. The US Food and Drug Administration's current guidance is based on dose by factor approach which uses the no observed adverse effect levels (NOAEL) of drug from preclinical toxicological studies to estimate human equivalent dose. Dose calculation always requires careful

consideration about the difference in pharmacokinetics and pharmacodynamics among species [193, 194].

Several parameters need to be taken into consideration to assess the pharmacokinetic properties of a drug. These are: half-life, clearance, volume distribution, bioavailability and systemic exposure of the drug. The half-life, i.e. the time required for the plasma concentration to decrease by one-half, characterizes the monoexponential decline in a drug concentration after drug input processes have been completed, assuming it follows a first–order elimination process. The half-life of a molecule provides also important information about other specific aspects of a drug's disposition such as how long it will take for its distribution in the body to reach an equilibrium between the multiple organs and tissues. Furthermore, as half-life determines the fluctuation between the minimum and maximum concentrations during a multiple dose regimen, it is the major determinant of the dosing interval between two consecutive doses, which is how often a dose should be administered [195].

In general, it is common to use radiolabeled pharmaceutical compounds to examine the pharmacokinetic properties of new drugs. Large molecules like proteins can be labeled with ^{125}I, which has a 60-day half-life, thus rendering easy to prepare reagents and perform experiments without tight time constraints, while its easy and very sensitive to detect. Therefore, we went on and administered intravenously (similar to the treatment dose and route of administration), a single dose of ^{125}I – labeled hα1 ECD, as to determine the period hα1 ECD is present in the animals' serum. The levels of the measured radioactivity were significantly reduced immediately after the intravenous administration (5 minutes post injection) and were almost diminished after 6 hours, indicating a very short half-life of hα1 ECD. At this point it is important to note that the amount of protein administered for the treatment protocol is at least 5- to 10-fold more that the r-nAChR serum antibodies. In detail, for the injected hα1 ECD 100μg correspond to about 560 nM while r-nAChR antibodies are virtually undetectable on day 7, approximately 45 nM on day 21 and 120 nM on day 45. Therefore, we assume that the protein's fast circulation elimination is not due to its binding to the antibodies. The protein's short half-life provides a possible explanation for the necessity of repeated high doses, in order to exert its therapeutic potency. These results, are a first step to further study hα1 ECD's biodistribution and other pharmacokinetic properties, in order not only to identify

tissues of interest, involved in the mechanism through which it elicits its therapeutic potency, but also to aid in determining the most efficacious dose amount and frequency.

Antigen-specific tolerogenic therapies are appealing from a safety point of view because they are not expected to induce global immunosuppression like systemic approaches. Results from animal models have shown that induction of tolerance to a single self-antigen can efficiently inhibit a polyclonal autoreactive response owing to mechanisms of "bystander suppression" and dominant antigen non-specific immunoregulation [196]. Several approaches have been used to induce tolerance in EAMG animal models. Nasal administration of soluble self-antigen, nAChR or its domains, efficiently prevented or suppressed disease through mucosal tolerance pathways of anergy, deletion or active suppression by induction of TGF-β or IL-10 producing Tregs. The data from these studies revealed suppression of Th1 and Th17 responses by down-regulation of IFN-γ or IL-17 expression and a skewing towards Th2 responses [169, 171, 181]. Moreover, expression of IL-2 and IL-12 cytokines, as well as costimulatory molecules on activated T-cells and APCs, such as the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and B7-1 and B7-2 respectively, had been compromised after nasal treatment with nAChR fragments [170, 171, 181]. Intravenous antigen-specific tolerance has not yet been reported in EAMG animal models. Nevertheless, efficient suppression of autoimmune disease by intravenous administration of disease-relevant peptides has been achieved in animal models of other autoimmune diseases, like EAE. These studies report mechanisms of clonal anergy and deletion and preferential induction of Th2-type cytokines [197]. Furthermore, their data indicate that effector cells from spleen are more susceptible to tolerization than lymph node effector cells, since when transferred to naïve rats, lymph node cells from tolerized rats caused the emergence of EAE whilst spleen cells did not. Another suggestion is that regulatory cells reside primarily in the spleen. It is hypothesized that the observed difference in encephalitogenicity of spleen and lymph node cells of rats, might reflect differences in cell trafficking between lymph node and spleen effector cells. Rostami and colleagues have efficiently suppressed EAE by intravenous administration of disease relevant antigen, reporting down-regulation of IL-12, IL-2 and Th1 response cytokines, while IL-10 characteristic cytokine of Tr1 cells was upregulated. They have also demonstrated the importance of cytokine IL-12,

since when administered to tolerized animals it abrogated the suppressive effect of the intravenous treatment [187, 189]. In any case, there are distinctions in the pathogenic mechanisms of autoimmune diseases, and subsequently for the mechanisms involved in the induction of tolerance when administering disease relevant peptides. Since our ultimate goal is to establish a treatment, elucidation of the mechanisms and the cells involved in reestablishing the immune tolerance in our EAMG animal model is pivotal.

In recent years, long-lived plasma cells have proved to play a key role in antibody-mediated autoimmune diseases. It has been shown in the mouse model of systemic lupus erythematosus (SLE), that they produce substantial amounts of autoantibodies and are resistant to standard immunosuppression [198], while they appear to maintain the autoimmune response in rheumatoid arthritis patients [199]. Persisting long-lived plasma cells are probably also responsible for the typically slow time course of serum autoantibody decline seen in most MG patients during treatment with steroids or other immunosuppressive drugs. Autoantibodies against the nAChR are thought to be produced by long-lived plasma cells, as patients treated with the anti-CD20 antibody rituximab did not show fluctuation in their nAChR antibody levels. On the contrary autoantibodies against MuSK were almost eliminated, leading to the hypothesis that their source are the short-lived plasma-cells that are continuously regenerated from autoreactive CD20 positive B cells [145, 200]. The long-lived plasma cells, in their survival niches in the spleen and in particular in the bone marrow are resistant to most therapies. Therefore, drugs targeting plasma cells may have a place in therapy in autoimmune diseases, like MG. Based on these evidence, it would be worth investigating in future experiments, whether targeting the long-lived plasma cells might contribute in increasing the observed efficacy of hα1 ECD. A suitable candidate drug in this category would be Bortezomib, whose mechanism of action is based in the induction of apoptosis through inhibition of the proteasome. Both short-lived and long-lived plasma-cells generated during the disease's progress are particularly sensitive to inhibition of the proteasome, due to their high-rate of Ig production [201]. Therefore, the elimination of pre-existing pathogenic plasma-cells might increase the effect of hα1 ECD in skewing the immune response to a more tolerogenic profile.

Despite the robust efficacy of hα1 ECD on preventing the manifestation of EAMG symptoms, its short half-life is a disadvantage as a candidate immunomodulatory drug. Compared with the conventional small-molecule drugs, protein therapeutics offer the advantages of higher specificity, greater activity and less toxicity [202]. Moreover, the susceptibility to enzymatic degradation, short circulation half-lives, and poor membrane permeability pose significant barriers for effective delivery of many therapeutic proteins to targeted disease sites. To counteract these unfavorable characteristics and achieve high therapeutic efficiency, appropriate protein delivery strategies and platforms need to be designed. The considerable success of nanoparticle formulations of small-molecules such as doxorubicin, paclitaxel and amphotericin B has paved the way for the exploration of nanoparticle technologies for protein delivery [203]. Such an approach could be an effective counter measure for hα1 ECD's short half-life, thus increasing its efficiency and at the same time reducing the amount required to exert its full therapeutic potency.

Nanoparticles, have considerable advantages as drug carriers. Nanoparticles have high surface-to-volume ratio that increases reactivity and possible biochemical activities. In protein delivery, nanoparticle technologies can: 1) protect proteins from premature degradation or denaturation in biological environment, 2) enhance systemic circulation half-life of proteins with poor pharmacokinetic properties, 3) control sustained release which can maintain drug concentration in the therapeutic range and 4) target diseased tissues, cells and intracellular compartments, thus improving the safety and efficacy of biologic therapeutics [202]. Iron oxide magnetic nanoparticles (IONPs) are stable, biocompatible, environmentally safe and difficult to be metabolized in the whole system, persisting in the circulation for a long period of time These unique characteristics render them appropriate for clinical applications. Furthermore, their surface tunability makes them multifunctional, allowing their fabrication with a variety of therapeutic or functional moieties [26]. Size, shape and surface characterization of the iron oxide nanoparticles determine their biological distribution which can involve opsonization, when interacting with serum proteins and particle cell interaction [204].

Tolerogenic nanoparticles, including IONPs, have been previously used for the induction of immune tolerance in autoimmune diseases, by providing the antigen alone to harness natural tolerogenic processes or environments. Santamaria and

colleagues created “synthetic APCs” by coating iron oxide nanoparticles with autoimmune diabetes disease relevant peptides bound to major histocompatibility class I molecules. These nanoparticles, present the antigen without costimulatory molecules and result in suppression of autoreactive CD8+ T cells and their conversion to a regulatory, anergic phenotype that controls pathogenic responses [205]. Furthermore, Kishimoto and colleagues have shown that administration of free antigen in combination with nanoparticles constructed from biodegradable polymers encapsulating rapamycin can inhibit antigen-specific T cell proliferation and induce antigen specific regulatory T cells in the EAE animal model [206, 207].

Based on the successful application of nanoparticles in reestablishing the immune tolerance in autoimmune diseases and taking into consideration the unique characteristics of iron oxide nanoparticles, we combined them with hα1 ECD in an attempt to improve the pharmacokinetic properties of hα1 ECD, thus further amplifying its efficacy. We have recently initiated studies with such NPs in our EAMG model. Although these experiments are ongoing, we have not seen any difference in the efficacy of treatment yet. Nevertheless, there haven’t been any indications of increased toxicity or disease symptoms exacerbations due to the nanoparticles. Therefore, we intend to further pursue this approach by using various nanoparticle formulations and nanoparticle-antigen combinations. One possible modification to increasing the circulation time of the nanoparticles in the blood stream is to coat them with hydrophilic polymers such as poly(ethyleneglycol) (PEG) to disperse them and minimize or eliminate the protein adsorption [208]. However, in order to be able to alter the nanoparticles’ specifications, in a way that will increase their specificity and efficacy, we also have to delineate the mechanisms and the cellular populations that are implicated in the induction of tolerance in our EAMG animal model.

The robust effect of hα1 ECD when administered at an early stage after the induction of the disease, leading to the prevention of EAMG onset paves the way for its further characterization as a therapy for MG. The results obtained from our experiments so far, even though at a preliminary stage, strongly support the development of a treatment that has the capacity to reprogram the immune system and eliminate the pathogenic factors that result in the manifestation of MG symptoms. Additional studies will address whether it will have the same efficacy on animals with

progressed EAMG, that have already disease manifestations. Any therapeutic approach has to target the suppression of ongoing disease rather than prevention and the upcoming results of these experiments will be of key importance to further understand the potency of hα1 ECD as a treatment for EAMG and MG.

Bibliography

1. JOHNSTON, J.D., *The Contributions of Dr. Mary Walker towards Myasthenia Gravis and Periodic Paralysis Whilst Working in Poor Law*

Hospitals in London. Journal of the History of the Neurosciences, 2005. **14**: p. 121-137.
2. Hughes, T., *The early history of myasthenia gravis.* Neuromuscular Disorders, 2005. **15**: p. 878-886.
3. Thanvi BR, L.T., *Update on myasthenia gravis.* Postgraduate Medicine Journal, 2004. **80**: p. 690-700.
4. Jon M. Lindstrom, e.a., *Antibody to acetylcholine receptor in myasthenia gravis.* Neurology, 1976. **26**: p. 1054-1059.
5. N. E.Gilhus, G.S., F. Romi, K. Lazaridis, P. Zisimopoulou and S. Tzartos, *Myasthenia gravis — autoantibody characteristics and their implications for therapy.* Nature Reviews-Neurology, 2016. **12**: p. 259-268.
6. M. N. Meriggioli, D.B.S., *Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity.* Lancet Neurology, 2009. **8**: p. 475-490.
7. Phillips LH, T.J., *Epidemiologic evidence for a changing natural history of myasthenia gravis.* Neurology, 1996. **47**: p. 1233-1238.
8. D. Grob, N.B., T. Namba, M. Pagala, *Lifetime course of myasthenia gravis.* Muscle Nerve, 2007. **37**: p. 141-149.
9. Philips, L.H., *The Epidemiology of Myasthenia gravis.* Annals of the New York academy of sciences, 2003. **998**: p. 407-412.
10. Yu YL, H.B., Ip MS, et al, *Myasthenia gravis in Hong Kong Chinese: 1. epidemiology and adult disease.* Acta Neurologica Scandinavica, 1992. **86**: p. 113-119.
11. Poulas K, T.E., Kokla A, Papanastasiou D, Tsouloufis T, Marinou M, Tsantili P, Papapetropoulos T, Tzartos SJ., *Epidemiology of seropositive myasthenia gravis in Greece.* Journal of Neurology, Neurosurgery, and Psychiatry, 2001. **71**: p. 352-356.
12. Robertson NP, D.J., Compston DA *Myasthenia gravis: a population based epidemiological study in Cambridgeshire, England.* Journal of Neurology, Neurosurgery, and Psychiatry, 1998. **65**: p. 492-496.
13. Melzer N, R.T., Fuhr P, et al, *Clinical features, pathogenesis, and treatment of myasthenia gravis: a supplement to the guidelines of the German Neurological Society.* Journal of Neurology, 2016. **263**: p. 1473-1494.
14. D, G., *Course and management of myasthenia gravis.* The Journal of the American Medical Association 1953. **153**: p. 529-532.
15. A.Trouth, e.a., *MyastheniaGravis: AReview.* Autoimmune Diseases, 2012. **2012**.
16. Grob D, B.N., Namba T, et al., *Lifetime course of myasthenia gravis.* Muscle Nerve, 2008. **37**: p. 141-149.
17. D.Grob, L.A., N.G.Brunner,and T.Namba, *Thecourse of myasthenia gravis and therapies affecting outcome.* Annals of the New York Academy of Sciences, 1987. **505**: p. 472-499.
18. H. Wu, W.C.X.a.L.M., *To build a synapse: signaling pathways in neuromuscular junction assembly.* Development, 2010. **137**: p. 1017-1033.
19. Hughes, B.W., M.L.M. De Casillas, and H.J. Kaminski, *Pathophysiology of myasthenia gravis.* Seminars in Neurology, 2004. **24**(1): p. 21-30.
20. B. W. Hughes, e.a., *Molecular Architecture Of The Neuromuscular Junction.* Muscle Nerve, 2005. **33**: p. 445-461.
21. James C. Ha, D.P.R., *Myasthenia gravis and related disorders: Pathology and molecular pathogenesis.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2015. **1852**: p. 651-657.

22. Robert E. Poage, S.D.M., *Presynaptic calcium influx, neurotransmitter release, and neuromuscular disease.* Physiology & Behavior, 2002. **77**: p. 507-512.
23. Sanes JR, L.J., *Induction, assembly, maturation and maintenance of a postsynaptic apparatus.* Nature Reviews Neuroscience volume, 2001. **2**: p. 791-805.
24. BL., P., *Basal lamina and the organization of neuromuscular synapses.* Journal of Neurocytology, 2003. **32**: p. 883-903.
25. Werner Hoch, J.m., Sigrun Helms, John Newsom-Davis, Arthur Melms & Angela Vincent, *Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies.* Nature Medicine, 2001. **7**: p. 365-368.
26. Y. Lee, e.a., *Rapsyn interacts with the muscle acetylcholine receptor via αhelical domains in the α, β, and ε subunit intracellular loops.* Neuroscience, 2009. **29**: p. 222-232.
27. Hoch, W., *Molecular dissection of neuromuscular junction formation.* Trends in Neuroscience, 2003. **26**: p. 335-337.
28. Ruff, R., *Neurophysiology of the neuromuscular junction: overview.* Annals of the New York academy of sciences, 2003. **998**: p. 1-10.
29. Chiawen Lucy Liang, S.H., *Neuromuscular Junction Disorders.* PM&R, 2013. **5**: p. 81-88.
30. Daniels, E.F.a.M.P., *Distribution of Na+ Channels and Ankyrin in Neuromuscular Junctions Is Complementary to That of Acetylcholine Receptors and the 43 kd Protein* Neuron, 1989. **3**: p. 163-175.
31. P. Zisimopoulou, T.B., N. Traka, S. J. Tzartos, *Serological diagnostics in myasthenia gravis based on novel assays and recently identified antigens.* Autoimmunity Reviews, 2013. **12**: p. 924-930.
32. DBerrih-Aknin S, F.-C.M., Eymard B *Diagnostic and clinical classification of autoimmune myasthenia gravis.* Journal of Autoimmunity, 2014. **48-49**: p. 1-6.
33. W. Hoch, J.M., S. Helms, J. Newsom-Davis, A. Melms & A. Vincent, *Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies.* Nature Medicine, 2001. **7**: p. 365-368.
34. Zhang B, T.J., Belimezi M, et al, *Autoantibodies to Lipoprotein-Related Protein 4 in Patients With Double-Seronegative Myasthenia Gravis.* Archives of Neurology, 2012. **69**: p. 445–451.
35. E. Gallardo, e.a., *Cortactin autoantibodies in myasthenia gravis.* Autoimmunity Reviews, 2014. **13**: p. 1003-1007.
36. H. H. Dale, H.W.D., *The presence of histamine and acetylcholine in the spleen of the ox and the horse.* Journal of Physiology, 1929. **68**: p. 97-123.
37. Karlsson, E., *Isolation Of The Nicotinic Acetylcholine Receptor By Biospecific Chromatography On Insolubilized Naja Naja Neurotoxin* FEBS Letters, 1972. **28**: p. 107-111.
38. Miledi R, M.P., Potter LT., *Isolation of the cholinergic receptor protein of Torpedo electric tissue.* Nature, 1971. **229**: p. 523-524.
39. Karlin, A., *Emerging Structure Of The Nicotinic Acetylcholine Receptors.* Nature Reviews Neuroscience, 2002. **3**: p. 102-114.
40. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., Numa, S. , *Primary structure of a-subunit*

precursor of Torpedo californica acetylcholine receptor deduced from cDNA sequence. Nature, 1982. **299**: p. 793-797.
41. Kalamida, D., *Muscle and neuronal nicotinic acetylcholine receptors Structure, function and pathogenicity.* FEBS Journal, 2007. **274**: p. 3799-3845.
42. Sine S M., E.A.G., *Recent advances in Cys-loop receptor structure and function.* Nature, 2006. **440**: p. 448-455.
43. V. Tsetlin, D.K., I. Kasheverov, *Assembly of nicotinic and other Cys-loop receptors.* Journal of Neurochemistry, 2011. **116**: p. 734-741.
44. Gaimarri, A., Moretti, M., Riganti, L., Zanardi, A., Clementi, F., and Gotti, C. , *Regulation of neuronal nicotinic receptor traffic and expression.* Brain Research Reviews, 2007. **55**: p. 134-143.
45. M. Mishina, T.T., K. Imoto, M. Noda, T. Takahashi and S. Numa, *Molecular Distinction Between Fetal and Adult Forms of Muscle Acetylcholine Receptor*. Nature, 1986. **321**: p. 406-411.
46. T. Barkas, A.M., B. Roth, C. Alliod, S. J. Tzartos, M. Ballivet, *Mapping the Main Immunogenic Region and Toxin-Binding Site of the Nicotinic Acetylcholine Receptor.* Science, 1987. **235**: p. 77-80.
47. Unwin, N., *Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution.* Journal of Molecular Biology, 2005. **346**: p. 967-989.
48. Verschuuren, J.J.G.M.et a., *Pathophysiology of myasthenia gravis with antibodies to the acetylcholine receptor, muscle-specific kinase, and low-density lipoprotein receptor-related protein 4.* . Autoimmunity Reviews, 2013. **12**: p. 918-923.
49. Kordas, G.et a., *Direct proof of the in vivo pathogenic role of the AChR autoantibodies from myasthenia gravis patients.* PLoS ONE, 2014. **9**.
50. R. Beroukhim, N.U., *Three-dimensional location of the main immunogenic region of the acetylcholine receptor.* Neuron, 1995. **15**: p. 323-331.
51. S.J. Tzartos, T.B., M.T. Cung, A. Mamalaki, M. Marraud, P. Orlewski, et al., *Anatomy of the antigenic structure of a large membrane autoantigen, the muscle-type nicotinic acetylcholine receptor.* Immunological Reviews, 1998. **163**: p. 89-120.
52.] J. Luo, P.T., M. Losen, M.H. de Baets, G.D. Shelton, J. Lindstrom, *Main immunogenic region structure promotes binding of conformationdependent myasthenia gravis autoantibodies, nicotinic acetylcholine receptor conformation maturation, and agonist sensitivity.* Journal of Neuroscience, 2009. **29**: p. 13898-13908.
53.] M.S. Saedi, R.A., W.G. Conroy, J. Lindstrom, *Determination of amino acids critical to the main immunogenic region of intact acetylcholine receptors by in vitro mutagenesis.* FEBS Letters, 1990. **267**: p. 55-59.
54. Gilhus, N.E.V., J. , *Myasthenia gravis: subgroup classification and therapeutic strategies.* . The Lancet Neurology, 2015. **14**: p. 1023-1036.
55. Heldal, A.T., Eide, G. E., Romi, F., Owe, J. F. & Gilhus, N. E. , *Repeated acetylcholine receptor antibody-concentrations and association to clinical myasthenia gravis development.* PLoS ONE 2014. **9**(12): p. e114060.
56. Cufi P, D.N., Weiss JM, Martinez-Martinez P, De Baets MH, Roussin R, et al., *Implication of doublestranded RNA signaling in the etiology of autoimmune myasthenia gravis.* Annals of Neurology, 2013. **73**: p. 281-293.
57. H. Shiono, Y.i.W., I. Matthews, Jian-Li Liu, W. Zhang, G. Sims, A. Meager, D. Beeson, A. Vincent, N. Willcox, *Spontaneous production of anti-IFN-a and*

anti-IL-12 autoantibodies by thymoma cells from myasthenia gravis patients suggests autoimmunization in the tumor. International Immunology, 2003. **15**: p. 903-913.
58. A. H. Maniaol, A.E., A. R. Lorentzen, Jone F. Owe et al, *Late Onset Myasthenia Gravis Is Associated with HLA DRB1*15:01 in the Norwegian Population.* PLoS ONE, 2012. **7**.
59. St. J. Burden, N.Y., W. Zhang, *The Role of MuSK in Synapse Formation and Neuromuscular Disease.* Cold Spring Harbor Perspectives in Biology, 2013. **5**.
60. DeChiara, T.M., et al., *The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo.* Cell, 1996. **85**(4): p. 501-512.
61. E. Bergamin, e.a., *The cytoplasmic adaptor protein Dok7 activates the receptor tyrosine kinase MuSK via dimerization.* Molecular Cell, 2010. **39**: p. 100-109.
62. N. Kim, A.L.S., T. O. Cameron, P. T. Hallock, A. M. Gomez, J. H. Huang, S. R. Hubbard, M. L. Dustin, S. J. Burden, *Lrp4 Is a Receptor for Agrin and Forms a Complex with MuSK.* Cell, 2008. **135**: p. 334-342.
63. Weston C, G.C., Teressa G, Hod E, Ren X-D, Prives J, *Cooperative regulation by Rac and Rho of agrin-induced acetylcholine receptor clustering in muscle cells.* Journal of Biological Chemistry, 2003. **278**: p. 6450-6455.
64. ZG Luo, e.a., *Implication of geranylgeranlyltransferase I in synapse formation.* Neuron, 2003. **40**: p. 703-717.
65. ZG Luo, e.a., *Regulation of AChR clustering by dishevelled interacting with MuSK and PAK1.* Neuron, 2002. **35**: p. 489-505.
66. P. Mittaud, e.a., *A single pulse of agrin triggers a pathway that acts to cluster acetylcholine receptors.* Molecular and Cellular Biology, 2004. **24**: p. 7841-7854.
67. L. Borges, e.a., *Identification of a motif in the acetylcholine receptor subunit whose phosphorylation regulates rapsyn association and postsynaptic receptor localization. .* Journal of Neuroscience, 2008. **28**: p. 11468-11476.
68. K. Otsuka, e.a., *Collagen Q and anti-MuSK autoantibody competitively suppress agrin/LRP4/MuSK signaling.* Nature Scientific Reports, 2015. **5**.
69. L. Kimbell, e.a., *C-terminal and heparin-binding domains of collagenic tail subunit are both essential for anchoring acetylcholinesterase at the synapse.* The Journal of Biological Chemistry, 2004. **279**: p. 10997-11005.
70. A. Cartaud, e.a., *MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction.* The Journal of Cell Biology, 2004. **165**: p. 505-515.
71. Hesser BA, H.O., Witzemann V., *Synapse disassembly and formation of new synapses in postnatal muscle upon conditional inactivation of MuSK.* Molecular and Cellular Neuroscience, 2006. **31**: p. 470-480.
72. A. Evoli, L.P., *Diagnosis and therapy of myasthenia gravis with antibodies to muscle-specific kinase.* Autoimmunity Reviews, 2013. **12**: p. 931-935.
73. Guptill, J.T., Sanders, D. B. & Evoli, A. , *Anti-MuSK antibody myasthenia gravis; clinical findings and response to treatment in two large cohorts.* Muscle Nerve 2011. **44**: p. 36-40.
74. van der Neut Kolfschoten M, S.J., Losen M, Bleeker WK, Martinez-Martinez P, Vermeulen E, et al. , *Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange.* Science, 2007. **317**: p. 1554-1557.
75. Plomp, J.J., Huijbers, M. G., van der Maarel, S. M. & Verschuuren, J. J. , *Pathogenic IgG4 subclass autoantibodies in MuSK myasthenia gravis. .*

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, 2012. **1275**: p. 114-122.
76. Cole RN, R.S., Gervasio OL, Phillips WD, *Anti-MuSK patient antibodies disrupt the mouse neuromuscular junction.* Annals of Neurology, 2008. **63**: p. 782-789.
77. Mori S, K.S., Akiyoshi T, Yamada S, Miyazaki T, Hotta H, et a, *Antibodies against muscle-specific kinase impair both presynaptic and postsynaptic functions in a murine model of myasthenia gravis.* American Journal of Pathology, 2012. **180**: p. 798-810.
78. Okada K, I.A., Okada M, Murata Y, Kakuta S, Jigami T, et al, *The muscle protein Dok-7 is essential for neuromuscular synaptogenesis.* . Science, 2006. **312**: p. 1802–1805.
79. Mori S, Y.S., Kubo S, Chen J, Matsuda S, Shudou M, et al, *Divalent and monovalent autoantibodies cause dysfunction of MuSK by distinct mechanisms in a rabbit model of myasthenia gravis.* Journal of Neuroimmunology, 2012. **244**: p. 1-7.
80. Kawakami, Y.et a., *Anti-MuSK autoantibodies block binding of collagen Q to MuSK.* Neurology, 2011. **77**: p. 1819-1826.
81. He, X., Semenov, M., Tamai, K. and Zeng, X, *LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way.* . Development, 2004. **131**(8): p. 1663-77.
82. Lu Y, T.Q., Endo S, Suzuki T. , *A role for LRP4 in neuronal cell viability is related to apoE-binding.* Brain Research, 2007. **1177**: p. 19-28.
83. S. D. Weatherbee, K.V.A., L. A. Niswander, *LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction.* Development, 2006. **133**: p. 4993-5000.
84. M. I. Leite, S.J.e.a., *IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis.* Brain, 2008. **131**: p. 1940-1952.
85. P. Zisimopoulou, P.E., J. Tzartos, K. Lazaridis *A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis.* Journal of Autoimmunity, 2014. **52**: p. 1-7.
86. Shen, C.et a., *Antibodies against low-density lipoprotein receptor-related protein 4 induce myasthenia gravis.* Journal of Clinical Investigation, 2013. **123**: p. 5190-5202.
87. Higuchi, O.et a., *Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis.* Annals of Neurology, 2011. **69**(2): p. 418-422.
88. Romi F, S.G., Aarli JA, Gilhus NE, *Muscle autoantibodies in subgroups of myasthenia gravis patients.* Journal of Neurology, 2000. **247**: p. 369-375.
89. M. N. Meriggioli, D.B.S., *Muscle autoantibodies in myasthenia gravis: beyond diagnosis?* Expert Review of Clinical Immunology, 2012. **8**: p. 427-438.
90. Marx A, W.N., Leite MI, et al., *Thymoma and paraneoplastic myasthenia gravis.* Autoimmunity., 2010. **43**: p. 413-427.
91. Wang K, M.J., Tu A., *Titin: major myofibrillar components of striated muscle.* PNAS, 1979. **76**: p. 3698-3702.
92. Liversage AD, H.D., Knight PJ, Tskhovrebova L, Trinick J, *Titin and the sarcomeresymmetry paradox.* Journal of Molecular Biology 2001. **305**: p. 401-409.
93. Labeit D, W.K., Witt C, et al., *Calcium-dependent molecular spring elements in the giant protein titin.* PNAS, 2003. **100**: p. 13716-13721.

94. Gautel M, L.A., Barlow DP, et al, *Titin antibodies in myasthenia gravis. Identification of a major immunogenic region of titin.* Neurology, 1993. **43**: p. 1581-1585.
95. F. Romi, e.a., *Striational Antibodies in Myasthenia Gravis.* Archives of neurology, 2005. **62**: p. 442-446.
96. Szczudlik, P.e.a., *Anti-titin antibody in early and late onset myasthenia gravis.* Acta Neurologica Scandinavica, 2014. **130**: p. 229-233.
97. F. Romi, e.a., *The severity of myasthenia gravis correlates with the serum concentration of titin and ryanodine receptor antibodies.* Archives of Neurology, 2000. **57**: p. 1596-1600.
98. Coronado R, M.J., Sukhareva M, Vaughan DM, *Structure and function of ryanodine receptors.* American Journal of Physiology, 1994. **266**: p. 1485-1504.
99. Benacquista BL, S.M., Samso M, Zorzato F, Treves S, Wagenknecht T., *Amino acid residues 4425-4621 localized on the three-dimensional structure of the skeletal muscle ryanodine receptor.* Biophysical Journal, 2000. **78**: p. 1349-1358.
100. Skeie GO, M.A., Treves S, Gilhus NE, Aarli JA, Zorzato F., *Ryanodine receptor antibodies in myasthenia gravis: epitope mapping and effect on calcium release in vitro.* Muscle Nerve, 2003. **27**: p. 81-89.
101. Mygland A, T.O., Aarli JA, Matre R, Gilhus NE, *IgG subclass distribution of ryanodine receptor autoantibodies in patients with myasthenia gravis and thymoma.* Journal of Autoimmunity, 1993. **6**: p. 507-515.
102. G. O. Skeie, S.A., A. Evoli, N. E. Gilhus, I. Illa, L. Harms, D. Hilton-Jones,A. Melms, J. Verschuuren & H. W. Horge, *Guidelines for treatment of autoimmune neuromuscular transmission disorders.* European Journal of Neurology, 2010. **17**: p. 893-902.
103. Suzuki, S.e.a., *Autoimmune targets of heart and skeletal muscles in myasthenia gravis.* Archives of Neurology, 2009. **66**: p. 1334-1338.
104. Romi, F.e.a., *Anti-voltage-gated potassium channel Kv1.4 antibodies in myasthenia gravis.* Journal of Neurology, 2012. **259**: p. 1312-1316.
105. Zoltowska Katarzyna M, B.K., Leite M, et al., *Collagen Q–a potential target for autoantibodies in myasthenia gravis.* Journal of the Neurological Sciences, 2015. **348**: p. 241-244.
106. Zhang, B.e.a., *Autoantibodies to agrin in myasthenia gravis.* PloS ONE, 2014. **9**: p. e91816.
107. Witzemann, V., Chevessier, F., Pacifici, P. G. & Yampolsky, P, *The neuromuscular junction: selective remodeling of synaptic regulators at the nerve/muscle interface.* Mechanisms of Development, 2013. **130**: p. 402-411.
108. Onodera, H., *The role of the Thymus in the Pathogenesis of Myasthenia gravis.* THE TOHOKU JOURNAL OF EXPERIMENTAL MEDICINE, 2005. **207**: p. 87-98.
109. Berrih-Aknin, S., *Role of the thymus in autoimmune myasthenia gravis* Clinical & Exeprimental Neuroimmunology, 2016. **7**: p. 226-237.
110. Giraud M, T.R., Vandiedonck C, Ke X, Levi-Strauss M, Pagani F, et al., *An IRF8-binding promoter variant and AIRE control CHRNA1 promiscuous expression in thymus.* Nature, 2007. **448**: p. 934-939.
111. Gavanescu I, K.B., Ploegh H, Benoist C, Mathis D. , *Loss of Aire-dependent thymic expression of a peripheral tissue antigen renders it a target of autoimmunity.* PNAS, 2007. **104**: p. 4583-4587.

112. Salmon AM, B.C., Cardona A, Changeux JP, BerrihAknin S., *An acetylcholine receptor alpha subunit promoter confers intrathymic expression in transgenic mice. Implications for tolerance of a transgenic self-antigen and for autoreactivity in myasthenia gravis.* The Journal of Clinical Investigation, 1998. **101**: p. 2340-2350.
113. Truffault F, d.M.V., Eymard B, Sharshar T, Le Panse R, Berrih-Aknin S, *Thymic Germinal Centers and Corticosteroids in Myasthenia Gravis: an immunopathological study in 1035 cases and a critical review.* Clinical Reviews in Allergy & Immunology, 2016. **52**: p. 108-124.
114. Derbinski J, G.J., Brors B, Tierling S, Jonnakuty S, Hergenhahn M,Peltonen L,Walter J,Kyewski B, *Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels.* Journal of Experimental Medicine, 2005. **202**: p. 33-45.
115. Melms A, S.B., Kirchner T, Muller-Hermelink HK, Albert E, Wekerle H. , *Thymus in myasthenia gravis. Isolation of T-lymphocyte lines specific for the nicotinic acetylcholine receptor from thymuses of myasthenic patients.* Journal of Clinical Investigation 1988. **81**: p. 902-908.
116. Scarpino S, D.N.A., Stoppacciato A, et al. , *Expression of autoimmune regulator gene (AIRE) and T regulatory cells in human thymomas.* Clinical & Experimental Immunology, 2007. **149**: p. 504-512.
117. J.H. O'Neill, N.M.M., J. Newsom-Davis, *The Lambert–Eaton myasthenic syndrome. A review of 50 cases.* Brain, 1988. **111**: p. 577-596.
118. M. Motomura, I.J., B. Lang, A. Vincent, J. Newsom-Davis, *An improved diagnostic assay for Lambert–Eaton myasthenic syndrome.* Journal of Neurology, Neurosurgery, and Psychiatry, 1995. **58**: p. 85-87.
119. P.W. Wirtz, N.W., A.R. van der Slik, B. Lang, P. Maddison, B.P. Koeleman, M.J. Giphart, A.R. Wintzen, B.O. Roep, J.J. Verschuuren, *HLA and smoking in prediction and prognosis of small cell lung cancer in autoimmune Lambert–Eaton myasthenic syndrome.* Journal of Neuroimmunology, 2005. **159**: p. 230-237.
120. Compston DAS, V.A., Newsom-Davis J, Batchelor JR., *Clinical, pathological, HLA antigen and immunological evidence for disease heterogeneity in myasthenia gravis.* . Brain, 1980. **103**: p. 579-601.
121. Giraud M, B.G., Yamamoto AM, et al, *Linkage of HLA to myasthenia gravis and genetic heterogeneity depending on anti-titin antibodies.* Neurology 2001. **57**: p. 1555-1560.
122. Giraud M, V.C., Garchon HJ, *Genetic factors in autoimmune myasthenia gravis.* . annals of New York Academy of Science, 2008. **1132**: p. 180-192.
123. Amdahl C, A.E., Gilhus NE, Nakkestad HL, Skeie GO, *Polygenic disease associations in thymomatous myasthenia gravis.* Archives of Neurology, 2007. **64**: p. 1729-1733.
124. Niks EH, K.J., Roep BO, et al, *Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5.* Neurology, 2006. **66**: p. 1772-1774.
125. Panse, S.B.-A.R.L., *Myasthenia gravis: A comprehensive review of immune dysregulation and etiological mechanisms.* Journal of Autoimmunity, 2014. **52**: p. 90-100.
126. N. E. Gilhus, J.F.O., J. M. Ho ff, F. Romi, G. O. Skele, and J. A. Aarli, *Myasthenia gravis: a review of available treatment approaches.* Autoimmune Diseases, 2011. **10**.

127. S. Berrih-Aknin, N.R., J. Bismuth, G. Cizeron-Clairac, E. Zelman, I. Shachar, P. Dartevelle, N. Kerlero de Rosbo, R. Le Panse, *CCL21 Overexpressed on Lymphatic Vessels Drives Thymic Hyperplasia in Myasthenia.* Annals of Neurology, 2009. **66**: p. 521-531.
128. R. M. Pascuzzi, *The edrophonium test.* Seminars in Neurology, 2003. **23**: p. 83-88.
129. M. N.Meriggioli and D. B. Sanders, *Advances in the diagnosis of neuromuscular junction disorders.* American Journal of Physical Medicine and Rehabilitation, 2005. **84**: p. 627-638.
130. Golnik KC, P., Lee AG, et al., *An ice test for the diagnosis of myasthenia gravis.* Ophthalmology, 1999. **106**: p. 1282-1286.
131. K. D. Sethi, M.H.R., and T. R. Swift, *Ice pack test for myasthenia gravis.* Neurology, 1987. **37**: p. 1383-1385.
132. Gilhus, N.E., *Myasthenia and neuromuscular junction.* Current Opinion in Neurology, 2012. **25**: p. 523-529.
133. Yang, L.e.a., *Non-radioactive serological diagnosis of myasthenia gravis and clinical features of patients from Tianjin, China.* Journal of the Neurological Sciences, 2011. **301**: p. 71-76.
134. Cruz, P.M.R.e.a., *Clinical features and diagnostic usefulness of antibodies to clustered acetylcholine receptors in the diagnosis of seronegative myasthenia gravis.* JAMA Neurology 2015. **72**: p. 642-649.
135. Chang, T.e.a., *Clinical and serological study of myasthenia gravis using both radioimmunoprecipitation and cell-based assays in a South Asian population.* Journal of the Neurological Sciences, 2014. **343**: p. 82-87.
136. Tsonis, A.I.e.a., *MuSK autoantibodies in myasthenia gravis detected by cell-based assay: a multinational study.* Journal of Neuroimmunology, 2015. **284**: p. 10-17.
137. S. J. Oh, D.E.K., R. Kuruoglu, R. J. Bradley, and D. Dwyer,, *Diagnostic sensitivity of the laboratory tests in myasthenia gravis.* Muscle and Nerve, 1992. **15**: p. 720-724.
138. Evoli, A., *Myasthenia gravis: new developments in research and treatment.* Current Opinion in Neurology, 2017. **30**: p. 464-470.
139. Gilhus NE, S.G., Romi F, Lazaridis K, Zisimopoulou P, Tzartos S. , *Myasthenia gravis - autoantibody characteristics and their implications for therapy.* Nature reviews Neurology, 2016. **12**: p. 259-268.
140. Palace, J., Newsom-Davis, J. & Lecky B., *A randomized double-blind trial of prednisolone alone or with azathioprine in myasthenia gravis.* Neurology, 1998. **50**: p. 1778-1783.
141. E. Kerty, A.E., Z. Argov, A. Evoli & N. E. Gilhus, *Guidelines for the treatment of ocular myasthenia.* European Journal of Neurology, 2014. **21**: p. 687-693.
142. R. Mantegazza, S.B., G. Camera, C. Antozzi, *Current and emerging therapies for the treatment of myasthenia gravis.* Neuropsychiatric Disease and Treatment, 2011. **7**: p. 151-160.
143. Jander, J.L.a.S., *Myasthenia gravis: recent advances in immunopathology and therapy.* Expert Review Of Neurotherapeutics, 2017. **17**: p. 287-299.
144. Hart IK, S.S., Sharshar T., *Immunosuppressive agents for myasthenia gravis.* The Cochrane Database of Systematic Reviews, 2007(4): p. CD005224.
145. Díaz-Manera J, M.-H.E., Querol L, et al, *Long-lasting treatment effect of rituximab in MuSK myasthenia.* Neurology, 2012. **78**: p. 189-193.

146. Iorio R, D.V., Alboini PE, et al, *Efficacy and safety of rituximab for myasthenia gravis: a systematic review and meta-analysis.* Journal of Neurology 2015. **62**(5): p. 1115-1119.
147. Gelfand, E.W., *Intravenous Immune Globulin in Autoimmune and Inflammatory Diseases.* The New England Journal of Medicine 2012. **367**: p. 2015-2025.
148. Mandawat A, K.H., Cutter G, et al, *Comparative analysis of therapeutic options used for myasthenia gravis.* Annals of Neurology, 2010. **68**: p. 797-805.
149. Barth D, N.N.M., Ng E, et al, *Comparison of IVIg and PLEX in patients with myasthenia gravis.* Neurology, 2011. **76**: p. 2017-2023.
150. Buckley RH, S.R., *The use of intravenous immune globulin in immunodeficiency diseases.* The New England Journal of Medicine, 1991. **325**: p. 110-117.
151. Koziolek MJ, K.B., Muhlhausen J, Muller GA, *Immunoadsorption in steroid-refractory multiple sclerosis.* Atherosclerosis Supplements, 2013. **14**: p. 167-173.
152. Pinching AJ, P.D., *Remission of myasthenia gravis following plasma-exchange.* Lancet, 1976. **2**: p. 1373-1376.
153. Gronseth, G.H.B., R. J., *Thymectomy for autoimmune myasthenia gravis (an evidence-based review).* Neurology, 2000. **55**: p. 7-15.
154. Marx, A.e.a., *The different roles of the thymus in the pathogenesis of the various myasthenia gravis subtypes.* Autoimmunity Reviews, 2013. **12**: p. 875-884.
155. Patrick J, L.J., Culp B, et al. Studies on purified eel acetylcholine receptor and anti-acetylcholine receptor *Studies on purified eel acetylcholine receptor and anti-acetylcholine receptor antibody.* PNAS, 1973. **70**: p. 3334-3338.
156. Lindstrom, J.M., et al, *Experimental autoimmune myasthenia gravis and myasthenia gravis: biochemical and immunochemical aspects.* Annals of New York Academy of Science, 1976. **274**: p. 254-274.
157. Baggi, F., et al., *Breakdown of tolerance to a self-peptide of acetylcholine receptor alpha-subunit induces experimental myasthenia gravis in rats.* Jouranl of Immunology, 2004. **172**: p. 2697-2703.
158. Lennon, V.A., J.M. Lindstrom, and M.E. Seybold, *Experimental autoimmune myasthenia gravis: cellular and humoral immune responses.* . Annals of New York Academy of Science, 1976. **274**: p. 289-289.
159. Stassen, M.H., et al., *Experimental autoimmune myasthenia gravis in mice expressing human immunoglobulin loci.* Journal of Neuroimmunology, 2003. **135**: p. 56-61.
160. Toro-Goyco, E., et al, *Induction of experimental myasthenia gravis in rhesus monkeys: a model for the study of the human disease.* Puerto Rico Health Sciences Journal, 1986. **5**: p. 13-18.
161. Link, H.a.B.G.X., *Rat models as tool to develop new immunotherapies.* Immunological Reviews 2001. **184**: p. 117-128.
162. Hoedemaekers, A.C., et al, *Age-related susceptibility to experimental autoimmune myasthenia gravis: immunological and electrophysiological aspects.* Muscle Nerve, 1997. **20**: p. 1090-1101.
163. Christadoss P, P.M., Deng C. , *Animal models of myasthenia gravis.* Clinical Immunology, 2000. **94**: p. 75-87.

164. Lindstrom, J.M., *Acetylcholine receptors and myasthenia.* Muscle Nerve, 2000. **23**: p. 453-477.
165. K. Lazaridis, e.a., *Characterization of a reproducible rat EAMG model induced with various human acetylcholine receptor domains.* Jouranl of Neuroimmunology, 2017. **303**: p. 13-21.
166. M. Losen, e.a., *Standardization of the experimental autoimmune myasthenia gravis (EAMG) model by immunization of rats with Torpedo californica acetylcholine receptors — Recommendations for methods and experimental designs.* Experimental Neurology, 2015. **270**: p. 18-28.
167. K. Lazaridis, e.a., *Specific removal of autoantibodies by extracorporeal immunoadsorption ameliorates experimental autoimmune myasthenia gravis.* Journal of Neuroimmunology, 2017. **312**: p. 24-30.
168. F. Baggi, e.a., *Oral administration of an immunodominant T-cell epitope downregulates Th1/Th2 cytokines and prevents experimental myasthenia gravis.* Journal of Clinical Investigation, 1999. **104**: p. 1287-1295.
169. Fu D. Shi, e.a., *Mechanisms of Nasal Tolerance Induction in Experimental Autoimmune Myasthenia Gravis: Identification of Regulatory Cells* The Journal of Immunology, 1999. **162**: p. 5757-5763.
170. D. Barchan, e.a., *Antigen-specific modulation of experimental myasthenia gravis: Nasal tolerization with recombinant fragments of the human acetylcholine receptor α-subunit.* PNAS, 1999. **96**: p. 8086-8091.
171. Consonni, A., *A Novel Approach To Reinstating Tolerance In Experimental Autoimmune Myasthenia Gravis Using A Targeted Fusion Protein, Mcta1–T146.* Frontiers in Immunology, 2017. **8**.
172. Wang, Z., *Suppression of experimental autoimmune myasthenia gravis by oral administration of acetylcholine receptor* Journal of Neuroimmunology, 1993. **44**: p. 209-214.
173. P. Maiti, S.F.e.a., *Immunosuppression of rat myasthenia gravis by oral administration of a syngeneic acetylcholine receptor fragment.* Journal of Neuroimmunology, 2004. **152**: p. 112-120.
174. K. Lazaridis, e.a., *Expression of human AChR extracellular domain mutants with improved characteristics.* International Journal of Biological Macromolecules, 2014. **63**: p. 210-217.
175. K. Kostelidou, e.a., *Expression and characterization of soluble forms of the extracellular domains of the β, γ and ε subunits of the human muscle acetylcholine receptor.* The FEBS Journal, 2006. **273**: p. 3557-3568.
176. A. Vincent, L.J., L. Curran, *a-Bungarotoxin binding to human muscle acetylcholine receptor measurement of affinity, delineation of AChR subunit residues crucial to binding, and protection of AChR function by synthetic peptides.* Neurochemistry International, 1998. **32**: p. 427-433.
177. S. Fuchs, e.a., *Role of Tolerogen Conformation in Induction of Oral Tolerance in Experimental Autoimmune Myasthenia Gravis* The Journal of Immunology, 2000. **165**: p. 3599-3605.
178. A. Rostami, e.a., *Mechanisms of Suppression of Experimental Autoimmune Encephalomyelitis by Intravenous Administration of Myelin Basic Protein: Role of Regulatory Spleen Cells.* Experimental and Molecular Pathology, 2000. **68**: p. 29-37.
179. A. M. Gomez, M.L., et al., *Antibody effector mechanisms in myasthenia gravis—Pathogenesis at the neuromuscular junction.* Autoimmunity, 2010. **43**: p. 353-370.

180. Panse, A.B.a.R.L., *New Pathways and Therapeutic Targets in Autoimmune Myasthenia Gravis.* Journal of Neuromuscular Diseases, 2018. **5**: p. 265-277.
181. Miriam C. Souroujon, e.a., *Mechanism of nasal tolerance induced by a recombinant fragment of acetylcholine receptor for treatment of experimental myasthenia gravis.* Journal of Neuroimmunology, 2000. **111**: p. 161-168.
182. S. Fuchs, e.a., *Suppression of ongoing experimental myasthenia by oral treatment with an acetylcholine receptor recombinant fragment.* Journal of Clinical Investigation, 1999. **104**: p. 1723-1730.
183. A. Hasselberg, e.a., *Role of CTA1R7K-COL-DD as aNovel Therapeutic Mucosal Tolerance–Inducing Vector forTreatment of Collagen-Induced Arthritis.* ARTHRITIS & RHEUMATISM, 2009. **60**: p. 1672-1682.
184. C. Hansson, e.a., *Feeding transgenic plants that express a tolerogenicfusion protein effectively protects against arthritis.* Plant Biotechnology Journal, 2016. **14**: p. 1106-1115.
185. Link, H., *Nasal tolerance in experimental autoimmune myasthenia gravis (EAMG): induction of protective tolerance in primed animals.* Clinical & Experimental Immunology, 1998. **111**: p. 506-512.
186. E. G. Spack, e.a., *Induction of Tolerance in Experimental Autoimmune Myasthenia Gravis with Solubilized MHC Class II:Acetylcholine Receptor Peptide Complexes.* Journal of Autoimmunity, 1995. **8**: p. 787-807.
187. G.X. Zhang, A.R., et al. , *The Role of IL-12 in the Induction of Intravenous Tolerance in Experimental Autoimmune Encephalomyelitis.* The Journal of Immunology, 2002. **168**: p. 2501-2507.
188. M. Gholamzad, e.a., *Intravenous Injection of Myelin Oligodendrocyte Glycoprotein-coated PLGA Microparticles Have Tolerogenic Effects in Experimental Autoimmune Encephalomyelitis.* Iranian Journal of Allergy, Asthma and Immunology, 2017. **16**: p. 271-281.
189. Brendan A. Hilliard, e.a., *Mechanisms of Suppression of Experimental Autoimmune Encephalomyelitis by Intravenous Administration of Myelin Basic Protein: Role of Regulatory Spleen Cells.* Experimental and Molecular Pathology, 2000. **68**: p. 29-37.
190. Howard L. Weiner, e.a., *Oral tolerance.* Immunological Reviews 2011. **241**: p. 240-259.
191. M. N. Sato, e.a., *Low dose of orally administered antigen down-regulates the T helper type 2-response in a murine model of dust mite hypersensitivity.* Immunology, 1999. **98**: p. 338-344.
192. X. Wang, e.a., *Mechanism of Oral Tolerance Induction to Therapeutic Proteins.* Advanced Drug Delivery Reviews, 2013. **65**: p. 759-773.
193. Anroop B. Nair, S.J., *A simple practice guide for dose conversion between animals and human.* Journal of Basic and Clinical Pharmacy, 2016. **7**: p. 27-31.
194. (CDER), U.S.D.o.H.a.H.S.F.a.D.A.C.f.D.E.a.R., *Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*, P.a. Toxicology, Editor. 2005. p. 1-27.
195. Meibohm, B., ed. *Pharmacokinetics and Half - Life of Protein Therapeutics*. Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives, ed. R. Kontermann. 2012, Wiley-VCH Verlag GmbH & Co. KGaA.: Wiley-VCH Verlag GmbH & Co.

196. Brian T. Fife, e.a., *Insulin-induced remission in new-onset NOD mice is maintained by the PD-1–PD-L1 pathway.* The Journal of Experimental Medicine, 2006. **203**: p. 2737-2747.
197. M. J. M. Jacobs, e.a., *Anergy of antigen-specific T lymphocytes is a potent mechanism of intravenously induced tolerance.* Immunology, 1994. **82**: p. 294-300.
198. Bimba F. Hoyer, e.a., *Short-lived Plasmablasts and Long-lived Plasma Cells Contribute to Chronic Humoral Autoimmunity in NZB/W Mice.* Journal of Experimental Medicine, 2004. **199**: p. 1577-1584.
199. O. Teng, e.a., *Induction of long-term B-cell depletion in refractory rheumatoid arthritis patients preferentially affects autoreactive more than protective humoral immunity.* Arthritis Research & Therapy, 2012. **14**.
200. A. Gomez, e.a., *Targeting plasma cells with proteasome inhibitors: possible roles in treating myasthenia gravis?* ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, 2012. **1274**: p. 48-59.
201. Gomez, A., *Proteasome Inhibition with Bortezomib Depletes Plasma Cells and Autoantibodies in Experimental Autoimmune Myasthenia Gravis.* The Journal of Immunology, 2011. **186**: p. 2503-2513.
202. M. Yu, e.a., *Nanotechnology for protein delivery: Overview and perspectives.* Journal of Controlled Release, 2016. **240**: p. 24-37.
203. R. Langer, e.a., *Nanocarriers as an emerging platform for cancer therapy.* Nature Nanotechnology, 2007. **2**: p. 751-760.
204. A. Ali, e.a., *Synthesis, characterization, applications, and challenges of iron oxide nanoparticles.* Nanotechnology, Science and Applications, 2016. **9**: p. 49-67.
205. P. Santamaria, e.a., *Reversal of Autoimmunity by Boosting Memory-like Autoregulatory T Cells.* Immunity, 2010. **32**: p. 568-580.
206. T. Kishimoto, e.a., *Tolerogenic nanoparticles induce antigen-specific regulatory T cells and Provide Therapeutic efficacy and Transferrable Tolerance against experimental autoimmune encephalomyelitis.* Frontiers in Immunology, 2018. **9**.
207. Maldonado, T.K.a.R.A., *Nanoparticles for the induction of Antigen-Specific immunological Tolerance.* Frontiers in Immunology, 2018. **9**.
208. M. Gupta, e.a., *Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications.* Biomaterials, 2004. **26**: p. 3995-4021.