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Standardization of the experimental autoimmune myasthenia gravis (EAMG) model by immunization of rats with *Torpedo californica* acetylcholine receptors — Recommendations for methods and experimental designs

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Abstract

Myasthenia gravis (MG) with antibodies against the acetylcholine receptor (AChR) is characterized by a chronic, fatigable weakness of voluntary muscles. The production of autoantibodies involves the dysregulation of T cells which provide the environment for the development of autoreactive B cells. The symptoms are caused by destruction of the postsynaptic membrane and degradation of the AChR by IgG autoantibodies, predominantly of the G1 and G3 subclasses. Active immunization of animals with AChR from mammalian muscles, AChR from *Torpedo* or *Electrophorus* electric organs, and recombinant or synthetic AChR fragments generates a chronic model of MG, termed experimental autoimmune myasthenia gravis (EAMG). This model covers cellular mechanisms involved in the immune response against the AChR, e.g. antigen presentation, T cell-help and regulation, B cell selection and differentiation into plasma cells. Our aim is to define standard operation procedures and recommendations for the rat EAMG model using purified AChR from the *Torpedo californica* electric organ, in order to facilitate more rapid translation of preclinical proof of concept or efficacy studies into clinical trials and, ultimately, clinical practice.

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Keywords

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Introduction

The serendipitous observation that immunization of rabbits with purified acetylcholine receptors (AChRs) led to MG-like symptoms has provided the basis for understanding the cause of myasthenia gravis (MG) and the mechanisms involved in its pathology (Patrick and Lindstrom, 1973). In this seminal work, experimental autoimmune MG (EAMG) was induced in rabbits by immunization with AChR from the electric organ of electric eels (*Electrophorus electricus*) in complete Freund's adjuvant (Patrick and Lindstrom, 1973). The immunization resulted in the production of antibodies to the *Electrophorus* AChR, binding of cross-reactive antibodies to the muscle AChR, and the subsequent paralysis and eventual death of the animals. EAMG has contributed to pre-clinical assessment and therapeutic discovery. Many variations of this animal model have been used since the 1970s. These later experiments included different amounts and sources of AChR, recipient species (see Table 1), sites for antigen injection (foot pads, base of the tail, hip and shoulder regions), and adjuvants [e.g. Titermax, incomplete Freund's adjuvant (IFA, based on mineral oil/water), complete Freund's adjuvant (CFA, IFA with additional heat killed *Mycobacterium tuberculosis*) or CFA with additional *Bordetella pertussis* toxin]. In each case, the animals mount an active immune response against the injected antigen; however only a small subset of the produced antibodies (~1%) cross-reacts with the animals' own muscle AChR (see Fig. 1) and this subset is responsible for the disease. Typically, muscle weakness occurs within 30–50 days after immunization. The EAMG model has been used extensively to analyze various aspects of MG pathology, and also experimental therapies to ameliorate MG (see Table 2). The chosen experimental parameters and procedures affect the disease time course, incidence and severity. EAMG scores can be increased using a susceptible strain, young animals, high amounts of AChR, a potent adjuvant and multiple injection sites for immunization. However, the disadvantages of a severe EAMG model are increased animal suffering, animal deaths, and an unrealistically stringent assessment of a therapeutic intervention. A mild EAMG model would be ineffective to demonstrate a beneficial effect of an experimental therapy, since little room exists for improvement of neuromuscular transmission. Below, the influence of various experimental parameters on the EAMG model is summarized and recommendations are offered for obtaining a robust and well-balanced EAMG model.

Animal care, safety and regulatory aspects

The use of the EAMG model is limited by ethical, environmental and safety regulations. The myasthenic muscle weakness itself constitutes an intrinsic discomfort and therefore the use of the EAMG model implies some degree of animal suffering that is unavoidable. Additional discomfort arises from stress while handling, anesthesia and injections. These aspects must be balanced against the expected benefit of new insights into the function of

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the neuromuscular junction, disease pathology or treatment efficacy of experimental drugs. We recommend that researchers planning to use the EAMG model seek advice from groups that have expertise in using it in order to reduce animal numbers and suffering to a minimum. Such an external review can be used for the application to institutional ethical boards which is in most countries required by law and also a prerequisite for publication in most journals. To minimize stress, the animals must be handled by experienced personnel. Lower stress was observed in rats that were caged with enrichment, such as, nestling, variety of objects and tunnels (Moncek et al., 2004).

Many reagents that interact with the proteins of the neuromuscular junction, and in particular with the AChR or the acetylcholine esterase (AChE) are highly toxic; e.g. alpha bungarotoxin, alpha cobratoxin, benzoquinonium, curare, sarin and neostigmine. Additionally, alpha bungarotoxin is frequently used in a ^{125}I radiolabeled form and any accidental physical contact might result in accumulation of ^{125}I in the thyroid gland. Careful planning of experiments, personal protection and working in dedicated laboratories reduce the risk to an acceptable level. Some of the reagents that are needed for realizing the EAMG model or for analyzing outcome measures involve wild living animals. These include the alpha toxin from the Indian cobra (*Naja naja*), the alpha bungarotoxin of the Taiwan banded krait (*Bungarus multicinctus*) and the AChR of the pacific electric ray (*Torpedo californica*). Import and export of these species, their tissues and proteins are in many countries restricted by national laws and/or need special permits of authorities. In many cases, however, it is possible to obtain access to the abovementioned purified proteins through collaborating research groups.

General animal care and housing

All care given to animals should be documented. To limit the stress and discomfort of the animals the following procedures are recommended. The number of personnel that handle the animals throughout the experiment should be kept to a minimum. A maximum of 2 researchers should be involved in immunizing the rats and assessing the clinical feature of EAMG. An inverted day–night cycle is advisable in order to perform the experimental procedures during the awake phase of the animals and avoid sleep-deprivation. The time of day that therapeutic drugs are administered and clinical scoring is performed should be kept constant. Cage change should take place 2–3 days before the initiation of experiment. Cages should be equipped with enriched environment supplies, nesting material, and a housing unit. We recommend social housing of young female Lewis rats (weight <300 g) in the cages with a floor area of 800 cm^2 and a height of >17.5 cm, with 3 animals per cage (National Research Council (U.S.) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011). If any animal becomes clinically weak (grade 2 or grade 3, see section ‘Clinical scoring’ below) all the cages should be supplied with water gel (e.g. HydroGel® or AQUA-JEL®) and soft food should be placed on the bottom of the cage. The same type of food should be administered to control animals and EAMG animals. Otherwise, the diet type should be kept constant throughout the study. Reporting the food vendor in published studies is recommended. Overgrown teeth can impair eating, ultimately causing starvation, and thus should be trimmed. Animals should be housed in specific pathogen free conditions. A health report including tested pathogens, and analytical

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methodology should be available (Kunstyr and Nicklas, 2000). When by accident some infection does occur, but disease symptoms are mild (e.g. a rotavirus infection resulting in diarrhea or staphylococcus infection at the immunization site), we suggest that the experiment can be continued under the following provisions: Animals should be treated as necessary and all infections, treatments and symptoms of each animal should be clearly documented in any resulting publication. If a suitable alternative exists, anti-inflammatory agents should be avoided due to potential obstruction with EAMG development (see also immunization section below).

Source and amount of AChR

The natural abundance of AChR in the electric organs of different fish species, such as *E. electricus*, *Torpedo californica* or *Torpedo marmorata*, confers an important practical advantage for generating sufficient amounts of purified AChR for the EAMG model. Other sources of AChR have been used successfully in various rat EAMG models (see Table 1), including AChRs from mammalian muscle and peptides corresponding to parts of the (human or rat) muscle AChR. The *T. californica* AChR (tAChR) has been used in most EAMG studies because it provides a reliable antigen for the induction of EAMG for which we describe the immunization standards. However, some antigen-specific therapies may depend on the exact amino acid sequence of the human AChR, such as immunodominant T or B cell epitopes, or on conformational epitopes that are specific for human MG. Other antigens such as the human AChR α1/1–210 peptides (Lennon et al., 1991), the recombinant chimeric *Aplysia* ACh-binding protein (AChBP) with human main immunogenic region (Luo and Lindstrom, 2012) or ectodomains of human AChR subunits (Niarchos et al., 2013) have also been used to induce EAMG. These human antigen models are clearly useful for answering specific research questions in exploratory studies. Nevertheless, we recommend the use of the *Torpedo* AChR for preclinical treatment efficacy studies wherever the drug mechanism allows this, since therapeutic testing requires a validated and standardized model for MG which is easily accessible for various laboratories. Table 2 summarizes various aspects of MG that can be studied in the model, e.g. proof of principle studies for immunosuppressive drugs.

Antibody titers, disease severity and disease incidence increase with higher amounts of tAChR used for immunization. We recommend using 40 μg tAChR for immunization since this dose results in a robust disease model (see Fig. 1 and Supplemental Tables). A characteristic of the EAMG model is the variable levels of autoantibodies mounted against the AChR by different animals, even within a single study.

The AChR from electroplaque tissue is purified by chromatography on a column containing α-cobratoxin linked to sepharose beads to which the AChR is bound. A second column is then used to concentrate the protein during the competitive elution with acetylcholine or benzoquinonium. The procedure outlined in Box 1 is a modification from (Wu et al., 2001). The amount and quality of the AChR are measured by radioimmunoassay using ^{125}I-alpha bungarotoxin. Denatured tAChR should not be used; important epitopes are conformationally dependent, including the main immunogenic region of the AChR which loses conformational specificity upon denaturation.

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Age, sex and strain determine susceptibility of rats to EAMG

Early studies showed that different rat strains vary in their ability to generate EAMG. Wistar Furth and Copenhagen strains fail to exhibit disease symptoms, whereas Wistar Munich and Fischer strain animals develop severe, fatal disease associated with impaired neuromuscular transmission (Biesecker and Koffler, 1988). Lewis and Brown Norway have a milder disease development. Additionally, since the incidence of MG depends on sex and age (Phillips, 2004), several studies explored the effect of these parameters in the development of EAMG. In the Brown Norway and Lewis strains it was observed that 8–10 weeks old rats are susceptible to EAMG but rats older than 100 weeks were clearly resistant (Hoedemaekers et al., 1997a, 1997b). Differences in antibody titers, isotype distribution, fine specificity or complement activation did not account for the observed resistance. The age-related resistance could be reproduced in the passive transfer MG (PTMG) model (Graus et al., 1993) and correlated with the density of s-laminin, agrin and rapsyn at the neuromuscular junction (Hoedemaekers et al., 1998). Increasing rapsyn-expression in susceptible muscles of 9-week old female Lewis rats prevented degradation of AChR by subsequent PTMG (Losen et al., 2005), thus demonstrating that rapsyn protects the AChR against antigenic modulation. The exact time point when the age-related resistance of the neuromuscular junction occurs has not been determined, but it seems likely that it is weight related. The effect of sex on EAMG has been studied in aged Brown Norway rats where muscle AChR-loss was more prominent in female compared to male rats (Hoedemaekers et al., 1997a). Lewis rats have been more commonly used in EAMG (de Silva et al., 1988; Gomez et al., 2011; Lennon et al., 1978; Lindstrom et al., 1976; Martinez-Martinez et al., 2007; Okumura et al., 1994). Since sex, age and strain are clearly major contributing factors to the severity of EAMG, we recommend standardizing the model by performing the immunization with tAChR in seven-week-old female Lewis rats.

Immunological differences of rat strains in the immune response to the AChR

Both in MG and in EAMG, most of the antibodies are directed toward conformation-dependent epitopes on the AChR, whereas T cells in contrast recognize also the denatured AChR. Immunodominant epitopes to the tAChR differ significantly between rat strains. In search of T cell epitopes in EAMG, synthetic peptides covering 62% of the tAChR alpha-subunit sequence were tested in a T cell proliferation assay with lymph node cells from rats immunized with tAChR (Fujii and Lindstrom, 1988). In Lewis rats, 2 of these peptides, alpha 100–116 and alpha 73–90, strongly stimulated T cells and, of these, alpha 100–116 was much more potent. Interestingly, the EAMG could be induced in the Lewis rats by immunizing with a synthetic peptide corresponding to the rat AChR-alpha 1 97–116 amino acid sequence (Baggi et al., 2004), but the time course of the disease was delayed as compared to tAChR immunizations. In three other strains of the rats (Brown Norway, Wistar Furth and Buffalo) completely different sets of peptides stimulated their T cells (see Table 3). Genetically restricted T cell recognition of AChR peptides in different rat strains suggested that T cells with different major histocompatibility haplotypes may recognize different AChR peptides (Fujii and Lindstrom, 1988). Also in MG patients, proliferative T

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cell responses to peptides of the AChR alpha 1 subunit correlated with human leukocyte antigen (HLA) types (Brocke et al., 1988). Experimental therapies in the EAMG model that are designed to modulate the immune response against specific AChR epitopes (such as immunodominant epitopes in human MG) are likely to be affected by the rat strain and corresponding MHC restriction.

Another immunological parameter that has been studied in different strains of the rat EAMG model is the Th1/Th2 balance. In the rat, $CD4^+$ T cells can be subdivided into two major subsets based on their different lymphokine production patterns. Th1 cells, which produce IL-2 and IFNγ, can transfer cell-mediated immunity. These cells also induce preferentially the synthesis of antibodies of the IgG2b isotype. Conversely, Th2 cells produce IL-4 and cause B cell proliferation and differentiation, eliciting mainly IgG1 and IgE production (Saoudi et al., 1999). This balance affects the isotype distribution of AChR antibodies. The rat subclasses IgG1, IgG2a, IgG2b and IgG2c can all activate rat complement; but their capacity to do so decreases in the following order IgG2b>IgG2a>IgG2c>IgG1 (Füst et al., 1980; Medgyesi et al., 1981). The Lewis and Brown Norway rats, differ markedly in their Th1/Th2 balance: the immune response to the tAChR led to a marked production of IL-2 and IFNγ in the Lewis rats, while the Brown Norway rats responded by producing more IL-4 (Saoudi et al., 1999). Consequently, the Lewis rats produced mostly IgG2b AChR antibodies, while the Brown Norway rats produced predominantly IgG1 AChR antibodies. Nevertheless, the EAMG incidence and severity were comparable in both strains. In this respect, the rat EAMG model is clearly different from the mouse EAMG model, where the polarization toward a Th2 immune response decreases muscle weakness because mouse IgG1 antibodies do not activate complement (reviewed in Gomez et al., 2010).

Treatment strategies that would rely on an immunologic property of a very specific rat strain, such as MHC class and cytokine profile are less likely to be translatable to a second species (e.g. mice) and eventually to MG patients. Investigators should be aware of immunological differences between human and rodent when designing the experiment. Therefore, we suggest that the Lewis rat is a suitable model for testing new therapies, including for example strategies to induce immunological tolerance to the AChR that hopefully can be used for treatment of AChR-MG patients in the future.

Adjuvants

When using 40 μg tAChR in CFA, chronic muscle weakness may develop at any time between 4 and 7 weeks after immunization. The use of either Titermax, or CFA with additional *B. pertussis*, will result in an additional acute phase of EAMG, a transient muscle weakness observed in 7–10 days after injection of tAChR.

A strong adjuvant such as CFA is needed to overcome the tolerance of the immune system to the muscle AChR. The *M. tuberculosis* in CFA enhances the Th1 immune response compared to IFA. CFA has been widely used for the EAMG model; increasing the concentration of *M. tuberculosis* in the CFA leads to higher incidence and average severity of EAMG. However, the use of CFA is controversial since it causes granulomas or adjuvant arthritis in some animals. Various alternative adjuvants are commercially available (e.g.

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Titermax™), but their proprietary composition and their limited use by different research groups using the EAMG model make them unattractive for a general recommendation at this point. Therefore, we recommend using CFA (containing 1 mg/mL *M. tuberculosis*). Higher amounts of *M. tuberculosis* (e.g. 2 mg/mL) can be used to reach clinical weakness in a larger percentage of animals, thus allowing a reduction of group sizes in studies that are powered for decreasing EAMG scores. Supplemental Table 1 (Exp 1. and Exp 2.), shows the EAMG disease scores using 40 μg tAChR and 0.1 mg *M. tuberculosis* per animal.

If the acute phase of EAMG in the period of 7/10 days after immunization is crucial for the experimental design/intervention, Titermax or CFA with additional *B. pertussis* may be used instead. The acute phase of EAMG provides a useful control showing that the treated and untreated rats were equally affected prior to therapy.

Keeping all other parameters of the rat EAMG model constant, as proposed here, might facilitate the identification of a safe and efficient alternative to CFA. In this respect it is important to remember that the chosen adjuvant determines the type of immune response; e.g. in mice it has been shown that using aluminum oxide as adjuvant can ameliorate AChR-EAMG by promoting a Th2 immune response (Milani et al., 2006).

Immunization

An important aspect in the immunization procedure is the utilization of skilled, competent, technical staff experienced in the handling of animals and in performing the technique. They must be knowledgeable and capable of recognizing signs of distress in all injected animals, and be responsible for taking action when necessary. EAMG is induced by injecting seven-week-old female Lewis rats at the base of the tail with tAChR. The injection volume is 200 μL consisting of 40 μg tAChR in 100 μL sterile phosphate buffered saline (PBS) mixed and emulsified with 100 μL CFA (containing 1 mg/mL *M. tuberculosis*). Preparations are emulsified using a blender (e.g. Sorvall). Prepared CFA can be purchased from well-known manufacturers. The control rats are injected with a similar volume of PBS and adjuvant without tAChR. For immunization, the rats are placed in an enclosed chamber with 4% isoflurane in air (or oxygen) and allowed to reach anesthetic depth. In order to reduce pain after the immunization, we recommend injecting animals with 0.01–0.05 mg/kg buprenorphine during the induction of anesthesia. The animal is placed on the surgical table with isoflurane flow (2–3% isoflurane in air or oxygen). Immunization is performed at the base of the tail and more proximally and laterally at the flanks. We do not recommend using additional or other injection sites, since immunization at the tail induces clinical EAMG in a large proportion of animals using the recommended amount of tAChR and CFA in Lewis rats (see Supplemental Table 1). To ensure proper delivery, the rat is shaved along the base of the tail. The site of injection is cleaned with 70% ethanol and a 26 gauge needle is inserted with a bevel side facing the skin. The CFA emulsion (200 μL per rat) is injected at 5–10 sites by moving the needle laterally below the skin (i.e. without intermediate removal of the needle from the injection site). By lifting up the skin, perforation by the needle tip is avoided. The needle is removed slowly and light pressure is applied to the injection site. After injection, the animal is allowed to recover on a heated pad or in a recovery cage under an infrared heating lamp. Once the animal is ambulatory, it is returned to the home cage.

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Footpad injections have been used by several groups when inducing the EAMG model. Due to ethical considerations (animal suffering) we recommend standardizing the model using the base of the tail injections. It is important to mention that EAMG incidence increases when footpad injections are used, and without footpad injections animal numbers need to be increased to achieve sufficient power if the EAMG scores are used for statistical comparison.

The injection site(s) must be observed by the investigators at least three times per week, for four weeks after the immunization. If an abscess, ulceration or dermatitis develops at the injection site, it must receive veterinary attention and treatment. Such lesions should be inspected at least three times per week until they are healed. Animals suffering from untreatable prominent dermatitis due to immunizations must be sacrificed.

Randomization and reduction in bias

The experimental design of the pre-clinical assessment of a therapeutic should detail the time and duration of drug delivery in relation to EAMG induction and observation of clinical signs. Randomization of animals should also be defined at this time. Grouping of animals should be reflective of the population, such as, weight (pre-treating) or weakness (post-treatment). Housing of controls and drug treated groups in separate cages may be necessary due to potential to transfer drug through normal grooming or eating of feces. The cages should be marked with letter or number designation to avoid bias during clinical scoring or strength testing. Blinding the experiment to animal handlers during assessment of the animals or masking the samples from the study can eliminate bias.

Clinical scoring

The severity of clinical signs is scored weekly by observational assessment of muscular weakness. The rats' muscular strength and fatigability are assessed by their ability to grasp and lift repeatedly a 300-gram rack from the table while suspended manually by the base of the tail for 30 s (Martinez-Martinez et al., 2007). Clinical scoring is based on the presence of tremor, hunched posture, muscle strength and signs of fatigue. Loss of body weight can be an additional indicator of muscle weakness, since chewing and swallowing is affected by EAMG; however, body weight loss can also occur as a side effect of treatment (see for example Gomez et al., 2011). Body weight is measured weekly. After weight loss or muscle weakness is observed, animals should be weighted daily since disease can progress rapidly in individual animals. Signs of EAMG are graded as follows: 0, no clinical signs observed; 1, no clinical signs observed before testing, appearance of weakness after exercise due to fatigue; 2, clinical signs present before testing, i.e. hunched posture, weak grip, or head down, 3; no ability to grip, hindlimb paralysis, respiratory distress/apnea, immobility; and 4, moribund. Clinical scores should be taken every 24 h or less if the animals demonstrate severe weakness (score 2). It might be possible to re-fine the disease score with a more detailed behavioral analysis of mild clinical symptoms and additional exercise challenges. These efforts are currently ongoing and might lead to a revision/redefinition of the disease scores in the future.

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Humane endpoints for rats

The rats undergoing induction of EAMG require daily observation to evaluate health status. An indication that an animal is suffering includes for instance: porphyrin secretion, hunched posture, lethargy, rough or ruffled hair coat (demonstrating inability to groom their hair coats), labored breathing, dehydration (evident of rapid weight loss). The rats are euthanized within 24 h if EAMG score 3 is observed. Any animal that persistently (over a period of more than 3 days) loses more than 15% of weight (as consequence of EAMG, treatment side effect, infection or otherwise) should also be sacrificed. Any animal that loses more than 20% of its weight over a shorter time frame should be sacrificed within 24 h. Methods of euthanizing animals are institution dependent, and investigators should be instructed by the IACUC Committee for proper procedures.

Electromyography

Decrement of compound muscle action potential (CMAP) can be measured in the tibialis anterior of EAMG animals. The rats are anesthetized with 60 mg/kg sodium pentobarbital or with 2.5% isoflurane in air. The animal must be kept warm (skin temperature between 35 and 37 °C) by means of an infrared heating lamp or a heat pad, but do not overheat (risk of myasthenic decompensation). For stimulation, two small monopolar needle electrodes are used. The cathode is inserted near the peroneal nerve at the level of the knee and the anode is more proximal and lateral (at a distance of 3–4 mm). For recording, a third monopolar needle electrode is inserted subcutaneously over the tibialis anterior muscle. A ring electrode distally around the relevant hind leg or a subcutaneous needle electrode at the distal tendon serves as a reference, and the animal is grounded by a ring electrode around the tail. Movement artifacts must be avoided. Stimulation and recording can be performed with the EMG systems that are also used in clinical practice. To detect a decrementing response, a series of 8–10 supramaximal stimuli are given at 3 Hz with a stimulus duration of 0.2 ms. The test is considered positive for decrement when both the amplitude and the area of the negative peak of the CMAP show a decrease of at least 10% (Kimura, 2001). To demonstrate reproducibility, at least three recordings are made of all investigated muscles.

In case only subclinical disease is present, the impairment of neuromuscular transmission can be quantified accurately by combining decrement measurements with intraperitoneal curare challenge (for rats of ~200 g: 20 μg/mL at a rate of 0.33 μg curare/minute). In this case, the elapsed time until decrement is observed (an equivalent of the cumulative curare dose) is a measure for the muscle weakness (Gomez et al., 2011). Because of the curare infusion and the resulting paralysis, this measurement can only be performed as a terminal experiment. Moreover, the infused curare might interfere with other assays such as RIAs for AChR antibody titers or immunofluorescent staining of tissue sections using alpha bungarotoxin. If the diaphragm of the animals is severely affected, curare infusion might result in respiratory failure before decrement is observed in the tibialis anterior muscles. This can be avoided by mechanical ventilation of the animal under anesthesia. The curare challenge strongly complements data from clinical scoring: because of the safety factor of neuromuscular transmission, disease scores change drastically over a narrow range of AChR-loss (60%–80% in the schematic example shown in Fig. 2). This means that disease

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scores cannot differentiate groups that have between 0 and 60% of functional AChR loss. Curare challenge can extend this range, but cannot detect differences over time since it has a too long half-life to allow recovery of the animals.

Detection of serum rat muscle AChR and tAChR antibodies

Blood samples of up to 200 μL can be taken weekly from the vena saphena, but the samples taken every other week are generally sufficient for determining the change in the antibody titer. Antibodies against rat AChR are detected as described (Lindstrom et al., 1976) with minor modifications (Martinez-Martinez et al., 2007). Briefly, 150 μL extract of denervated rat muscle extract (containing ~5 nM AChR) is labeled with an excess of ^{125}I-α-BT (e.g. NEX126, PerkinElmer) and incubated with 5 μL of rat serum at 4 °C overnight. Antibodies against tAChR are detected by labeling 0.05 μg tAChR with ^{125}I-α-BT. These labeled tAChRs are mixed with 5 μL serum diluted 1:200 in PBS and with 2.5 μL normal rat serum as a co-precipitant/carrier.

The formed immune-complexes are then precipitated using 100–150 μL of secondary goat anti-rat antibody serum during 4 h and then centrifuged at >14,000 *g* for 5 min. Pellets are washed three times in PBS with 0.5% Triton X-100. Finally, radioactivity is measured in a γ-counter. Titers are expressed in nmol/L toxin binding sites.

Measurement of total muscle AChR concentration

Total muscle AChR is measured with a radioimmunoassay as described (Lindstrom et al., 1976). For analysis of dissected muscles, such as the tibialis anterior or other similarly sized muscles, the following modification of the methods can be used (Losen et al., 2005). After dissection, muscles are weighed and stored at −80 °C (muscles may lose weight over time due to dehydration but AChR content is preserved). Muscles are cut in ~1 mm sections using a scalpel and homogenized using a dispersion instrument (e.g. Ultra-Turrax, 3 times for 30 s, with for 30 s intervals) at 4 °C in 10 mL of extraction buffer (PBS, 10 mM NaN_3, 10 mM iodoacetamide and 1 mM phenylmethyl sulfonyl fluoride). The homogenate is centrifuged at 22,000 *g* (or higher) for 30 min and the resulting pellet is resuspended in 2.5 mL extraction buffer supplemented with 2% Triton X-100. AChR is extracted from the membrane with the detergent using a reciprocal shaker during 1 h at 4 °C, followed by centrifugation at N22,000 *g* for 30 min at 4 °C. The AChR in the supernatant is then incubated with an excess of ^{125}I-α-bungarotoxin with high specific activity (e.g. NEX126H, PerkinElmer) and an excess of serum from the EAMG rats. Immune-complexes are then precipitated using the goat anti rat-antibody serum as described above. Total muscle membrane AChR concentration is calculated per gram of fresh muscle and thus expressed in fmol/g.

ELISA for measurement of serum anti-AChR antibody isotypes

The isotype distribution of *Torpedo* AChR antibodies can be determined by ELISA using anti-rat Ig isotype specific antibodies, as described (Saoudi et al., 1999). High binding microtiter plates are coated overnight at 4 °C with purified tAChR in PBS (50 μL, 5 μg/mL) followed by washing 3 times with 100 μL ELISA buffer (PBS with 0.5% Tween 20) and

blocking for 15 min with 0.5% bovine serum albumin dissolved in the ELISA buffer. The tAChR should not be denatured and controlled by RIA for ^{125}I-α-BT binding. Rat sera diluted in ELISA buffer are added and incubated for 1 h at room temperature. Each serum is tested in duplicate and assessed in 4 dilutions (1:317, 1:1000, 1:3170 and 1:10,000). Fifty μL of the monoclonal AChR antibodies mAb 35 (IgG1), mAb 155 (IgG2a) and mAb 22 (IgG2b) is used as a concentration of 2 μg/mL to prepare standard curves, using the same dilutions as for the sera (Loutrari et al., 1992; Osborn et al., 1992). Subsequently, mouse anti-rat γ1, γ2a or γ2b monoclonal antibodies and an anti-mouse IgG secondary antibody are used for detection.

Immunofluorescence analysis of neuromuscular junctions

The density of AChR, its associated proteins or deposition of complement factors on the postsynaptic membrane can be analyzed by immunofluorescence. As a reference, a presynaptic marker is used. Isolated muscles (e.g. tibialis anterior or diaphragm) of the EAMG and control animals are frozen on melting isopentane. Cryosections of 10 μm are dried, fixed and blocked with PBSA (phosphate-buffered saline with 2% bovine serum albumin). Sections can then be incubated with primary antibodies against the vesicular acetylcholine transporter (VAChT) or the synaptic vesicles protein 2 (SV2) to localize the NMJ. To determine the deposition of complement, antibodies to C3, C9 or membrane attack complex (C5b-9) can be used. Subsequently the sections are incubated with fluorescent-conjugated α-bungarotoxin and the corresponding secondary antibodies. Since the antibodies are deposited at a high density at the NMJ in EAMG, it is important that secondary antibodies do not cross-react with rat immunoglobulins. This can easily be controlled by performing a staining of EAMG muscles with the secondary antibodies only (omitting the primary antibodies). An excess of primary and secondary antibodies, and bungarotoxin should be used so these do not limit the staining intensity. All the sections are stained and processed in parallel to avoid inter-assay variations.

For quantitative analysis, pictures of muscle sections are taken using a fluorescent microscope with a digital camera and analysis software. The exposure time is set to a constant value for each channel ensuring that no saturation of the pictures occurs. Also all other microscope settings are maintained constant. Endplate areas are identified by pre-synaptic markers and the mean intensity of staining in each channel is measured in the corresponding area. The presynaptic marker can be used to normalize the expression of the postsynaptic proteins. Multiple NMJs should be assessed for staining intensity. All the sections are stained and processed in parallel to avoid inter-assay variations (Losen et al., 2005).

Electron microscopy

The EAMG and control rats are anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg) and transcardially perfused with a Tyrode solution (0.1 M) followed by a fixation buffer (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The tibialis anterior muscles are removed and sectioned on a vibratome at 1 mm. The sections are postfixed for 1 h with 1% osmium tetroxide in a 0.1 M phosphate buffer, pH 7.4, dehydrated through a

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graded ethanol series and embedded in epoxy resin. Endplates are located in toluidine blue-stained semi-thin sections from the central region of each muscle. Ultra-thin sections from selected areas are contrasted with uranyl acetate and lead citrate and viewed with a transmission electron microscope. At least five endplate regions are photographed from each muscle. Pictures are scanned for morphometric analysis using the ImageJ software. The key parameter to be analyzed for EAMG by morphometric analysis is the folding index, i.e. the ratio of the length of the postsynaptic membrane per length of the adjacent presynaptic membrane in each nerve bouton (Engel et al., 1976; Losen et al., 2005; Wood and Slater, 1997).

Considerations for experimental designs

Outcome parameters of prevention and treatment studies

In MG patients, a therapeutic drug generally would be used to treat a disease. However, prevention of disease relapse is also an important objective. The design of a preclinical study in the rat EAMG can include a preventive arm (starting at the time of immunization), a therapeutic arm (starting after the acute phase of the EAMG or alternatively, 4 weeks after immunization), or both. Treatments that affect early mechanisms of the immune response (e.g. antigen presentation, clonal expansion) will only be effective when applied preventively in the EAMG model; in patients such treatments are also effective, but even broad-spectrum immune-suppressive drugs might take months, or even years before their beneficial effect become evident (Gomez et al., 2012). Conversely, therapies that act against the later stages of the autoimmune response (e.g. complement inhibitors) have the potential to act more rapidly (Soltys et al., 2009). Nevertheless, a significant reduction of AChR autoantibody titer does not necessarily lead immediately to an improved EAMG score for two reasons: The NMJ needs considerable time to recover completely from autoantibody attack (we observed a significantly increased susceptibility to curare >2 weeks after a single injection of the AChR-specific mAb 35 in young female Lewis rats; ML, PM and PM-M, unpublished observations) and even a very low titer of AChR antibodies (<1 nM) can impair NMJ transmission in the rat EAMG model (Janssen et al., 2008). Therefore we suggest that clinical EAMG scores should be combined with at least one relevant biological EAMG parameter in order to determine the efficacy of a treatment. The outcome parameters should be defined before the study.

The marked muscle weakness that can be observed in the rat EAMG model makes it possible to choose this parameter as a primary outcome for testing the effect of a disease-modulating intervention. A percent of survival analysis should not be used as a primary EAMG outcome parameter and animals that reach EAMG grade 3 need to be sacrificed. Defining the EAMG score as outcome parameter is useful when the treatment affects the muscle strength or the neuromuscular junction directly, independent of autoantibody titers. However, it should be kept in mind that muscle weakness changes over a rather narrow range of AChR density at the neuromuscular junction because of the safety factor (see Fig. 2). If novel immunosuppressive or -modulatory drugs are tested, antibody titers against rat muscle AChR can be used as a relevant biomarker instead. Since the titer against rat AChR can be measured accurately and calculated as an absolute SI unit (nmol/L), we recommend

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that this measurement is included in all studies using the EAMG model in order to allow comparison of experiments. The same is true for the weight of animals: weight is a crucial parameter of general health of animals and should therefore always be measured. It is reduced in EAMG animals but can also be affected by therapies (e.g. Gomez et al., 2011). For this reason it is important to include groups of the untreated and treated (healthy) control rats in the study.

Measurement of functional AChRs using curare infusions is especially useful to detect any remaining subclinical damage to the neuromuscular junction after treatment. Because of the high amount of extra-synaptic AChRs in the muscle membrane, curare resistance is more informative than total muscle AChR content by radioimmunoassay which detects extrajunctional AChR as well. However, total muscle AChR can valuably complement other measurements, especially when the treatment is expected to affect AChR turnover or synthesis (e.g. Martinez-Martinez et al., 2007). Similarly, quantitative immunofluorescence and electron microscopic analysis can optionally be used to corroborate other outcome measures. These measures are relatively labor intensive and restricted to the last time point of the experiment; therefore these parameters are less suitable as primary outcome measurements than EAMG scores or antibody titers. In conclusion, we recommend the measurement of the following outcome parameters:

Standard primary outcome parameters with multiple testing:

- EAMG score (weekly measurements)
- antibody titers against rat muscle AChR (every other week)
- weight (weekly).

Secondary (optional) outcome parameters:

- decrement measurements with curare (endpoint)
- muscle AChR concentration (endpoint)
- antibody titers against tAChR (every other week)
- tAChR antibody isotype distribution (every other week)
- NMJ folding index (endpoint)
- NMJ quantitative immunofluorescence (endpoint).

Power calculations and statistical analysis of results

Depending on the precise research question any of the aforementioned outcome parameters can be useful for the determination of sample size. Animal studies are typically powered at 80% or higher to detect a statistically significant difference between groups with $p < 0.05$. The supplemental data of this manuscript might be useful for power calculations based on clinical scores, tAChR antibody titers, or rat muscle AChR antibody titers, since they provide information on the typical variability of the EAMG model. Additional outcome parameters that are based on the biological mechanism of the tested therapeutic intervention can be used for power calculations instead, provided that data on effect size are available

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through proof-of-concept experimental outcome. The power analysis dictates the minimal number of animals necessary to register a statistical effect. Careful calculations of the "n" required in a study will ensure that the outcome result reflects the significance between the groups and not the lack of power to justify the result (Steward and Balice-Gordon, 2014).

In outlining the design to determine the statistical significance of therapeutic efficacy, several factors must be considered; proper controls, number of outcome measurements, distribution of the outcome measurements, and statistical analysis to be performed. Documentation of the results should adequately described statistical methods, provide a complete listing of all analyses done even if values did not reach statistical significance.

Concluding remarks

The tAChR induced rat EAMG model has a high validity for many aspects of human MG, including immunological, neuromuscular and symptomatic parameters. Therefore, it is ideally suited for development of new or improved MG therapies. The standardization of various parameters of the model will help to make studies comparable and thereby increase confidence in the results. Effect sizes can then be used as a basis for deciding about further preclinical or clinical studies. It is important to mention that such standardization is a dynamic process and will likely need future adjustments to meet new insights.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.expneurol.2015.03.010.

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Box 1

Method for isolation of Torpedo AChR

Frozen *Torpedo californica* electroplaque tissue is crushed in mortar and pestle and added to cold homogenization buffer (1 mM sodium phosphate, pH 7.5, 0.1 M NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM iodoacetamide and 1 mM PMSF). The slurry is grinded in a homogenizer or blender and the homogenate is then centrifuged at high speed to collect non-soluble membranes. It is preferable to spin this homogenate at 100,000 g at 4 °C for 30 min. The pellets are resuspended in cold homogenization buffer containing 1% (v/v) of Triton® X-100. Sample is rotated or agitated overnight at 4 °C. Homogenate is then centrifuged at high speed, as described above, and the supernatant containing the AChR is collected for column chromatography and stored on ice. Neurotoxin affinity column is prepared by coupling of α-cobratoxin (*Naja naja kauthia*) to CNBR-agarose. CNBR-agarose resin is placed into 250 mL Erlenmeyer flask and allowed to settle; the supernatant is then aspirated. The resin is washed with 1 mM hydrochloric acid swirl twice by resuspending resin, allowing resin to settle, and aspirating supernatant. The resin is then washed once with a coupling buffer (10 mM sodium carbonate [Na_2CO_3], 90 mM sodium bicarbonate [$NaHCO_3$], 500 mM NaCl, pH 8.3). The resin is resuspended in coupling buffer and α-cobratoxin, dissolved in coupling buffer, is added. The resin and α-cobratoxin solution is rotated overnight at 4 °C. The resin is then washed twice in coupling buffer. The resin is resuspended in a blocking buffer (100 mM Tris–HCl, pH 8.0) for 2 h at 4 °C. The resin is allowed to settle and the buffer is removed. Acetate buffer (100 mM Na acetate trihydrate, 500 mM NaCl, pH 4.0) is added to resuspend the resin and allowed to settle. The buffer is removed. Resin is washed four times in a column buffer (100 mM Tris–HCl, pH 8.0, 500 mM NaCl). The resin can be added to a 1.5 by 20 cm column (35 mL volume) affixed with a 2-way stopcock on the outlet port placed in the stop flow position. First, the column buffer is added to column and then the stopcock is opened. The resin is poured in slowly. Additional column buffer may be used to resuspend resin in flask and add to column. The column is allowed to run until all resin is added. The column stopcock is closed and resin is allowed to settle. The column is washed five times in NaCl/Triton buffer (500 mM NaCl, 0.1% (v/v) Triton X-100 in PBS, pH 7.4). The column is now ready to use.

Microcrystalline hydroxyapatite in cross-linked 4% beaded agarose column is prepared by combining with equal volume 10 mM sodium phosphate (NaPi) buffer, pH 7.5 in an Erlenmeyer flask. The resin is washed twice in NaPi buffer. Resin is poured slowly into a 1.5 by 20 cm column (35 mL volume) affixed with a 2-way stopcock on the outlet port placed in the stop flow position. Residual resign in the flask may be resuspended in additional column buffer. The column is opened and allowed to run until all resin is added. The column stopcock is closed and resin is allowed to settle. When resin is completely settled, the stopcock is opened to let the buffer drain, by gravity, to just below the top of the column. The column is now ready to use.

To load the *T. californica* electroplaque tissue supernatant on the neurotoxin affinity column: the cap from neurotoxin affinity column is removed and the stopcock is opened to allow the column buffer to drain out just until it reaches the top of the resin. The resin

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is gently overlayed with 10 mM NaPi buffer and the column is opened until the buffer is drained just to the top of the resin. The column is gently refilled with a NaPi buffer to the top, filling the remaining head space of the column. The inlet cap is replaced and Luer tubing or similar (male luer connector, short silicon tubing and non-luer Teflon tubing) is attached at the top. The tubing is connected to a reservoir of NaPi buffer that is placed above to allow gravity to fill the column for constant free flow to exchange the buffer. The flow is stopped, the tubing is removed from the reservoir and the remaining buffer is removed from the top of the resin with a Pasteur pipette. The *T. californica* electroplaque tissue supernatant is overlayed on top of the column, filling the column headspace. The column's stopcock is opened and the homogenate is allowed to run into the resin. The addition of more homogenate is applied to the top until all the homogenate samples are contained in the resin of the column. The NaPi buffer is gently overlayed on top of the resin to completely fill the column. The column is re-connected with a cap and tubing. The reservoir of the NaPi buffer that is placed above to allow gravity to fill the column. The column is washed with 2 column volumes of 10 mM NaPi buffer to remove non-bound proteins from the neurotoxin affinity column. The column's stopcock is closed.

The neurotoxin affinity column is attached to the hydroxyapatite column to begin collection of AChR. A carbachol buffer (1 M carbamylcholine chloride, 10 mM Tris pH 7.4, 0.1% Triton X-100) is added to the system by first washing the carbachol buffer through hydroxyapatite column. The buffer in the space above the resin in the neurotoxin affinity column is replaced with the carbachol buffer. The neurotoxin affinity column's stopcock is connected to the top of the cap of the hydroxyapatite column by tubing. The stopcock of the hydroxyapatite column runs to the pump which will connect back to the top of the neurotoxin affinity column. The pump is set at the highest speed possible without developing leaks from high back pressure and run in a refrigerated chromatography cabinet or cold room for 24 h.

AChR can be eluted from the hydroxyapatite column using a 152 mM NaPi buffer [102 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 7.4]. Fractions of approximately 1 mL (10–15 mL per run) are collected and tested for the presence of protein (colorimetric assay or absorbance reading). All the fractions that contain protein are pooled. The purified AChR is then dialyzed in dialysis tubing overnight at 4 °C with stirring against PBS. Glycerol is added to the AChR as 10% (v/v). The final preparation stored in small aliquots at −80 °C. The protein concentration amount should be determined. A SDS-PAGE gel stained by Coomassieblue should be performed to determine the purity of the AChR sample.

Regenerating the neurotoxin affinity column and the hydroxyapatite column: the columns are disconnected. The columns can be regenerated by attaching a reservoir and flushing the column with 10 column volumes of NaCl/Triton buffer [1 × PBS, pH 7.4, 0.5 M NaCl, 0.5% Triton X-100]. When complete, turn off the stopcocks and store at 4 °C until next use.

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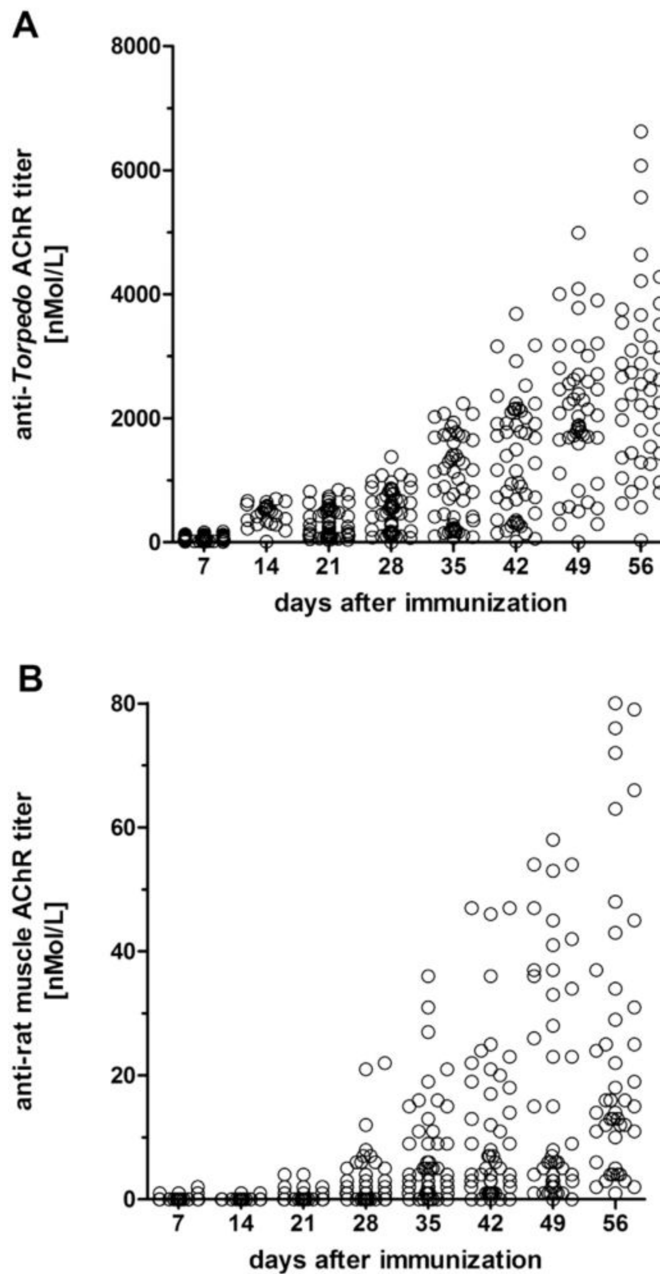

Fig. 1.

Representative anti-tAChR (A) and anti-rat muscle AChR titers (B) after immunization with 40 μg tAChR in CFA (with 1 mg/mL *Mycobacterium tuberculosis*) on day 0 in 7-week old female Lewis rats. Anti-tAChR titers were detected approximately 2 weeks before anti-rat muscle AChR titers were measured. In the period between 35 and 56 days after immunization, anti-tAChR titers were two orders of magnitude higher compared to rat muscle AChR antibody titers. The variability of antibody titers seen here is typical of the EAMG model. The raw data used for the graph are available in Supplemental Table 2.

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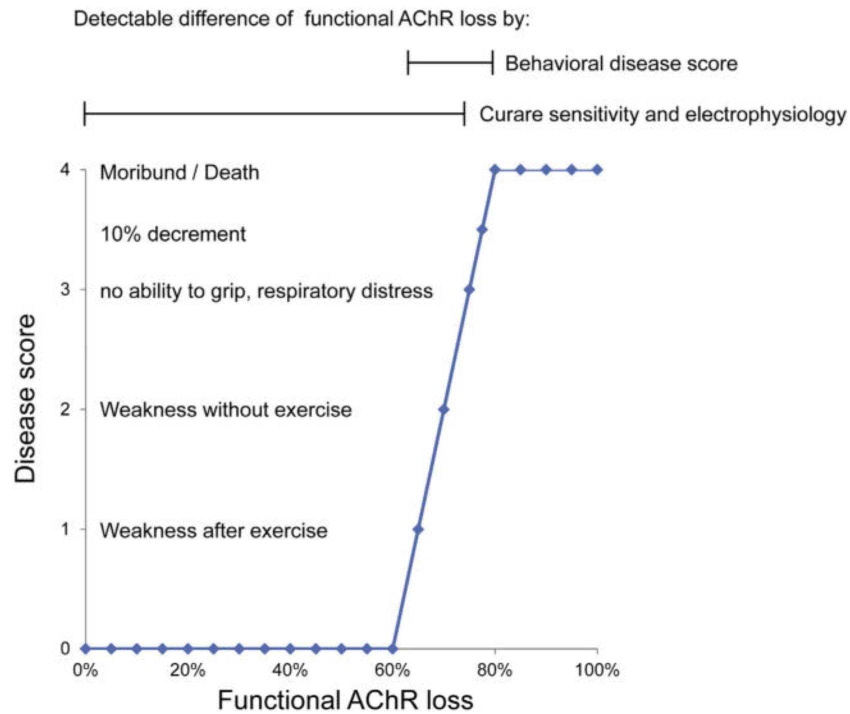

Fig. 2.

Schematic representation of the relation between AChR loss and muscle weakness. Because of the safety factor of neuromuscular transmission (3 in this example), animals with an average AChR loss of up to 60% have no disease symptoms. Challenge of neuromuscular transmission with curare can reveal subclinical damage to the neuromuscular junction. The effect of therapeutical interventions can thereby be studied much more sensitively.

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Table 1

AChR sources and species for EAMG induction.

Source of AChR	Recipient animal	Reference
Torpedo californica (electric organ)	Rat (*Rattus norvegicus*)	Lennon et al. (1978)
	Mouse (*Mus musculus*)	Berman and Patrick (1980)
	Pig (*Sus scrofa domesticus*)	De Haes et al. (2003)
	Rhesus monkey (*Macaca mulatta*)	Tarrab-Hazdai et al. (1975)
	Frog (*Rana ripiens*)	Nastuk et al. (1979)
	Guinea pig (*Cavia porcellus*)	Lennon et al. (1975)
Torpedo marmorata (electric organ)	Rat (*Rattus norvegicus*)	Elfman et al. (1983)
	Rabbit (*Oryctolagus cuniculus*)	Barkas and Simpson (1982)
Electrophorus electricus (electric organ)	Rabbit (*Oryctolagus cuniculus*)	Patrick and Lindstrom (1973)
	Rat (*Rattus norvegicus*)	Lennon et al. (1975)
	Guinea pig (*Cavia porcellus*)	Lennon et al. (1975)
Rat AChR (syngeneic muscle)	Rat (*Rattus norvegicus*)	Lindstrom et al. (1976)
Cat (denervated muscle)	Rabbit (*Oryctolagus cuniculus*)	Dolly et al. (1983)
Chicken (denervated muscle)	Rabbit (*Oryctolagus cuniculus*)	Dolly et al. (1983)
Human AChR (denervated muscle)	Rat (*Rattus norvegicus*)	Lennon et al. (1991)
1–210 sequence of the human AChR-α1 subunit (*Escherichia coli*)	Rat (*Rattus norvegicus*)	Lennon et al. (1991)
97–116 sequence of the rat AChR-α1 subunit (synthetic)	Lewis Rat (*Rattus norvegicus*)	Baggi et al. (2004)
Chimeric Aplysia ACh-binding protein (AChBP)/human muscle AChR	Lewis Rat (*Rattus norvegicus*)	Luo and Lindstrom (2012)

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Table 2

Published studies using the rat EAMG model.

Studied disease mechanism or treatment	Reference
Electrophysiological changes	Barone et al. (1980); Engel et al. (1976); Hohlfeld et al. (1981b); Kelly et al. (1978); Lennon et al. (1975); Molenaar et al. (1979); Olsberg et al. (1987); Plomp et al. (1995); Ruff and Lennon (1998); Takamori et al. (1984); Thompson et al. (1992); Verschuuren et al. (1990); Zahm et al. (1983)
Reduction in AChR	De Baets et al. (1988); Engel et al. (1977); Fumagalli et al. (1982); Lindstrom et al. (1976); Merlie et al. (1979)
Role of complement/complement inhibition	Lennon et al. (1978); Sahashi et al. (1978); Soltys et al. (2009)
Immune response to AChR	Asthana et al. (1993); Brown and Krolick (1988); De Baets et al. (1982); Fujii and Lindstrom (1988); Hohlfeld et al. (1981a); Li et al. (1998); Noguchi et al. (1980); Wang et al. (1993a); Zhang et al. (1988, 1996); Zoda and Krolick (1993)
Immunosuppression	Barone et al. (1980); Drachman et al. (1985); Duan et al. (2003); Duplan et al. (2002); Gomez et al. (2011); Ishigaki et al. (1992); Janssen et al. (2008); Kim et al. (1979); Luo and Lindstrom (2014); Menon et al. (2008); Pestronk et al. (1983); Ubiali et al. (2008); Zhang et al. (1997)
Antigen-specific drug conjugates	Killen and Lindstrom (1984); Olsberg et al. (1985)
Neonatal Fc-receptor blockade to decrease autoantibody stability	Liu et al. (2007)
Lymphocyte depletion by irradiation	de Silva et al. (1988)
Immune modulation	Brenner et al. (1984); Karussis et al. (1994); Kong et al. (2009); Yarilin et al. (2002); Zhu et al. (2006)
Targeting of cytokines	Aricha et al. (2011); Duan et al. (2002); Im et al. (2001)
Tolerance by administration of AChR (orally)	Maiti et al. (2004); Okumura et al. (1994); Wang et al. (1993b, 1994); Yi et al. (2008)
Tolerance by administration of AChR (nasally)	Ma et al. (1995)
Tolerance by administration of AChR (dendritic route)	Li et al. (2005); Xiao et al. (2003)
Removal of plasma cells (vaccination against surviving)	Kusner et al. (2014)
Removal of plasma cells (proteasome inhibition)	Gomez et al. (2011)
Inhibition of T cells	Araga et al. (2000); Aricha et al. (2008); McIntosh et al. (1995); Wauben et al. (1996); Xu et al. (2001); Yoshikawa et al. (1997)
Amplification of neuromuscular signaling	Brenner et al. (2003); Kim et al. (1980)
Overexpression of the AChR-anchor protein rapsyn	Martinez-Martinez et al. (2007)

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Table 3

Immunodominant T cell epitopes in different rat strains based on published results (Fujii and Lindstrom, 1988).

Peptide	Position in AChR alpha 1 subunit	Lewis	Brown Norway	Wistar Furth	Buffalo
SEHETRLVANY	1–11				
YVNQNETNVRLRQQ	45–59				+++
TNVRLRQQWIDVRLRWNGY	52–70				+++
RWNPADYGGIKKIRLPSY	66–83				
GGIKKIRLPSDDVWLPGY	73–90	++			
IRLPSDDVWLPDLVLY	78–93				
LVLYNNADGDFAIVY	89–104				
YAIVHMTKLLLDYTGKI	100–116	+++			
YTGKIMWTPPAIFKSY	112–127				
YCEIIVTHFPFDQQNCT	127–143				
DGTKVSISPESDRPDG	152–167	+		+++	
SPESDRPDLSTY	159–170				
ESGEWVMKDYRGWKHWTCCPDTPYLDITYHF	172–205		+++	+	+
KHWYYTCCPDTPYL	185–199				
LPTDSGEK	235–242				
VELIPSTSSAVPLIGKY	261–277				
DRASKEKQENKIFADDIY	330–347				
SKEKQEVK	333–340				
SDISGKQVTGEVIFQTY	349–365				
TGEVIFQTY	357–365				
VIFQTPLIKNPDVKSAIEGY	360–379				
DVKSAIEGVKYIAEHY	371–386				
DEESSNAAEEWKYVAMVIDHY	389–409				
YGRLIELSQUEG	427–437				