Analysis of native acetylcholine receptor-associated proteins in TE 671 cells

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Preface

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List of abbreviations

1-DE	One-dimensional gel electrophoresis
2-DE	Two-dimensional gel electrophoresis
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
BT	Bungarotoxin
ChAT	Choline acetyltransferase
ColQ	Collagenic tail
Da	Dalton
DAP	Dystrophin/Utrophin associated glycoprotein complex
DIGE	Differential in-gel electrophoresis
DMD	Duchenne muscular dystrophy
EAMG	Experimental autoimmune myasthenia gravis
ESI	Electrospray ionization
FCS	Foetal calf serum
IF	Intermediate filament
Ig	Immunoglobulin
IMDM	Iscove's modified dulbecco's medium
LEMS	Lambert-Eaton myasthenic syndrome
LGMD	Limb girdle muscular dystrophy
NMJ	Neuromuscular junction
NRG	Neuregulin
MAb	Monoclonal antibody
MG	Myasthenia gravis
MIR	Main immunogenic region
MS/MS	Tandem mass spectrometry
MuSK	Muscle-specific kinase
OBB	Odyssey blocking buffer
PBS	Phospate buffered saline
PMSF	Phenylmethanesulphonylfluoride
PVDF	Polyvinylidene difluoride
Rapsyn	Receptor-aggregating protein of the synapse
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src-family kinase
VAChT	Vesicular acetylcholine transporter
VGCC	Voltage-gated calcium channel

Abstract

Myasthenia Gravis (MG) is an autoimmune disease characterised by auto-antibodies directed against the acetylcholine receptor (AChR) or the muscle-specific kinase (MuSK) at the NMJ. The postsynpatic membrane of the NMJ is the main target for the damage induced by the antibodies. A proportion of MG patients neither have antibodies against the AChR nor to MuSK. The auto-antigen in these seronegative patients is still unknown. Antibodies interfering with proteins that play an important role in the stability or maturation of the NMJ might disturb the molecular organization of the NMJ and, thereby, cause myasthenic symptoms. The aim of this project was to characterize AChR associated proteins that can be a candidate for the unknown auto-antigens. To analyse the proteins of the NMJ, the TE671 rhabdomyosarcoma cell line was used as an alternative source for NMJ components. First, we analysed the presence of some NMJ proteins in the TE671 cells by immunoblotting experiments. Antibodies recognized many NMJ associated proteins in the TE671 cell extract. As a next step, we investigated the efficiency of mild detergent conditions in solubilizing the AChR from the cell membrane by radioimmunoassay. Our experiments showed that low concentrations of the detergents NP-40, triton-X100 and brij-97 solubilized the best amounts of AChR. Subsequently, the AChR was precipitated together with AChR-associated proteins using alfa-bungarotoxin (α-BT) and with the anti-AChR antibody IgG1-637. The precipitated proteins were identified by immunoblotting using antibodies and by mass spectrometry. The presence of AChR subunits was demonstrated by Western blot in the immunoprecipitation experiments with IgG1-637. Additionally the mass spectrometry analysis revealed the presence of cytoskeleton proteins and keratins in α-BT precipitated samples. Thus, although these results have, disappointingly, demonstrated little presence of AChR complexes in the precipitated samples, they do give clue about which further experiments could be performed to identified AChR-associated proteins.

Samenvatting

Myasthenia Gravis (MG) is een auto-immuunziekte waarbij er auto-antilichamen gericht zijn tegen de acetylcholine receptor (AChR) of tegen muscle-specific kinase (MuSK) ter hoogte van de neuromusculaire junctie (NMJ). De auto-antilichamen induceren schade aan de postsynaptische membraan van de NMJ. Bij een klein aantal van de MG-patiënten worden noch antilichamen tegen AChR noch tegen MuSK teruggevonden. Het auto-antigen van deze seronegatieve patiënten is nog onbekend. Wanneer antilichamen interfereren met eiwitten die een belangrijke rol spelen in de stabiliteit of de maturatie van de NMJ, kan dit de moleculaire organisatie van de NMJ verstoren en symptomen van MG veroorzaken. Het doel van deze studie was om eiwitten die geassocieerd zijn met de AChR te karakteriseren om vervolgens kandidaat eiwitten te vinden voor de ongekende auto-antigenen in MG. De TE671 rhabdomyosarcoma cellijn werd gebruikt als alternatieve bron voor de NMJ om de eiwitten te bestuderen. Eerst werd de aanwezigheid van NMJ eiwitten in de TE671 cellen geanalyseerd door middel van Western blot experimenten. Antilichamen herkenden in het TE671 celextract veel eiwitten die geassocieerd zijn met de NMJ. Vervolgens werd de efficiëntie van milde detergenten om de AChR uit de celmembraan op te lossen nagegaan met behulp van een radioimmunoassay. Dit experiment toonde aan dat lage concentraties van NP-40, triton-X100 en brij-97 de grootste hoeveelheden AChR oplosten. Daarna werden de AChR en AChR-geassocieerde eiwitten geprecipiteerd met alfa-bungarotoxine (α-BT) en met anti-AChR IgG1-637 antilichamen. De geprecipiteerde eiwitten werden geïdentificeerd door gebruik te maken van immunoblotting met antilichamen en van massaspectrometrische analyses. De aanwezigheid van subeenheden van de AChR werd aangetoond door Western blot in de immunoprecipitatie-experimenten met IgG1-637. De massaspectrometrische analyses bevestigden de aanwezigheid van eiwitten van het cytoskelet en keratines in de stalen die geprecipiteerd werden met α-BT. Desondanks deze resultaten slechts weinig AChR complexen aantoonden in de geprecipiteerde stalen, geven ze aan welke verdere experimenten uitgevoerd dienen te worden om AChR-geassocieerde eiwitten te identificeren.

1 Introduction

1.1 Neuromuscular transmission

The neuromuscular junction (NMJ) is an anatomically and functionally differentiated chemical synapse. Its main function is to efficiently communicate the electrical impulse from the motor neuron to the skeletal muscle to signal contraction [1-3]. The NMJ contains three important structural components, namely the presynaptic nerve terminal, the synaptic cleft and the postsynaptic membrane of the muscle [1, 2, 4]. The neurotransmitter acetylcholine (ACh) is synthesized in the presynaptic nerve terminal and is released into the synaptic cleft on arrival of a nerve impulse. The structures located on the postsynaptic region are thrown into deep folds to increase the surface area of the muscle membrane. The membrane infoldings optimize the transmission of the chemical signal ACh. At the tips of these folds, acetylcholine receptors (AChRs) are clustered at concentrations of more than 10.000 receptors per μm^2 [1, 2, 4, 5]. Other proteins, including sodium channels, are localized in the troughs of these folds [6]. ACh binds to the AChRs at the postsynaptic membrane, which causes the membrane to depolarise, initiating the muscle action potential. The action potential then spreads along the length of the muscle fibre, triggering release of calcium from internal stores and leading to muscle contraction. The synaptic cleft contains a high concentration of the enzyme acetylcholinesterase (AChE), which ultimately terminates synaptic transmission by rapidly hydrolyzing free ACh [1, 7, 8]. Other proteins such as proteoglycans, involved in the stabilization of the NMJ structure, are also present in the synaptic cleft [4]. In the following paragraphs, the role of specific signalling pathways and proteins involved in NMJ development and AChR clustering and maintenance will be summarized. The pathways and proteins that are discussed throughout the text are depicted in figure 1. Since the NMJ is a site of well-characterised autoimmune and hereditary disorders, a short summary will be given of these diseases. Finally, the aim of this project will be described.

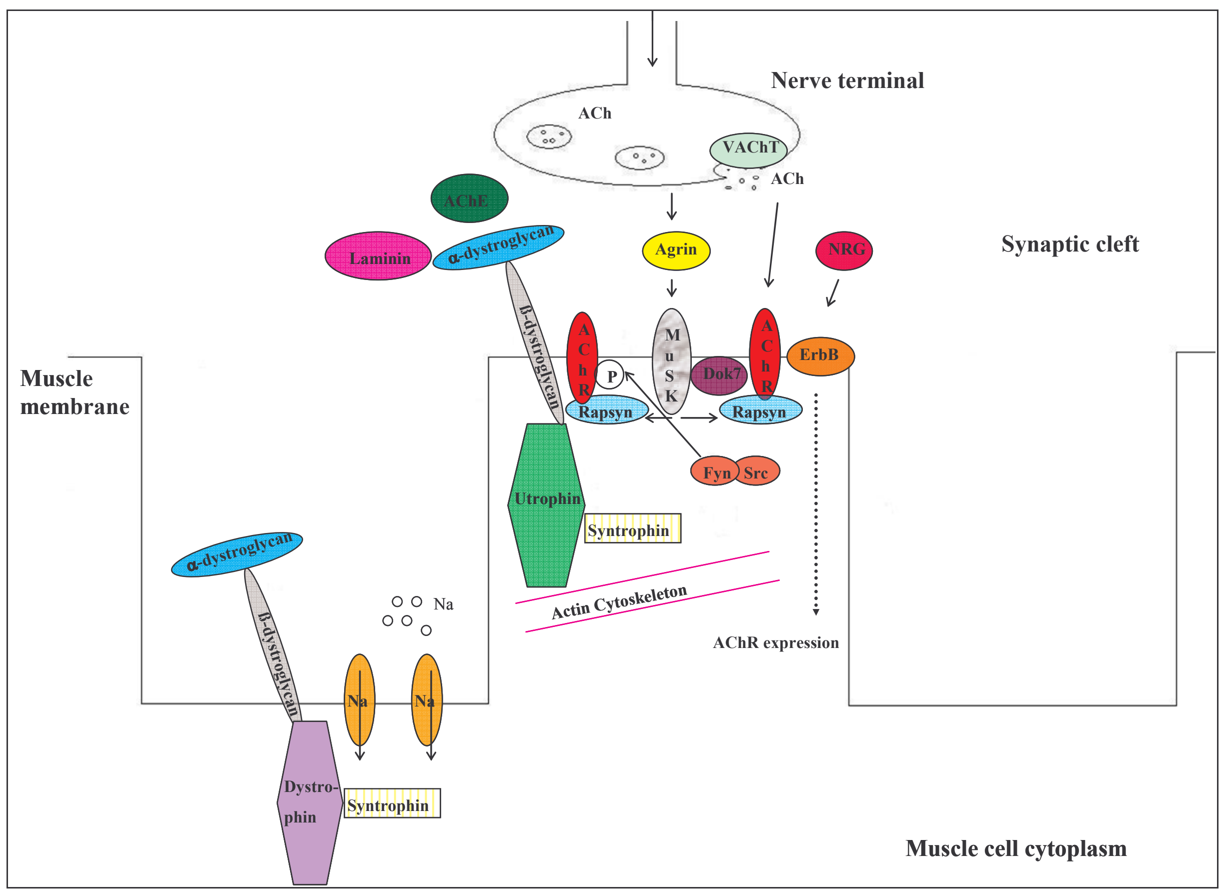

Figure 1: Schematic model of the molecular organization of the neuromuscular junction
Details are discussed throughout the text.

1.2 Clustering of the AChRs at the NMJ

From a functional point of view, the AChR is the most important protein at the NMJ. This glycoprotein spans the postsynaptic membrane and consists of five homologous subunits, present in the stoichiometry $\alpha_2\beta\gamma\delta$. In adult muscle, the embryonic γ-subunit is replaced by the homologous ε-subunit [1, 9, 10]. The molecular weights of the subunits and other NMJ proteins are summarized in appendix 1. Clustering of these AChRs in the postsynaptic membrane is a well regulated process that is critical for efficient synaptic transmission. The formation of AChR clusters is an early event in postsynaptic differentiation during neuromuscular synaptogenesis [5]. An important role in the synaptogenesis is played by agrin, a protein released by the presynaptic motor nerve terminal into the synaptic cleft, and its downstream signalling pathways [4, 11]. Mice deficient of neural agrin lack differentiated synapses and nerve-associated clusters of postsynaptic proteins [12]. During NMJ formation, agrin activates the receptor muscle-specific tyrosine kinase (MuSK), which is located in the postsynaptic membrane [4, 11]. In the absence of MuSK, mice lack differentiated NMJs, clusters of AChRs and other postsynaptic proteins, and thus they show phenotypic similarities to mice lacking agrin [13]. In addition to agrin, there is another component that can achieve MuSK activation and trigger postsynaptic specializations at the NMJ. This protein, Dok-7, is a member of the Dok family of cytoplasmic proteins and can induce clustering of AChR via activation of MuSK [11, 14]. Mice lacking Dok-7 neither form AChR clusters nor neuromuscular synapses [11]. Thus, Dok-7 is essential for neuromuscular synaptogenesis through its interaction

with MuSK. The downstream pathway of agrin/MuSK signalling leading to AChR clustering includes the receptor-aggregating protein of the synapse (rapsyn) as a central element [15]. Rapsyn, a 43kD protein, is present at the cytoplasmic surface of the postsynaptic membrane from the initial stages of synapse formation and is co-localized precisely with AChRs [1, 16, 17]. Rapsyn plays many important roles in neuromuscular synapse formation, including AChRs clustering [16, 17], sequestration of AChR to the postsynaptic apparatus and linking AChRs to the subsynaptic cytoskeleton [16]. Rapsyn deficient mice fail to cluster AChRs and other postsynaptic proteins such as utrophin, dystroglycan and ErbB receptors and lack differentiated NMJs [15]. Mutations in rapsyn can cause congenital myasthenic syndrome in humans and gradual loss of synaptic AChRs [18]. These studies suggest that rapsyn is essential for anchoring AChR clusters to the subsynaptic cytoskeleton. Via the MuSK/rapsyn pathway, agrin also induces clustering of many other postsynaptic proteins, leading to aggregates of these proteins with AChRs.

Clustering of AChRs is also induced by components of the extracellular matrix, in particular laminin-1 and laminin-2/4 (merosin). These forms of laminin are all expressed in the muscle during NMJ formation and play an important role in AChR clustering during NMJ development [5, 19]. An important characteristic of laminins is to make extracellular matrices by forming an organized network with other proteins, and, thus, stabilizing cells by interaction with cytoskeleton-linked surface receptors. Whereas agrin promotes AChR cluster formation primarily through a MuSK-dependent mechanism, laminin induces AChR clustering via a dystroglycan-dependent mechanism [20, 21]. However, intracellularly, the laminin pathway requires tyrosine kinases downstream of MuSK as well as rapsyn to cluster AChRs in myotubes. Thus, the signalling mechanism of laminin overlaps with the agrin pathway [19].

In addition to these post-translational pathways for postsynaptic differentiation, the high synaptic density of NMJ components, particularly of the AChR, is also achieved through a specific upregulation in the transcription of their genes in subsynaptic nuclei. This activity originates at least in part from neuregulin-1 (NRG-1), a protein expressed by motorneurons and skeletal muscle and which is concentrated at the NMJ [22, 23]. NRG-1 binds and activates ErbB receptor tyrosine kinases which are expressed in muscle tissue and are concentrated at the NMJ. Activation of these receptors by NRG-1 is thought to stimulate signalling pathways that activate transcription of certain genes of NMJ components [24, 25]. In mice lacking one copy of the NRG-1 gene, the density of AChRs at the postsynaptic membrane is reduced and the mice are myasthenic, showing that NRG-1 is indeed required to maintain a high receptor density *in vivo* [26]. Interestingly, agrin and NRG-1 signalling pathways, both contributing to the high synaptic density of AChRs at the NMJ, may be linked. Agrin acts as a master organizer of the NMJ by activating its own MuSK-based signalling pathway and by organizing, through aggregation, the NRG-1 pathway. Through multiple levels of cross-talk, both the agrin and NRG-1 signals appear important to lead to full differentiation of the NMJ, including synaptic transcription of many of its components [5].

1.3 Stabilization of AChR clusters and postsynaptic maturation

Neuromuscular synaptogenesis does not only involve initial formation of the NMJ during embryogenesis but also synaptic maturation and stabilization. Since proteins at the NMJ degrade while the synapse itself remains stable, signalling mechanisms that maintain the appropriate number and distribution of synaptic proteins must function throughout the life of a synapse. The molecular mechanisms mediating the postnatal NMJ stabilization differ from those involved in NMJ formation, and much less in known about them [5, 22, 27]. MuSK is again required [28] and antibodies against MuSK occur in patients with myasthenia gravis [29], implying that MuSK function keeps NMJs intact. However, some of the pathways for NMJ and AChR cluster maintenance and maturation may not be essential in initial NMJ formation. In mice lacking components of the dystrophin/utrophin associated glycoprotein complex (DAP), containing next to utrophin and dystrophin also sarcoglycans, dystrobrevin, syntrophins and α-, and β-dystroglycan, NMJs form but fail to mature properly. The components of the DAP link the muscle cytoskeleton to the extracellular matrix and play a role in stabilization of AChRs in clusters, formation of postjunctional folds and postnatal maturation of the postsynaptic membrane [5, 16]. The proteins utrophin and dystrophin are the largest DAP components and play an important role in the maintenance of muscle fiber integrity, but they are not required for NMJ formation [5, 30]. Dystrophin resides on the cytoplasmic side of the plasma membrane and serves as a molecular link between the subsarcolemmal actin-cytoskeleton and the surrounding basal lamina, providing mechanical stability to the cells [16, 31]. Whereas dystrophin is co-localized with sodium channels in the troughs of junctional folds, utrophin is co-localized with AChR at the crests of postsynaptic junctional folds [1, 16]. Utrophin links the DAP complex to actin filaments at the region of high AChR concentration in the neuromuscular synapses [16, 32]. In the NMJ of utrophin mutant mice, dystrophin, β-dystroglycan, dystrobrevin, rapsyn and syntrophin are all present, suggesting that utrophin is not essential for localizing these proteins to the neuromuscular synapse. However, utrophin appears to be important for maturation of the tertiary structure of the postsynaptic apparatus at the NMJ [30]. Mice deficient for both dystrophin and utrophin develop a muscular dystrophy resembling human Duchenne muscular dystrophy (DMD) [33]. Thus, the DAP complex may not be required for initial formation of the NMJ and AChR clusters, but it is critical for their maturation [5].

A more important role in consolidation and maintenance of AChR clusters and the NMJ is played by the dystroglycans. Dystroglycans maintain muscle structure and serve signalling functions. α-Dystroglycan, found extracellularly, binds laminin in the extracellular matrix and binds to β-dystroglycan, which spans the muscle membrane [1, 20]. Rapsyn serves to anchor β-dystroglycan to the NMJ. β-dystroglycan binds to intracellular utrophin at sites of AChR clustering or to dystrophin at extrasynaptic regions of the sarcolemma [1, 16]. Thus, dystroglycans provide a molecular link between the muscle basal lamina and the intracellular cytoskeleton [16]. Jacobson *et al* found that dystroglycans are necessary for the condensation of AChR clusters and stabilization of AChRs in the plasma membrane, as well as for assembling normal synaptic components [20].

Finally, syntrophins might play an important role in the stabilization of AChRs at the NMJ. Syntrophins are a family of cytoplasmic proteins that are associated with dystrophin, utrophin and dystrobrevin and that have signalling and structural roles at the NMJ. Skeletal muscles express

different homologous isoforms of syntrophin [16, 32], but α-syntrophin is the major isoform in adult muscle. It co-localizes with dystrophin at the troughs of the junctional folds, and with utrophin at the crests [32].

Several studies have shown that besides the DAP proteins, stabilization of AChR clusters also requires tyrosine kinases. First, the kinase inhibitors herbimycin and staurosporine disperse preformed AChR clusters from myotubes [34], showing that tyrosine kinase activity is necessary to maintain AChR clusters. Second, although not required for AChR clustering, Src-family kinases (SFKs) are necessary for stabilization of AChR clusters. The SFKs Src, Fyn, and Yes are expressed in numerous cell types, including neurons and muscle. Smith *et al* showed that Src and Fyn deficient mice all show normal muscle development, motor axon pathfinding, clustering of postsynaptic proteins and synapse-specific transcription. However, agrin-induced AChR clusters are less stable in cultured myotubes lacking Src and Fyn, demonstrating that the stability of AChR clusters depends on Src and Fyn [35]. Sadasivam *et al* found that Src and Fyn maintain clusters of rapsyn and DAP components, they maintain AChR-rapsyn interactions and AChR β-phosphorylations, they mediate AChR-cytoskeletal linkage and they control rapsyn protein levels [36]. Taken together, these studies show that tyrosine kinases are essential to keep the postsynaptic apparatus of the NMJ intact and reveal that complex signalling pathways underlie stabilization. It remains to be investigated how these kinases act in this process.

1.4 Pathology of the NMJ

Neuromuscular transmission in humans can be compromised by pathology at the presynaptic regions, at the synaptic cleft, and at the postsynaptic regions of the NMJ. A short summary of both genetic and acquired disorders that alter the NMJ architecture and function is given here.

1.4.1 Congenital myasthenic syndromes

Congenital myasthenic syndromes (CMS) are a heterogenous group of genetic disorders that impair the neuromuscular transmission. Causal defects in presynaptic nerve terminal, synaptic cleft and postsynaptic apparatus have been identified [37]. The presynaptic CMS are caused by mutations in the CHAT gene, coding for the choline acetyltransferase (ChAT) protein. ChAT catalyzes the reversible synthesis of ACh from acetyl-CoA and choline at cholinergic synapses. Mutations in the ChAT can cause defects in ACh resynthesis and therefore, impaired neuromuscular transmission [38, 39]. Synaptic CMS can be caused by mutations in the collagenic tail subunit (ColQ) of AChE, which leads to an absence of AChE from the synaptic cleft. AChE is the enzyme responsible for the rapid hydrolysis of ACh released at cholinergic synapses [37, 40]. Neuromuscular transmission is impaired by desensitization of AChR by prolonged exposure to ACh and by an endplate myopathy caused by cationic overloading of the postsynaptic region [41]. The most common CMS is a postsynaptic reduction in the number of AChRs. Most postsynaptic CMS are caused by mutations in subunits of the AChR. Mutations have now been discovered in all AChR subunits and in different domains of the subunits, the most common of which is a mutation in the ε subunit [42]. The mutations can be divided in two major groups, namely those that alter the kinetic properties and those that decrease the expression of AChR. The kinetic mutations divide further in slow-channel

mutations that increase, and fast-channel mutations that decrease the synaptic response to ACh [40]. In a subset of CMS patients without mutations in AChR, endplate AChR deficiency is caused by mutations in rapsyn, the protein that plays a critical rol in concentrating AChRs in the postsynaptic membrane [18]. Recently, Beeson *et al* demonstrated that recessive inheritance of mutations in Dok-7 affect the size and structure of the NMJ and can thus underlie a CMS [14].

1.4.2 Muscular dystrophies

Muscular dystrophies are a group of genetic disorders characterised by progressive muscle degeneration and weakness. Mutations in various muscle genes have been shown to be responsible for such disorders. DMD is inherited through an X-chromosome-linked transmission and involves mutations in the DMD gene, which regulates the expression of dystrophin [43]. The absence of dystrophin results in the disruption of the linkage between the sarcolemal cytoskeleton and the glycoprotein complex in muscle of patients with DMD. This, in turn, may render the sarcolemma susceptible to damage from muscle contraction and thus lead to muscle cell necrosis. In addition, the absence of dystrophin can lead to a reduction in all of the dystrophin-associated proteins in the sarcolemma [31].

The limb girdle muscular dystrophies (LGMD) are another important subgroup of muscular dystrophies. The LGMD autosomal disorder, characterised by predominant effects on the proximal limb muscles, can result form mutations in at least 19 different genes. The proteins involved are very diverse and include sarcomeric, sarcolemmal and enzymatic proteins. The mutations of several LGMD deficient proteins can lead to very different phenotypes [44].

1.4.3 Acquired neuromuscular transmission disorders

Myasthenia gravis (MG) is an autoimmune disorder of the NMJ characterized by muscle weakness. In approximately 85% of MG patients, pathogenic auto-antibodies directed against the AChR are present and cause defective neuromuscular transmission [7, 8, 42, 45]. In most cases, the antibody is directed against the main immunogenic region (MIR) on the α subunit of the AChR [8]. The auto-antibodies are thought to lead to changes on the NMJ by three mechanisms. First, there is a muscular weakness in MG as a consequence of a reduced number of functional AChRs on the postsynaptic membrane. Antibodies crosslink with the AChR, resulting in accelerated endocytosis and degradation of the AChRs by muscle cells. A second important mechanism that is responsible for the neuromuscular defect is complement-mediated focal lysis of junctional folds of the postsynaptic membrane. By binding the AChR, antibodies trigger a complement cascade resulting in a focal destruction of the postsynaptic membrane by the membrane attack complex. The damage to the postsynaptic membrane is only partially restored, resulting in a reduced number of postsynaptic folds. Thirdly, antibodies against the ACh-binding site or the ion channel may inhibit AChR function [7, 8, 42]. The structure of the NMJ in a healthy person and a MG patient are shown in figure 2 and 3, respectively.

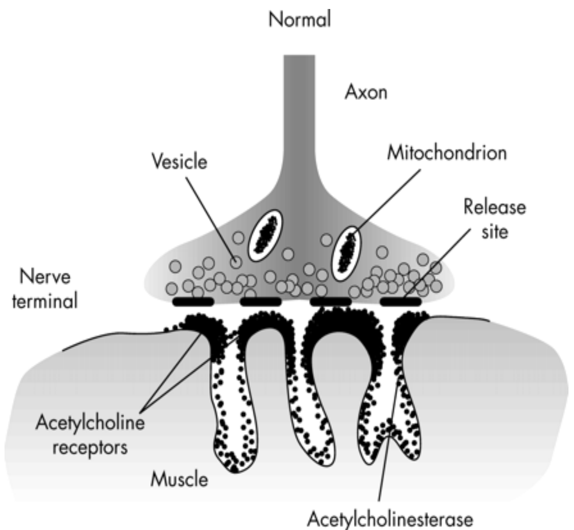

Figure 2: Schema of NMJ
ACh released from the motorneuron terminal binds to AChRs on the postsynaptic membrane, which causes the membrane to depolarise, initiating the muscle action potential [8].

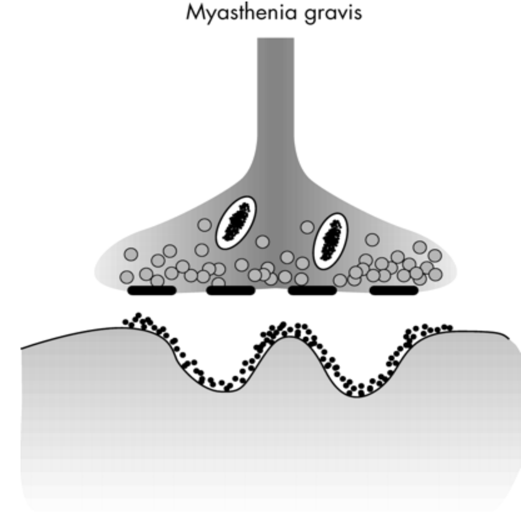

Figure 3: Schema of NMJ in MG
A widened synaptic cleft, a reduced number of AChRs and a simplification of the postsynaptic membrane are depicted [8].

Further, Hoch *et al* have described MuSK antibodies in a subgroup of MG patients who do not have antibodies against the AChR [29]. As described, MuSK is one of the proteins involved in anchoring and clustering of AChRs at the postsynaptic membrane. It is no surprise that interfering with these processes by antibodies can impair transmission at the NMJ. Finally, antibodies that target other components of the NMJ, but that are not detectable by the currently available tests may be responsible for the remaining cases of myasthenia gravis.
Secondly, the Lambert-Eaton myasthenic syndrome (LEMS) is an acquired condition resulting from auto-antibodies directed against voltage-gated calcium channels (VGCC) at the motor nerve terminal. Binding of antibodies to the VGCCs results in a decrease in nerve-evoked calcium ion entry and a reduction in neurotransmitter release, leading to transmission failure and thus muscle weakness [1, 46, 47].
Finally, acquired neuromyotonia, also known as Isaac's syndrome, is another autoimmune disorder, frequently caused by antibodies to the voltage-gated potassium channels present on the presynaptic nerve terminals. This disorder can co-exist with neuropathies or with myasthenia gravis, and muscle weakness can be the major compliant [42, 48].

1.5 Study outline

During this project, the proteins of the NMJ were analysed. As mentioned before, a proportion of MG patients neither have detectable antibodies against the AChR nor to MuSK. A first reason may be that these patients express a modification in the AChR or MuSK protein. Therefore, auto-antibodies will develop against these foreign proteins. Because of the modification in the antigen, these antibodies are not detectable by the currently available laboratory techniques. Second, an unknown antigen can be the target for auto-antibodies in these seronegative MG patients. Probably, the myasthenic symptoms can be caused by auto-antibodies interfering with a protein that plays an important role in the development or stabilization of the NMJ. Proteins that are

associated with the AChR are suspected to play an important role in the maintenance of the NMJ, and, therefore, they can be a candidate for the unknown target of the antibodies. Our goal was to characterize AChR associated proteins that can be a candidate for the unknown auto-antigen. To identify AChR associated proteins of the NMJ, proteomic techniques were performed. First, the AChRs were isolated and precipitated using α-bungarotoxin (BT). Proteins that are associated with the AChR will be coprecipitated together with the receptor. Then, one-dimensional gel electrophoresis (1-DE) was performed followed by silver staining. To identify the precipitated proteins, immunoblotting experiments and mass spectrometry analyses were performed. By using immunoblotting, only the known proteins can be detected when antibodies are available. Additionally, to characterize the unknown AChR associated proteins, mass spectrometry analyses were performed. Since the precipitaton technique was not used before in our lab, it first had to be optimized. To achieve this, the TE671 cell line was used as an alternative to the muscle and potentially more abundant source of AChR. Mainly the optimization steps will be discussed throughout this report.

2 Materials and methods

2.1 Cell culture

TE671 cells were maintained in 162 cm^2 flasks in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Invitrogen, Belgium) with L-glutamin and 25 mM Hepes, supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate (Gibco) and 0.02% dexamethasone. The cell lines were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2 and were split once a week. Harvesting cells was performed once a week. First, the medium was removed and the cells were washed with phosphate buffered saline (PBS). Subsequently, cells were scraped in PBS, and washed with PBS supplemented with 1.85% iodine acetate and 0.001% 0.1M phenylmethanesulphonylfluoride (PMSF). Following centrifugation, cell pellets were stored at -80°C.

2.2 Antibodies

Different antibodies were used to detect the presence of neuromuscular proteins in the TE671 cell line. To detect the ErbB2, ErbB3 and ErbB4 proteins, respectively rabbit anti-ErbB2 antibody (C-18) (Santa Cruz Biotechnology Inc., CA, USA), rabbit anti-ErbB3 antibody (C-17) (Santa Cruz Biotechnology Inc.) and rabbit anti-ErbB4 antibody (C-18) (Santa Cruz Biotechnology Inc.) were used. Antibodies against the α-AChR (monoclonal antibody (mAb) 155), α-AChR main immunogenic region (MIR) (mAb 198), β-AChR (mAb 148), δ-AChR (mAb 141) and ε-AChR (mAb 154 and mAb 168) were a kind gift of Dr. SJ Tzartos (Hellenic Pasteur Institute, Greece). To detect the MuSK protein, a rabbit anti-MuSK antibody (PA1-1740, Affinity Bioreagents, Golden, USA) was used. Mouse anti-s-laminin IgG1 mAb was purchased form the Developmental Studies Hybridoma Bank (University of Iowa, IA, US). Mouse anti-utrophin mAb MANCHO7 is kindly donated by Prof. G.E. Morris (North East Wales Institute, Wrexham, UK). A mouse monoclonal anti-syntrophin antibody (SYN1351) against syntrophin was purchased from Novus Biologicals, Littleton, CO, USA. To detect rapsyn, a monoclonal mouse anti-rapsyn antibody (clone 1234, Affinity Bioreagents) was used. Additionally, the goat anti-human rapsyn K-16 was purchased form Santa Cruz, Biotechnology Inc. The anti-Dok7 (H-77) antibody was purchased from Santa Cruz Biotechnology Inc. Finally, dystrophin was detected by mouse anti-dystrophin antibody (clone 19 8F6), purchased from the Developmental Studies Hybridoma bank.

2.3 Radioimmunoassay

To measure the amount of AChR present in different TE671 cell extracts, a radioimmunoassay (RIA) was performed as described by Lindstrom *et al* and by Losen [49, 50]. Shortly, TE671 cells from one 162 cm^2 flask were lysed with 1 ml lysis buffer (50 mM NaCl, 30 mM triethanolamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 2 mM Na-orthovanadate, 10 mM *p*-nitrophenylphosphate, 50 µM phenlyarsine-oxide, 1 mM benzamidine, 1 mM *N*-ethylmaleimide, 1 mM Na-tetrathionate, 25 µg/ml aprotinin and 25 µg/ml leupeptin). Different detergents (NP-40, cholate, brij-97, triton-X100, digitonin, chaps, and saponina) were added to the lysis buffer. Following cell lysis, the insoluble material, such as nuclei and cytoskeletal elements, was removed by centrifugation at 14000 rpm for 5 min. Then, AChRs were radiolabelled with ^{125}I-α-BT (Amersham Pharmacia Biotech Benelux, the Netherlands) and incubated with human IgG1 anti-

human receptor antibody (IgG1-637) (1mg/ml) overnight at 4°C. The IgG1-637 antibody was produced by Stassen *et al* [51]. Samples were then incubated with goat-anti-human Ig (polyclonal serum) for 4 h at 4°C. Following two washing steps with 0.5% Triton-X100 in PBS, radio-activity was measured using a γ-counter.

2.4 Isolation of AChR using α-bungarotoxin

To examine association of postsynaptic proteins with the AChR, AChRs were isolated using α-BT as described by Fuhrer *et al* [52] with the following modifications. TE671 cells from one 162 cm^2 flask were lysed with 1 ml lysis buffer (50 mM NaCl, 30 mM triethanolamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 2 mM Na-orthovanadate, 10 mM *p*-nitrophenylphosphate, 50 µM phenlyarsine-oxide, 1 mM benzamidine, 1 mM *N*-ethylmaleimide, 1 mM Na-tetrathionate, 25 µg/ml aprotinin and 25 µg/ml leupeptin). Different detergents (NP-40, cholate, brij-97, triton-X100, digitonin, chaps, and saponina) were added to the lysis buffer. Following cell lysis, the insoluble material was removed by centrifugation at 14000 rpm for 5 min. The protein concentration was measured (Biorad, California, USA) by reference to a calibration line obtained with BSA as a standard. The cell lysates containing 1000 µg protein were pre-cleared by adding 50 µl of pre-washed streptavidin-agarose beads (Molecular Probes, Invitrogen, Leek, The Netherlands). Proteins that bind aspecifically to the streptavidin-coupled agarose beads were removed by centrifugation at 6000 rpm for 2 min. To precipitate the AChR, the cleared cell lysates were incubated with biotin-conjugated α-BT (1mg/ml) (Molecular Probes) for 1h on rotating device. As negative controls, free α-BT was added. Free α-BT can not bind the streptavidin on the beads, and therefore, the immunecomplexes will not be precipitated. Immunecomplexes were precipitated with pre-washed streptavidin-coupled agarose beads for 1 h with constant rotation. Next, immunoprecipitates were collected by centrifugation. All proteins that did not bind the α-BT remained in the supernatant and were also used for further analysis. Beads were washed twice with a wash buffer 1 (0.4% detergent, 470 mM NaCl, 30 mM triethanolamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 2 mM Na-orthovanadate, 10 mM p-nitrophenylphosphate, 50 µM phenylarsine-oxide, 50 mM NaF) and twice with wash buffer 2 (same as 1 but containing 120 mM NaCl). Following the washing steps, the pellet was resuspended in SDS loading buffer (5% v/v 1M Tris-HCl, 3% v/v glycerol, 6% w/v SDS, 6% v/v β-mercaptoethanol, and a bit bromophenolblue). Finally, the samples were boiled to dissociate the immunecomplexes from the beads. The beads were collected by centrifugation and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed with the supernatant.

2.5 Isolation of AChR using antibodies

To examine association of postsynaptic proteins with the AChR, AChRs were isolated using an anti-AChR antibody. TE671 cell pellets were lysed with 1 ml lysis buffer (detergent, 50 mM NaCl, 30 mM triethanolamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 2 mM Na-orthovanadate, 10 mM *p*-nitrophenylphosphate, 50 µM phenlyarsine-oxide, 1 mM benzamidine, 1 mM *N*-ethylmaleimide, 1 mM Na-tetrathionate, 25 µg/ml aprotinin and 25 µg/ml leupeptin). The detergents cholate, NP-40 or brij-97 were added to the lysis buffer. Following cell lysis, the insoluble material was removed by centrifugation at 14000 rpm for 5 min. The protein concentration was measured (Biorad, California,

USA) by reference to a calibration line obtained with BSA as a standard. The cell lysates containing 1000 µg protein were incubated with 20 µl of IgG637 (1mg/mg), anti-human antibody binding the α-subunit of the AChR [51], and 10 µg of IVIg coprecipitant overnight at 4°C with constant rotation. Next day, 100 µl goat anti-human Ig secondary antibody (polyclonal serum) was added for 4 hours on rotator device. Next, immunoprecipitates were collected by centrifugation. The supernatant, containing the unbound proteins, was also used for further analysis. Immunecomplexes were then washed twice with a wash buffer 1 (0.4% detergent, 470 mM NaCl, 30 mM triethanolamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 2 mM Na-orthovanadate, 10 mM p-nitrophenylphosphate, 50 µM phenylarsine-oxide, 50 mM NaF) and twice with wash buffer 2 (same as 1 but containing 120 mM NaCl). Following the washing steps, the pellet was resuspended in SDS loading buffer (5% v/v 1M Tris-HCl, 3% v/v glycerol, 6% w/v SDS, 6% v/v β-mercaptoethanol, and a bit bromophenolblue). Finally, the samples were boiled to dissociate the immunocomplexes and a SDS-PAGE was performed.

2.6 Western blot

For Western Blot analysis, the cell lysates and the precipitated samples were subjected to electrophoresis on a 10% SDS/PAGE. Proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, USA) using an electroblotting apparatus. After blocking with 1:1 Odyssey blocking buffer / PBS (OBB/PBS)(LiCor Biosciences, Lincoln, USA), the membrane was cut into pieces and incubated with different primary antibodies to detect different proteins. The protein blots were probed with antibodies diluted 1:1000 in OBB/PBS. After several washes, the membrane was incubated with 1:10000 diluted IGDye 800 goat anti-rabbit (Rockland Incorporated) or IGDye 700 goat anti-mouse (Molecular Probes). Following washes, the antigen-antibody complexes were detected by the enhanced chemiluminescence system (LiCor Scanner, Westburg). The molecular masses of proteins were estimated relatively to the electrophoretic mobility of cotransferred prestained molecular mass markers (LiCor Biosciences).

2.7 Staining

To visualize protein bands on the gel, a silver staining was performed according the method described by Shevchenko *et al* [53]. Gels were fixed for 1 h in a solution containing 50% methanol and 5% acetic acid, followed by incubations for 10 min both in 50% methanol and in water. Then, gels were incubated in 0.02% sodiumthiosulfate for 3 min. Following two washing steps in water for 1 min, gels were incubated in 0.1% silver nitrate for 20 min. Next, gels were washed twice in water for 1 min and gels were developed in 2% sodium carbonate containing 0.04% formaldehyde. Development was stopped with 5% acetic acid. Gels were rinsed with water and scanned with a HP Scan Jet 6300 scanner.

2.8 Digestion

Gel bands were cut and digested manually as described by Shevchenko *et al* [53]. Briefly, bands were dehydrated in 50% acetonitrile, dried and kept at -20°C until further analysis. Following reduction during 1 h at 56°C in a solution containing 10 mM DTT in 100 mM NH_4HCO_3, bands were

incubated in an alkylation solution (55 mM iodoacetamide in 100 mM NH_4HCO_3) during 45 min in the dark at RT. Two steps of consecutive washing in 100 mM NH_4HCO_3 and dehydration in acetonitrile were performed. Following the washing steps, bands were dried by *in vacuo* centrifugation. Incubation in 100 mM NH_4HCO_3 containing trypsin (12.5 ng/µl, Promega, Leiden, The Netherlands) for 45 min on ice was followed by an overnight incubation at 37°C in 50 mM NH_4HCO_3. Supernatant was removed and collected from this point onwards. Bands were washed with 20 mM NH_4HCO_3 and proteins were extracted in three consecutive steps using 5% formic acid in 50% acetonitrile. The pooled supernatant was dried by *in vacuo* centrifugation. The pellet was dissolved in 5% acetonitrile in 100 mM acetic acid containing 4 pg/µl of cortisone. Finally, the pellet was stored at -20°C until mass spectrometric analysis was performed.

2.9 Mass spectrometry

Analysis of the protein digests was performed with an electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a LCQ Classic (ThermoFinnigan, San Jose, USA) equipped with a nano-LC column switching system. The trapped sample was separated on a reversed phase-high performance liquid chromatography analytical column (Biosphere C18, 200mm x 0.05mm I.D.m Nanoseparations, Nieuwkoop, The Netherlands) using a linear gradient for 5 to 60% v/v acetonitrile in water containing 100 mM acetic acid in 120 min. Then, the eluate from the analytical column was introduced in a nanoelectrospray device (ThermoFinnigan) and sprayed from a gold-coated fused silica emitter (5µM I.D.m Nanoseparations). To optimize the ion optics and the calibration parameters, the LCQ automated protocols were used. The mass spectrometer was operated in a data-dependent acquisition mode to automatically switch between MS (m/z 300-1500 Thopson in centroid mode at a maximum injection time of 150 ms) and MS/MS acquisition on the three most intense precursor ions, controlled by Xcalibur 1.3 sofware. To monitor the flow stability and overall performance of the ion trap mass spectrometer, cortisone served as an internal analytical standard. Peptide masses were screened against the GenBank human-specified protein database using Mascot (Matrix Sciences, London, UK) and Sequest (ThermoFinnigan Electron Corporation). Sequest compared the found MS/MS spectra with the theoretical MS/MS spectra in a database and it determined if the spectra were matching. To confirm the results, the data of the MS/MS spectra could also be analysed by Mascot. This computer program is using other algorithms, but has the same goal as Sequest.

3 Results

3.1 Presence of NMJ proteins in TE671 cells

The aim of this project was to isolate the AChR out of the TE671 cell membrane together with associated proteins. To achieve this, precipitation experiments were performed and the precipitates were analysed by immunological methods. First, the presence of some NMJ proteins in the total cell extract before precipitation was tested by immunoblotting experiments. A table describing the molecular weights and locations of proteins in the NMJ can be found in appendix 1.

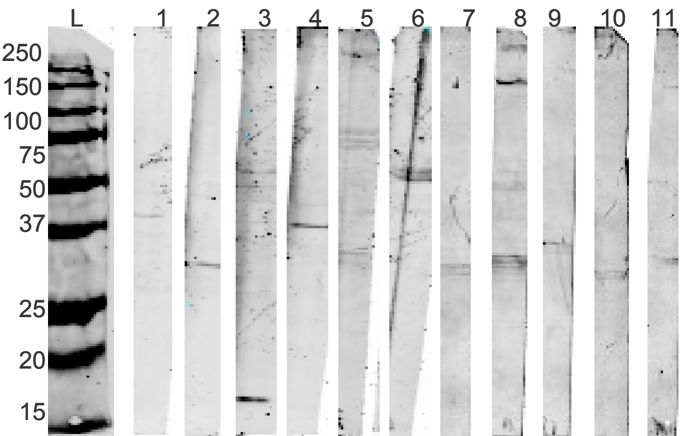

Figure 4: Presence of NMJ proteins in the TE671 cells

TE671 cells were lysed with lysis buffer containing 0.5% cholate for 3 hours. Cell extracts were subjected to electrophoresis on 10% SDS gels. Following transfer to a PVDF membrane, the membrane was cut into pieces to test the presence of NMJ proteins: ε-AChR (1, 4), δ-AChR (2), β-AChR (3), S-laminin (5), Syntrophin (6), MuSK (7), ErbB2 (8), Dok-7 (9), Rapsyn (10) and ErbB3 (11). The ladder (L) indicates the molecular marker. The membranes were incubated with primary antibody, as listed in the materials and methods section. Following incubation with secondary antibody, 1:10000 diluted, the scan was made with the Odyssey scanner.

Table 1: Expected and detected size of NMJ proteins by immunoblotting

Protein	Expected size (kDa)	Detected size (kDa)
α-AChR	40	/
β-AChR	50	17
ε-AChR	60	40
δ-AChR	65	30
S-laminin	190	200 and 75
Syntrophin	58	55
Rapsyn	43	30
MuSK	97	30
ErbB2	185	150 and 30
ErbB3	180	35
Dok-7	53	35
Utrophin	400	/
Dystrophin	430	/

In this experiment, the presence of the AChR subunits, S-laminin, syntrophin, utrophin, MuSK, ErbB, dystrophin, Dok-7 and rapsyn was tested. The expected and detected molecular weights of all tested proteins are listed in table 1. In figure 4, protein bands were detected when using the antibodies against the ε, β and δ subunits of the AChR (lane 1 to 4). However, these bands were much smaller than the molecular weights of these proteins, especially in the case of the β subunit. Then, using the anti-S-laminin antibody, a band of approximately 200 kDa was detected, in addition to some smaller, probably unspecific bands (lane 5). Further, a 55 kDa band was seen when blotting with the antibody against the syntrophin protein (lane 6). Although Musk has a size of approximately 100 kDa, a band with a size of 30 kDa was visible here (lane 7). Then, the ErbB2 antibody detected a 150 kDa band, in addition to some unspecific 30 kDa bands (lane8). When analysing the presence of Dok7, a 35 kDa band was detected (lane 9) To test the presence of rapsyn, two different antibodies were used, mouse anti-rapsyn and goat anti- rapsyn K-16, described in materials and methods. The first antibody gave rise to a 30 kDa band (lane 10), whereas no band was detected by the second antibody (data not shown). Finally, some bands were detected by the anti-ErbB3 antibody (lane 11). However, a 180 kDa band was not seen in this immunoblotting experiment. Since no bands were seen when blotting with antibodies against utrophin, dystrophin, and the α-subunits of the AChR, these results are not shown.

3.2 Solubilization of AChR

To isolate the AChR out of the cell membrane in an efficient way, a suitable detergent had to be selected. To test the efficiency of different detergents in solubilizing the AChR, a radioimmunoassay was performed using ^{125}I-α-BT. Seven detergents were tested in three different concentrations to solubilize the AChR.

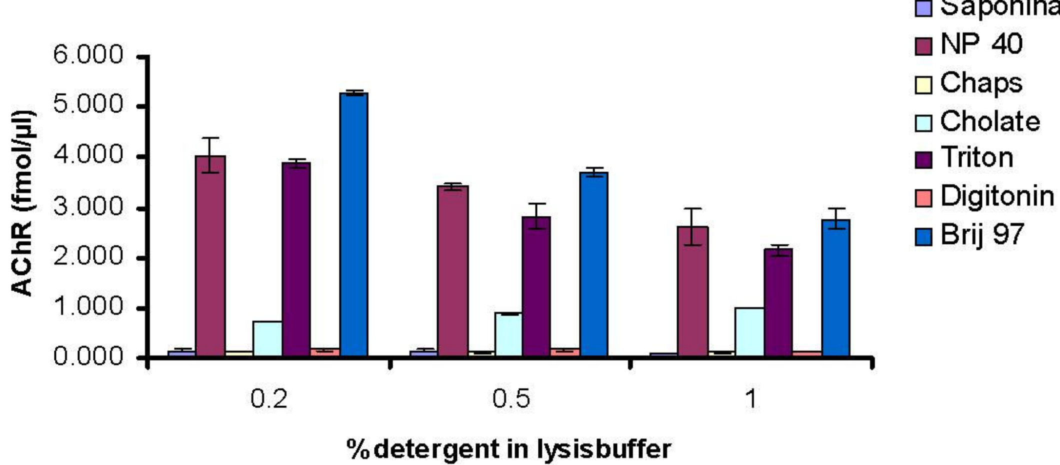

Figure 5: Solubilization of AChR by different detergents

TE671 cells were lysed during one hour with lysis buffer containing 0.2, 0.5 or 1% of different detergents. The AChR concentration solubilised during the lysis was measured by RIA using ^{125}I-labelled α-BT, as described in the materials and methods section.

Figure 5 shows that different amounts of AChR were solubilised using different detergents. The detergents NP-40, triton and brij-97 solubilised the highest amounts of AChR in each of the concentrations. Cholate solubilised an intermediate amount of AChR, whereas saponina, chaps and digitonin solubilised a very low amount of AChR. When comparing the detergent concentrations in the case of NP-40, triton and brij-97, 0.2% of detergent solubilised the highest amounts of AChR. The raw data of this experiment are listed in appendix 2. A table describing the calculations concerning the RIA and the precipitation experiments can be found in appendix 3.

3.3 Isolation of AChRs using α-BT

To examine the association of AChRs with other proteins, the AChRs were extracted from TE671 cells and precipitated with α-BT-biotin in combination with streptavidin-coupled agarose beads. To assess nonspecific protein binding to the beads, samples were incubated with the beads for half an hour prior to the precipitation. To test whether proteins were bound to the resin specifically through their association with the AChR, free α-BT was used as a negative control. To maintain the protein-protein interactions in a protein complex, many protease inhibitors were added to avoid protein degradation. Following the precipitation reaction, the samples were analysed by silver staining.

To optimize the precipitation experiments, both the amounts of 10 µl and 50 µl of the beads were tested.

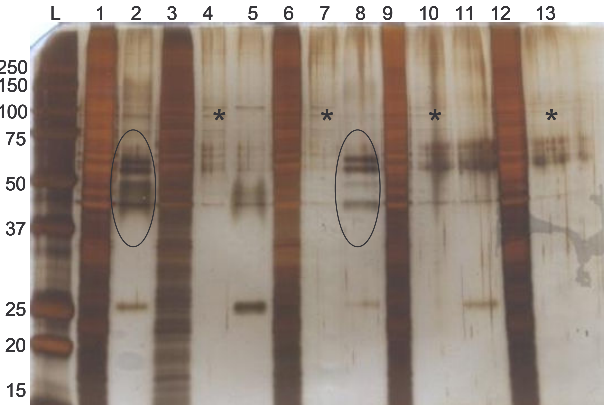

Figure 6: Precipitation of AChRs using α-BT and variable amounts of beads

Cholate extraction was performed on the TE671 cells. Total membrane fraction before precipitation was loaded as a control (lane 1). AChRs were isolated using α-BT-biotin combined with streptavidin-agarose beads (lane 2, 8). As a negative control, free α-BT was added to the cell lysates (lane 5, 11). 50 µl of beads was used in the samples 2 and 5, whereas only 10 µl of beads was added to samples 8 and 11. Additionally, total cell extracts after precipitation, containing all proteins that were not binding the α-BT, were analysed (lane 3, 6, 9, 12). The supernatant of the washing steps was loaded on the gel (lane 4, 7, 10, 13). The molecular marker is indicated by L. Samples were analysed by SDS-PAGE and silver staining as described in the materials and methods section.

As depicted in figure 6, more protein bands appear if 50 µl of beads were used compared to 10 µl of beads (lane 2 versus 8). When comparing the protein amount present in the supernatant of the washing steps, indicated with an asterisk, more proteins were lost when only 10 µl of beads was used (lane 4 and 7 versus lane 10 and 13). In addition, differences in protein bands were present

when α-BT (lane 2, 8) or free α-BT (lane 5, 11) was used to precipitate the AChR. In this experiment, a cholate extraction was performed on the TE671 cells to separate the cytosolic and membrane proteins. Only the membrane proteins were analysed in this precipitation experiment. Because no cytosolic proteins were present in the samples anymore, less unspecific binding to the beads could occur. However, the cholate extracted samples were tested only once to validate the specificity of the precipitation. In further experiments, total cell lysates were used in order to include important cytosolic membrane-associated proteins.

As shown in figure 5, the detergents chaps, digitonin, and saponina were not able to solubilize high amounts of the AChR. However, these detergents were used in some of the precipitation experiments. From this time on, the total cell extracts were used to perform the precipitations. Following the cell lysis, the nonsolubilised proteins and waste material present in the pellet of the centrifugation step, which was performed to separate the solubilised and nonsolubilised proteins, were resuspended in lysis buffer. These primarily nonsolubilised proteins were used as a kind of control sample during these experiments. Performing this extra step, one can analyse if some membrane proteins become solubilised due to a longer incubation in lysis buffer. The precipitation experiments using digitonin, chaps or saponina as detergent all gave comparable results. Following, the results of the detergent digitonin are shown.

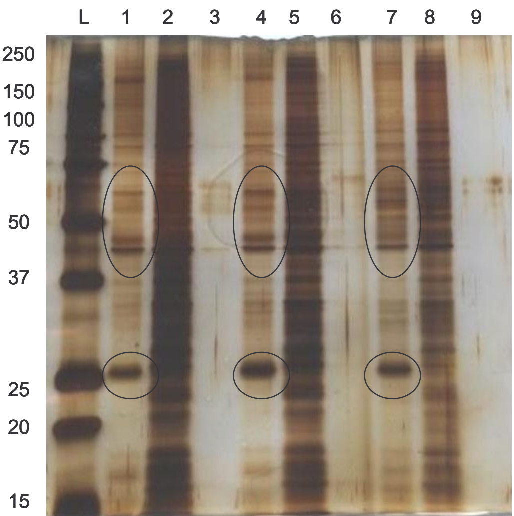

Figure 7: Precipitation of AChRs using α-BT and detergent digitonin

TE671 cells were lysed in a buffer containing 1% digitonin. AChRs were isolated using α-BT-biotin and streptavidin-agarose beads (lane 1, 7). The nonsolubilised material (lane 7-9) was re-incubated in lysis buffer and analysed in the same way as the solubilised proteins. As a negative control, free uncoupled α-BT was added to the cell lysates (lane 4). Total cell lysates after precipitation, containing all proteins that were not binding the α-BT, were analysed (lane 2, 5, 8). Supernatant of the washing steps was also loaded on the gel (lane 3, 6, 9). The molecular marker is indicated by L. Samples were analysed by SDS-PAGE and silver staining as described in the materials and methods section.

In figure 7, no differences were detected when comparing the samples precipitated with α-BT-biotin or with free α-BT (lane 1 versus 4). Since the nonsolubilised proteins also stayed in the lysis buffer during the entire experiment, some proteins became solubilised during the precipitation experiment. Lane 7 shows that many proteins were still present in the nonsolubilised material.

These precipitates showed the same band pattern as the precipitates of the primary solubilised proteins (lane 7 versus 1). Comparable results were seen in the precipitations using the detergents chaps and saponina. Since no differences were detected between the precipitated and the negative control samples, the precipitations probably did not occur in a specific way.

To continue the optimization steps, new precipitations were performed using NP-40 and digitonin to solubilize the proteins. To precipitate the protein complexes, 50 µl of beads was used.

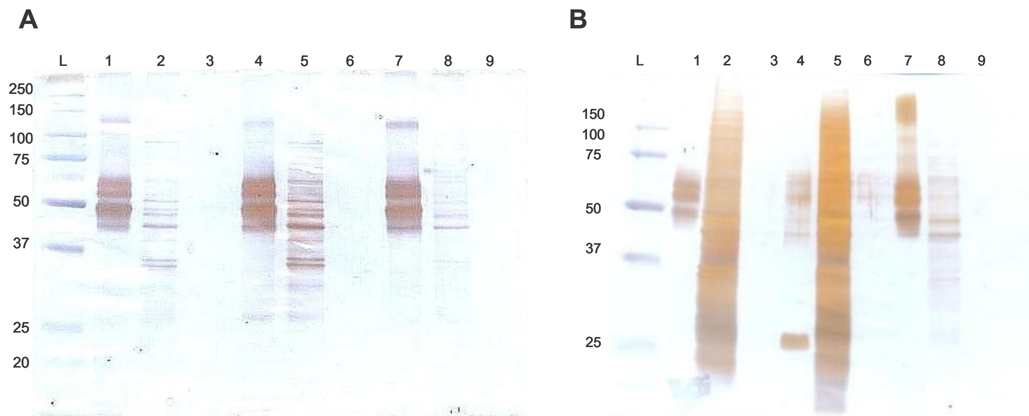

Figure 8: Precipitation of AChRs using α-BT

TE671 cells were lysed in a buffer containing 1% digitonin (A) or 1% NP-40 (B). AChRs were isolated using α-BT-biotin combined with streptavidin-agarose beads (lane A1, A4, A7 and B1, B7). The nonsolubilised material was re-incubated in lysis buffer and analysed in the same way as the solubilised proteins (lane A7-9 and B7-9). As a negative control, free uncoupled α-BT was added to the cell lysates (lane B4). Total cell lysates after precipitation, containing all proteins that were not binding the α-BT, were analysed (lane A2, A5, A8 and B2, B5, B8). Supernatant of the washing steps was also loaded on the gel (lane A3, A6, A9 and B3, B6, B9). The molecular marker is indicated by L. Samples were analysed by SDS-PAGE and silver staining as described in the materials and methods section.

In figure 8A, no negative control sample was taken into account. In the precipitates (lane 1, 4), bands with a molecular weight of about 130 kDa were seen, in addition to many bands with a size between 40 and 60 kDa. The nonsoluble material that was re-incubated in lysis buffer gave rise to the same pattern of protein bands (lane 7). It is not clear why much less proteins are present in the total cell lysate after precipitation, in comparison with the other precipitation experiments. In this experiment, the band pattern was more clear than in previous precipitation using digitonin as detergent. In figure 8B, differences were visible in the precipitates with α-BT-biotin and free α-BT (lane 1 versus 4). These differences could be seen more clearly when comparing the control sample to the precipitate of the nonsoluble material. Both in figure A and B, a 130 kDa band was present more pronounced in the precipitate of the nonsolubilised sample than in the solubilised sample (lane 7 versus 1). This protein probably needs more time to get solubilised, and, therefore, was less detectable after one hour of lysis.

In the following optimization steps of the precipitation experiments, triton, cholate and brij-97 were used to solubilize the proteins. Since previous results showed that many proteins still can be solubilised when the nonsolubilised material is incubated in lysis buffer for a longer period, the incubation time in lysis buffer was variable during the following experiment. Starting the experiment, lysis buffer containing detergent was added to the cell pellets for one hour. Then, the sample was centrifugated at maximum speed, and the supernatant with the solubilised proteins was used to start the first precipitation (lane 2-6 in figure 9). The remaining cell pellet, containing the nonsolubilised material, was resuspended in lysis buffer and incubated for another hour. Again, the centrifugation step was performed and the second precipitation was started using this supernatant (lane 7-10). The pellet was resuspended for a third time in order to start a third precipitation (lane 11-14). The next picture shows the results of the precipitation using brij-97 as detergent.

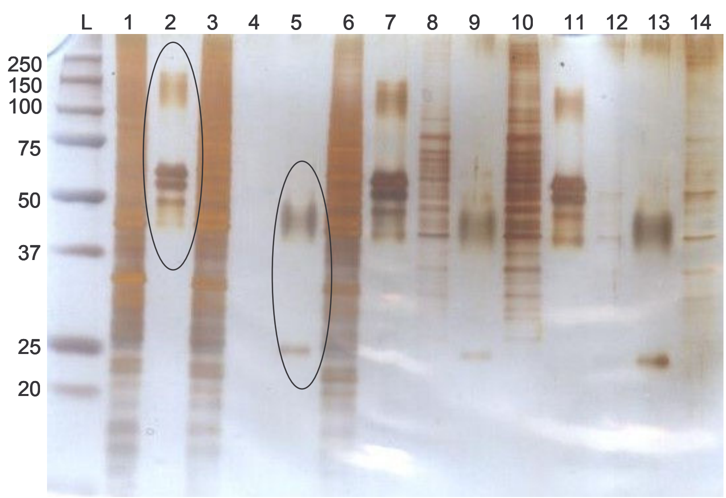

Figure 9: Precipitation of AChRs using α-BT with brij-97 as detergent

TE671 cells were lysed in a buffer containing 0.5% brij-97. Total cell lysate before precipitation was loaded as a control (lane 1). AChRs were isolated using α-BT-biotin combined with streptavidin-agarose beads (lane 2, 7, 11). As a negative control, free uncoupled α-BT was added to the cell lysates (lane 5, 9, 13). Total cell lysates after precipitation, containing all proteins that were not binding the α-BT, were also analysed (lane 3, 6, 8, 10, 12, 14). Supernatant of the washing steps was loaded on the gel once (lane 4). The molecular marker is indicated by L. Samples were analysed by SDS-PAGE and silver staining as described in the materials and methods section.

As can be seen in appendix 1, the AChR subunits α, β, γ/ε and δ have a molecular weight of 40, 50, 60 and 65 kDa respectively and the δ-dimer has a weight of 130 kDa. Bands of these sizes were present in all precipitated samples (lane 2, 7, 11), but they were absent in the control samples (lane 5, 9, 13). Differences were thus detected when comparing the precipitated sample and the negative control, indicated by the circles. These differences were even seen in the samples of the second and third lysis (lane 7 and 11 versus lane 9 and 13). The total cell lysate after precipitation contained much more protein in the samples of the first lysis when compared to the other lysis steps (lane 3 versus 8 and 12). This is due to the fact that only membrane proteins were left in the samples of the second and third lysis, while in the first lysis all cytosolic proteins were still present. Very similar results were seen in the precipitation experiments using cholate or triton as a

detergent, although the differences between precipitated and the negative control sample were less clear in the case of cholate (data not shown).

Finally, an additional experiment was performed to compare the length of the protein bands in this experiment with the bands of a *Torpedo californica* sample. The AChRs were purified from the *Torpedo californica* electric organ, which is very similar to the mammalian NMJ, as described by Patrick and Lindstrom [54]. Then, both the AChRs of the *Torpedo californica* and the precipitated sample of the TE671 cell line were loaded on the same gel.

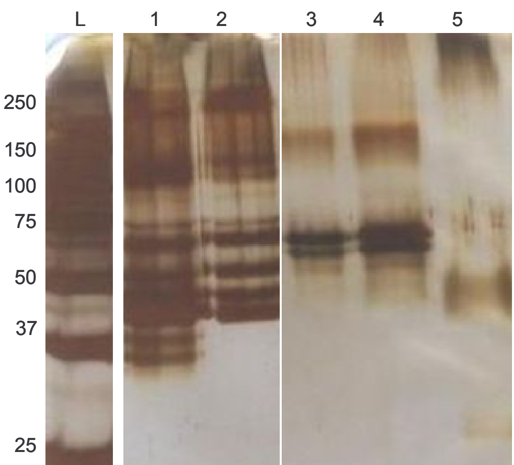

Figure 10: Comparison between Torpedo californica AChRs and precipitated sample
AChRs were isolated out of the Torpedo as described by Patrick et al [54]. TE671 cells were lysed in a buffer containing 0.5% Brij-97. AChRs were isolated using α-BT-biotin combined with streptavidin-agarose beads (lane 3,4). As controls, free uncoupled α-BT was added to the cell lysates (lane 5). The molecular marker is indicated by L. Samples were analysed by SDS-PAGE and silver staining as described in the materials and methods section.

In the *Torpedo californica* sample, bands with the molecular weight of the AChR subunits can be detected (lane 1, 2). Since these samples were not boiled before they were loaded on the gel, the AChRs did not completely break down into the subunits, and, therefore, also bigger protein bands were present. The results of the precipitated samples were the same as in the previous figure. As depicted in this figure, the bands present in the precipitated samples were comparable to the *Torpedo californica* AChR subunits. Since the AChR of the *Torpedo califonica* and the mammalian NMJ have a high similarity, they should be present at exactly the same location. Bands both of the *Torpedo californica* and the precipitated sample were cut out, digested and analysed by mass spectrometry. In section 3.4.2, these results will be described.

3.4 Identification of precipitated proteins

To characterize the precipitated proteins, both immunoblotting experiments and mass spectrometry analyses were performed.

3.4.1 Immunoblotting

The presence of AChR and associated proteins in the precipitated samples was tested by performing immunoblots with the relevant antibodies. Since bands with the same molecular weight as the AChR subunits were seen in the silver staining experiments, the AChR was expected to be present in the precipitated samples.

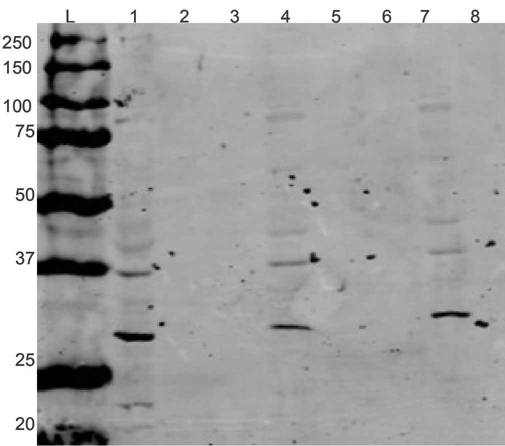

Figure 11: Immunoblotting of AChR subunits

TE671 cells were lysed in a buffer containing 1% NP-40. Total cell lysate before precipitation was loaded as a control (lane 1). AChRs were isolated using α-BT-biotin combined with streptavidin-agarose beads (lane 2, 3). As a negative control, free uncoupled α-BT was added to the cell lysates (lane 6). Total cell lysates after precipitation, containing all proteins that were not binding the α-BT, were analysed (lane 4, 7). Supernatant of the washing steps was also loaded on the gel (lane 5, 8). The molecular marker is indicated by L. Samples were resolved by SDS-PAGE, transferred to PVDF membrane and probed with specific antibodies against the ε- and δ-AChR subunits and with goat anti-mouse Ig secondary antibody.

The presence of the ε- and δ-subunits was tested using specific antibodies, as described in materials and methods. Two bands with a molecular weight between 20 and 37 kDa were clearly present in the total cell extracts before and after precipitation, as depicted in lane 1, 4, and 7 of figure 11. No bands at all were detected in the precipitated samples. Although the 50 kDa bands, expected to be the AChR subunits, were visible in the silver staining experiment using the same samples (data not shown), no AChR subunits could be detected here in the precipitates by the anti-AChR antibodies.

In addition to this experiment, many other Western blots were performed. Antibodies specific for rapsyn, S-laminin, syntrophin, ErbB2, ErbB3, and the AChR subunits were used in order to detect those proteins in the precipitates. However, in none of the immunoblotting experiments, any of these proteins could be detected. Even in the total cell lysate after precipitation, no protein bands were detected at all (data not shown).

3.4.2 Mass spectrometry

In parallel with the immunoblotting experiments, mass spectrometry analyses were performed to identify the precipitated proteins. The gel, shown in figure 12, was cut into sections and the proteins present in each gel section were digested by trypsin. Tryptic peptide mixtures were separated and identified by ESI-MS/MS on a LCQ iontrap followed by database searching.

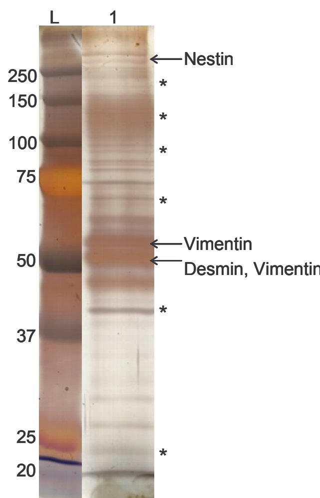

Figure 12: Identification of precipitated proteins

TE671 cells were lysed in a buffer containing 0.5% NP-40. AChRs were isolated using α-BT-biotin combined with streptavidin-agarose beads (lane 1). The molecular marker is indicated by L. Samples were analysed by SDS-PAGE and silver staining Finally,.28 bands were cut out, digested and analysed by mass spectrometry as described in materials and methods. The identified proteins are indicated by an arrow, the asterisks indicate the bands containing actin.

Mass spectrometry analysis of the precipitated fraction revealed the presence of cytoskeleton proteins including desmin, vimentin, nestin and different forms of actin, as indicated on figure 12. The size of these proteins corresponded to their location in the gel. However, AChR could not be identified in the protein bands corresponding to the molecular masses of the AChR subunits. In addition, keratins were detected in all analysed bands. As an example, the MS/MS spectrum of desmin is shown in the following figure.

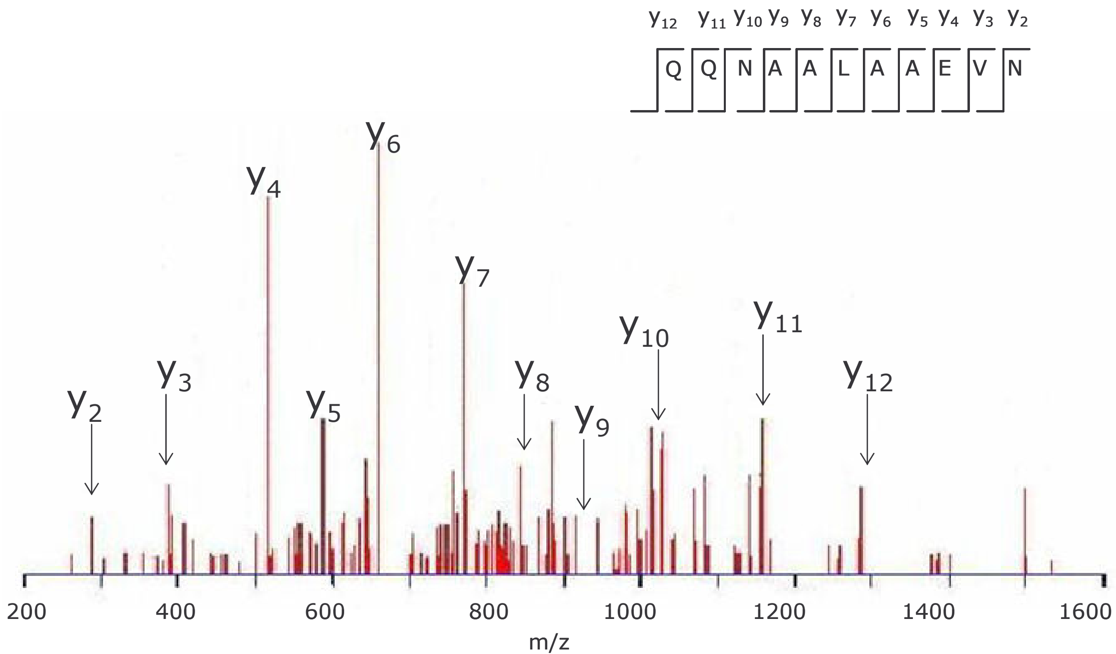

Figure 13: Proteomic analysis of precipitated proteins by MS/MS

Protein bands were isolated, subjected to in-gel trypsin digestion and analysed by peptide mass fingerprinting using the ThermoFinnigan LCQ mass spectrometer. ESI-MS/MS analysis of the ions followed by database searching identified the ions as desmin. The inset shows the peptide sequence with assigned fragment ions.

The mass spectrometer was connected to a computer on which a spectrum was visible after the analysis. This spectrum showed the parent-ions. Then, three parent-ions with the highest peaks were fragmented into daughter-ions, and again a mass spectrum was generated. An example of such a MS/MS spectrum is shown in figure 13, with the mass-over-charge ratio on the x-axis and the relative abundance of the peptides on the y-axis. Some high peaks were filtered by the computer software and were not analysed. For instance, some peaks coming from human keratin with known m/z ratios were ignored by the mass spectrometer. These peaks would disturb the spectrum and other important peaks to identify would be missed. Ignoring some keratin peaks increased the chance to identify a protein. Further, small ions were formed during fragmentation. Peptide fragments that are charged at the C-terminal are called y-ions, whereas fragments with the charge at the N-terminal are called b-ions. A series of b- and y-ions forms a certain sequence that can belong to one or more proteins. In the MS/MS spectrum of figure 13, the y-ions with m/z ratios 289.16, 388.23, 517.27, 588.31, 659.35, 772.43, 843.47, 914.51, 1028.55, 1156.61, and 1284.67 corresponded to the sequence NVEAALAANQQ. This sequence, together with sequences from other spectra, indicated that the protein was desmin. The computer program Sequest compared these MS/MS spectra with theoretical peptide spectra in a human database and gave a score to this comparison. Finally, the program indicated the proteins in which the spectra were found.

In addition to this experiment, protein bands were isolated from other gels and identified by mass spectrometry. The samples shown in figure 8 were analysed. Mass spectrometry of these bands resulted repetitively in the identification of keratin species corresponding to the molecular weight of the protein bands. Further, the samples shown in figure 9 were analysed. Since there is a high chance to contaminate the gel with keratins during a silver staining, no silver staining was

performed to avoid contamination on these samples. Then, they were analysed by applying direct digestion and mass spectrometry analysis. However, keratins were identified in all samples. Finally, samples of the *Torpedo californica* and the precipitated samples shown in figure 10 were collected, digested, and analysed by mass spectrometry. The MS/MS spectrum of the α-AChR subunit of the *Torpedo californica* is shown in the following figure.

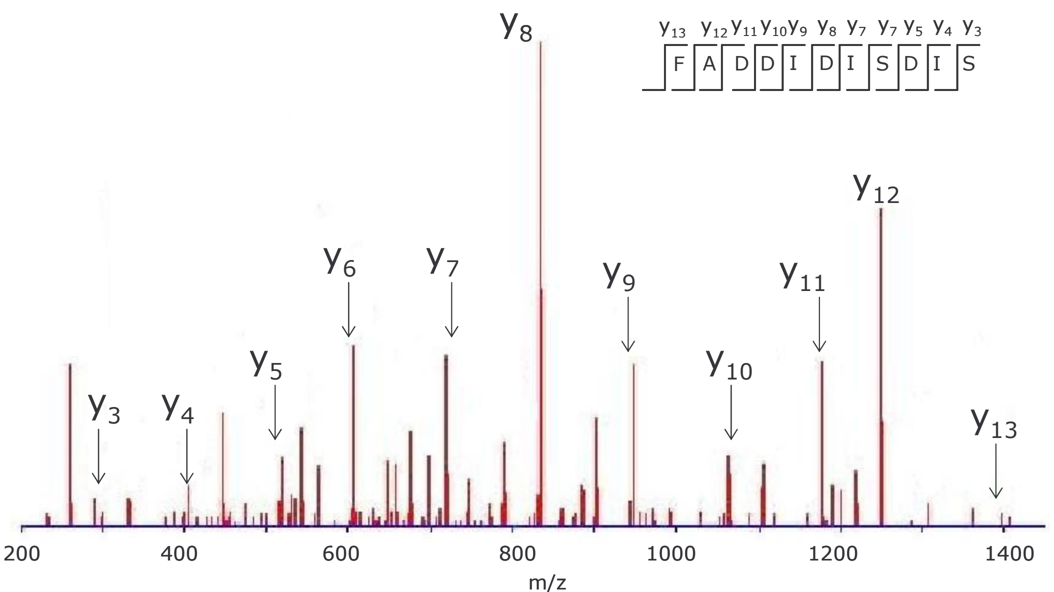

Figure 14: Proteomic analysis of Torpedo californica by MS/MS

Protein bands were isolated, subjected to in-gel trypsin digestion and analysed by peptide mass fingerprinting using the ThermoFinnigan LCQ mass spectrometer. ESI-MS/MS analysis of the ions followed by database searching identified the ions as α-AChR subunit. The inset shows the peptide sequence with assigned fragment ions.

In the MS/MS spectrum of figure 14, the y-ions with m/z ratios 291.17, 404.25, 519.28, 606.31, 719.39, 834.42, 947.50, 1062.53, 1177.56, 1248.60, and 1395.66 corresponded to the sequence SIDSIDIDDAF. This sequence corresponded to the α-subunit of the AChR from *Torpedo californica*. In addition to the α-subunit, the other AChR subunits were identified by mass spectrometry and their location in the gel corresponded to their molecular weight. Keratins were identified in both the precipitated and the negative control samples of the TE671 cell line.

3.5 Isolation of AChR using antibodies

From previous experiments, there is still no evidence that the AChR subunits were present in the precipitates. The presence of the AChR could not be confirmed yet, neither by immunoblotting, nor by mass spectrometry. Therefore, an alternative immunoprecipitation technique was performed using IgG637 antibodies to bind the AChR.

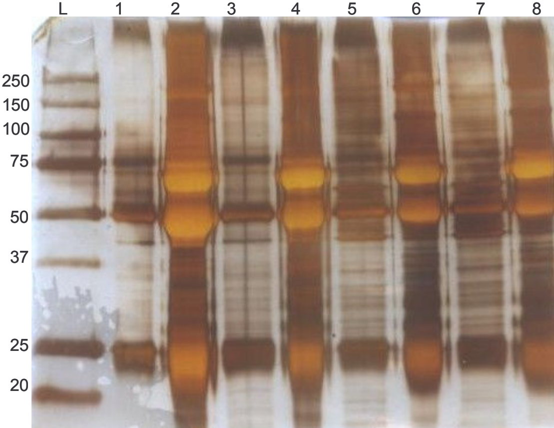

Figure 15: Precipitation of AChR using IgG673 antibodies

TE671 cells were lysed in a buffer containing 0.2% brij-97 (lane 1-4) or cholate (lane 5-8). AChRs were isolated using IgG637 combined with IVIg and secondary goat anti-human Ig (lane 1, 5). As a negative control, IgG637 was not added to the cell lysates (lane 3, 7). Total cell lysates after precipitation, containing all proteins that were not binding the antibodies, were also analysed (lane 2, 4, 6, 8). Precipitates were analysed by SDS-PAGE and silver staining as described in the materials and methods section.

When comparing the precipitated samples and the control samples, differences were hardly seen, neither when brij-97 (lane 1 versus 3) nor when cholate (lane 5 versus 7) was used to solubilize the proteins. When cholate was used as a detergent, more proteins were detected in the precipitates by silver staining (lane 5 versus 1). However, the precipitation of these proteins could not be confirmed to be specific. The intense gold bands present in the total cell extract after precipitation represented the heavy and light chains of the secondary antibody.
In addition to the silver staining, an immunoblotting was performed on these samples using anti-AChR antibodies.

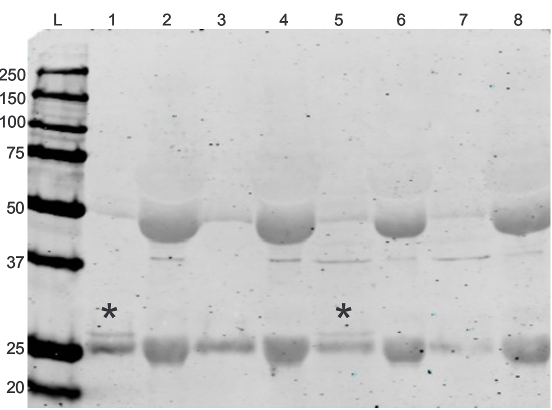

Figure 16: Immunoblotting of samples precipitated with IgG637 antibodies

TE671 cells were lysed in a buffer containing 0.2% brij-97 (lane 1-4) or cholate (lane 5-8). AChRs were isolated using IgG637 combined with IVIg and secondary goat anti-human Ig (lane 1, 5). As a negative control, no IgG637 was added to the cell lysates (lane 3, 7). Total cell lysates after precipitation, containing all proteins that were not binding the antibodies, were also analysed (lane 2, 4, 6, 8). Precipitates resolved by SDS-PAGE, transferred to PVDF membrane and probed with specific antibodies against the α-, ε- and δ-AChR subunits and with goat anti-mouse Ig secondary antibody.

The only difference that was seen when comparing the precipitates with the negative controls is the small 25 kDa band, indicated in figure 16 with an asterisk. The protein bands present in the control

samples probably derived from the precipitating antibodies itself, since no AChR proteins could be precipitated when no IgG637 was added. Antibodies against the α-, ε-, and δ-AChR subunits were used to perform the immunoblotting. Although the subunits have a molecular weight of approximately 50 kDa, a 25 kDa band was detected by the antibodies. In addition, heavy and light chains of the precipitating antibodies were seen, especially in the total cell extract after precipitation.

In figure 15, many proteins were present with a molecular weight of approximately 50 kDa, including parts of the precipitating antibodies. Therefore, it was more difficult to see differences with the control samples. To keep the precipitating antibodies intact, no β-mercaptoethanol was added to the sample buffer. The heavy and light chains of the antibodies will stay together, and will not migrate that far in the gel anymore. This would make it more obvious to analyze the 50 kDa bands.

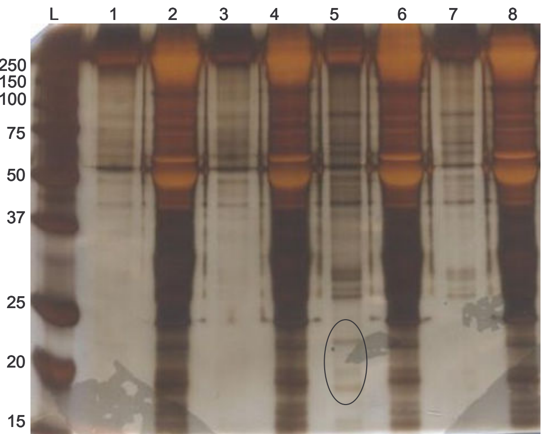

Figure 17: Precipitation of AChR using IgG673 antibodies without β-mercaptoethanol

TE671 cells were lysed in a buffer containing 0.2% NP-40 (lane 1-4) or cholate (lane 5-8). AChRs were isolated using IgG637 combined with IVIg and secondary goat anti-human Ig (lane 1, 5). As a negative control, IgG637 was not added to the cell lysates (lane 3, 7). Total cell lysates after precipitation, containing all proteins that were not binding the antibodies, were also analysed (lane 2, 4, 6, 8). Precipitates were denatured in a buffer with SDS but without β-mercaptoethanol. Then they were analysed by SDS-PAGE and silver staining as described in the materials and methods section.

Using NP-40 as a detergent to solubilize the proteins, no differences were detected between the precipitated sample and the negative control (lane 1 versus 3 in figure 17). However, when proteins were solubilised with cholate, some differences were seen when comparing the precipitated and the control sample (lane 5 versus 7). Protein bands with a molecular weight of approximately 20 kDa were present in the samples precipitated with IgG1-637, indicated with a circle, whereas these bands were absent in the negative control sample. However, these bands were smaller than the AChR subunits. In addition to the silver staining, an immunoblotting experiment was performed on the samples. The presence of α-, ε- and δ-AChR was analysed using the specific antibodies as described in the materials and methods section. However, no AChR subunits could not be detected, neither in the precipitates nor in the total cell lysates after precipitation (data not shown). Finally, a mass spectrometry analysis was performed on the samples of both the precipitation experiments. These results are not finished yet, and therefore, they can not be shown here.

4 Discussion

The molecular organization of the NMJ is designed for optimal efficiency of neuromuscular transmission. This signal transduction between motor nerve terminal and muscle membrane is disturbed in MG patients. In MG, the AChR is the main auto-antigen and the postsynaptic membrane of the NMJ is the target for the damage induced by the anto-antibodies. In a subgroup of MG patients, MuSK is the target for the auto-antibodies. However, in a proportion of MG patients, the auto-antigen is still unknown. To find a new candidate auto-antigen, we studied the AChR and associated proteins of the NMJ. The NMJ itself has been studied as a prototypical synapse for decades, and considerable knowledge exists concerning the molecular structure and function. However, it is likely that many, if not most, components of the NMJ remain undiscovered. Our hypothesis is that such an unknown NMJ component can be a candidate for the missing auto-antigens in double seronegative MG patients. Antibodies interfering with proteins that play an important role in the stability or maturation of the NMJ might disturb the molecular organization of the NMJ and, thereby, the process of neuromuscular transmission. AChR associated proteins can play an important role in the processes of NMJ stabilization and maturation, and therefore we focussed on the study of these proteins. To analyse the proteins of the NMJ, the TE671 cell line was used as an alternative source for NMJ components. The different techniques we performed will be discussed here.

4.1 Presence of NMJ proteins in TE671 cells

Studies of the NMJ are complicated by the small size of the region (35-90 µm) and the low abundance of them in muscle fiber (one per myofiber) [55]. Additionally, there are difficulties in applying standard fractionation techniques for NMJ isolations. Therefore, we used the TE671 cell line instead of real NMJs to optimize the different techniques. Several studies have shown that the human cell line TE671, originally identified as a rhadbomyosarcoma, expresses muscle-type AChRs [56, 57]. This cell line has been suggested as a source of human AChR for diagnostic RIAs to detect anti-AChR antibodies in MG [56]. Additionally, the cell line is used to show that MG patient sera can modulate surface AChRs [58]. Therefore, the TE671 cell line is a good alternative to study the AChR and associated proteins of the NMJ. To increase the expression level of AChR in the cells, dexamethasone was added to the culture medium [59]. Agrin could also have been added to the culture medium to induce the formation of AChR clusters, but in our experiments no agrin was used. Of course, the TE671 cell line shows several important differences with the real NMJ. For example, different proteins will be expressed in the TE671 cell line compared to the NMJ. To test which proteins of the NMJ are present in the TE671 cell line, Western blot experiments using specific antibodies against the NMJ proteins were performed. In figure 4, the presence of S-laminin, syntrophin, utrophin, MuKS, ErbB, dystrophin, Dok-7, rapsyn, and all AChR subunits was tested. Antibodies against s-laminin, syntrophin, MuSK, rapsyn, ErbB2, ErbB3, Dok-7, and the ε, β and δ subunits of the AChR all gave rise to one or more bands. However, in many cases the sizes of these bands differed from the expected sizes. Probably, the proteins were degraded or fragmented during the extraction procedure and/or further steps of the experiment. When the epitope of the protein remains intact, the antibody still can bind this part of the protein, and a smaller protein band will be detected. The protein bands visible in this figure can also be due to unspecific binding of the

antibody to another protein present in the cell extract. On the other hand, no bands were detected by antibodies against the α-AChR subunit, utrophin or dystrophin. Since utrophin and dystrophin have a molecular weight of approximately 400 kDa, these proteins were too big to migrate into the 10% gel. The reason why no α-AChR subunits were detected, is not completely clear yet. Although the TE671 cells express an abundance of partially mature α-AChRs [60] and although two different antibodies were used, no α-subunit could be detected. The antibodies against the AChR subunits can detect denaturated proteins [61], thus, this can not be the explanation for the absence of the α-subunit. Since the Western blot system should be sensitive enough (appendix 3), the antibodies probably did not work well. Further, the variable conditions in the immunoblotting experiment, such as dilution of primary antibody or concentration of the gel, were not optimized for these proteins. This can be a reason why some proteins were not detected. In future experiments, the conditions of the immunoblotting experiments have to be optimized for each protein separately.

4.2 Solubilization of AChR

The biochemical properties of integral membrane proteins and of water-soluble proteins are very different, as the native membrane surrounding integral membrane proteins must be disrupted by detergents without causing denaturation. Once membrane proteins are solubilized in an aqueous environment, they can be studied by biochemical techniques [62]. A detergent suitable for a native solubilization of membrane proteins must meet several requirements. It must be as mild as possible but still be able to disrupt lipid-lipid interactions without disturbing interactions between protein components in complexes. Disruption of certain lipid-protein associations is usually not desired because it may weaken protein complexes, especially if lipids are a structural component of the protein complex. The detergents used in the RIA experiment for the solubilization of AChR included triton-X100, digitonin, cholate, NP-40, brij-97, chaps and saponina. Figure 5 showed that NP-40, triton and brij-97 were able to solubilize the highest amount of AChRs out of the TE671 cell membranes. Therefore, these detergents were used in further precipitation experiments. Then, cholate might be the best detergent to keep the AChR in a more functional conformation when compared to other detergents [63]. Because cholate can solubilize proteins in a more native conformation, this detergent was also be used in our experiments. Although in our experiments digitonin was almost not able to solubilize any AChRs, this detergent is often used by other researchers to solubilize the AChR and associated proteins [52]. However, these investigators used the proteins of C2C12 myotubes instead of TE671 cells. To be able to compare the results, also digitonin was used in our precipitation experiments. The detergents might display different characteristics influencing the efficiency of the solubility of the membrane proteins, and therefore, the tested detergents showed different results in the RIA.
When comparing the different concentrations of detergent in the lysis buffer, it was seen in figure 5 that the 0.2% concentration solubilised the highest amounts of AChRs. However, it should be mentioned that the cells harvested in the same conditions, but containing different amounts of total protein (data not shown), were used for each concentration of detergent. Since the cell extract contained different total protein concentrations, different concentrations of AChR were present in the starting material. Therefore, different amounts of AChR were seen when comparing the detergent concentrations.

Nevertheless, it is important to realize that this RIA experiment only provides us with information about the solubilization of the AChR and not of other membrane proteins. Finally, it should be mentioned that, when using muscle extracts instead of TE671 cells, new RIA experiments have to be performed to find the optimal detergent for the solubilization of the membrane proteins. The different membranes within eukaryotic cells each contain a distinct lipid composition and lipid to protein ratio. Therefore, different detergents in different concentrations will be needed for the optimal solubilization of the protein complexes.

4.3 Isolation of AChRs using α-BT

To investigate the interactions between the AChR and other NMJ proteins, precipitation reactions were performed. AChRs were isolated using α-BT combined with streptavidin-coupled agarose beads. Since this experiment was not performed in our lab before, the protocol first had to be optimized, and, of course, some difficulties were found during the precipitation experiments. They will be discussed here together with the optimization steps.

4.3.1 Optimization of the protocol

Performing the precipitation protocol, many steps had to be taken into account. First, the total protein concentration had to be measured before starting the experiment. According to manufacture's instructions, 1 mg of total protein was needed to start the precipitation. However, the detergents and protease inhibitors present in the lysis buffer were interfering with the BioRad system, which was used to measure the protein concentration. Due to this, the protein concentration could not be measured exactly at the beginning of each experiment. Since cells of one confluent 162 cm^2 flask were used for each precipitation experiment, a good estimation of the protein concentration could be made based on previous protein measurements. Although a different lysis buffer was used, the total amount of protein in one culture flask was estimated to have a value between 1 and 2 mg. This amount should be sufficient to start the precipitation with. However, a different method to measure the protein concentration, not influenced by the detergents, should be applied in future experiments. Second, a pre-clearing step was included into the protocol to minimize unspecific binding of proteins to the agarose beads. Beads were added to the cell extract for half an hour, followed by a centrifugation step in order to remove the beads together with the unspecifically bound proteins. Then, in order to distinguish between specific precipitations and unspecific ones, free α-BT was used as a negative control. Free α-BT is not labelled with the biotin molecule, and therefore, it can not bind to the streptavidin-coupled beads. The AChR can bind to the free α-BT, but these complexes can not be precipitated by the beads. However, in all precipitation experiments we performed, many proteins were still present in the negative control samples. Despite the application of the pre-clearing step, it is possible that still some proteins were present that could bind to the beads in an unspecific way. These proteins should be visible in both the precipitated and the negative control samples. However, clear differences were detected between the precipitates and the negative controls, particularly in figure 9. Additionally, in figure 6, only membrane proteins were analysed, and proteins were still present in the negative control samples. Thus, the presence of protein bands in the negative controls can not be explained exclusively by unspecific binding of proteins to the beads. The protein

bands also did not derive from α-BT or from streptavidin, because then they would be visible in both the precipitated and the negative control samples. Additionally, these molecules have a low molecular weight, so they migrated off the 10% SDS gel. In future experiments, the negative controls and the proteins removed by preclearing can also be analysed by immunoblotting or mass spectrometry to compare them with the precipitated proteins. Finally, following the precipitation reaction itself, the samples were analysed by SDS-PAGE and silver staining. With a silver staining experiment, one can detect 1 ng of protein in a gel, which is much more sensitive compared to the coomassie staining. Since low amounts of proteins were expected to be present in the precipitated samples, the silver staining was preferred to the coomassie staining.

Besides the standard steps, some optimizing steps were performed. First, the amount of beads necessary to precipitate the protein complexes was tested. As depicted in figure 6, more proteins were lost during the washing steps when only 10 μl of beads was used. Although also 10 μl of beads is an excess to precipitate the immunecomplexes, probably there were not enough bindingsites avaibable. This protein loss has to be avoided, and therefore, we decided to use 50 μl of beads in further experiments. Subsequently, the duration of lysis and its influence on the amount of solubilised proteins was tested. Since proteins still were solubilised after a longer time period of lysis, as depicted in figure 7, 8, and especially 9, we decided to increase the duration of lysis in future experiments. This will probably increase the amount of solubilised membrane proteins, and thus, the chance to precipitate the AChR together with known or unknown proteins of interest will be higher. Once the presence of the AChR can be confirmed, further optimizations will be necessary.

4.3.2 Precipitated protein bands

When those optimization steps were performed, clear differences were detected between the precipitated samples and the negative controls. Figure 9 showed that bands with the same molecular weight as the AChR subunits were present in the precipitated samples whereas they were absent in the negative controls. The α, β, γ/ε, and δ subunits of the AChR have a molecular weight of 40, 50, 60, and 65 kDa respectively (appendix 1). When the δ subunits form a dimer, it gives rise to a 130 kDa protein. In addition to the AChR subunits, other NMJ proteins such as rapsyn, syntrophin or β-dystroglycan, also have a molecular weight about 50 kDa. Thus, the AChR or other NMJ proteins were expected to be present in those bands. The identification of the protein bands is described later on. It is important to mention that our precipitation experiments were performed as described by Fuhrer *et al* [52], except for some minor modifications. Using this technique, these investigators were able to show associations of the AChR with rapsyn, β-dystroglycan and utrophin [52], with the SFKs Src and Fyn [64] and with MuSK [65] in myotubes. Although biochemical co-purification of the AChR and rapsyn is difficult, they were able to coprecipitate rapsyn and the AChR. The difficulties in co-purification of these proteins from muscle extracts most likely originate from difficulties in extracting rapsyn from the cytoskeleton and from its protease sensitivity. Fuhrer *et al* also showed that some protein interactions are very fragile and that many protein complexes are disrupted even during the mildest extraction procedures [52]. An important difference between both studies is the cell line that is used for the experiments. Fuhrer *et al* studied the associations of AChR with NMJ proteins in the C2C12 myotubes, whereas we used

the TE671 cell line. However, these studies confirm the possibility to precipitate the AChR and associated proteins using this technique. A possibility to improve our results is to use these C2C12 myotubes for further analyses.
Finally, the precipitated samples were compared to AChRs isolated from *Torpedo californica*. The electric organ of *Torpedo californica* has modified muscle fibers, primarily used to shock a prey by the production of a high amount of electricity. The postsynaptic membrane of *Torpedo californica* is very similar to the mammalian NMJ. The *Torpedo* rays have been used as model organisms for molecular biology research because of the high natural abundance of some proteins in its electrical organs, mainly AChE and AChR (reviewed by [3]). The subunits of the *Torpedo californica* AChR also have a molecular weight of 40, 50, 60 and 65 kDa. The purification of the AChR subunits from the *Torpedo californica* was performed as described by Patrick and Lindstrom [54]. To compare these subunits with the proteins we isolated during the precipitation, both samples were loaded together on a gel. In the *Torpedo californica* sample, the four AChR subunits can clearly be distinguished as depicted in figure 10. Because this sample was not boiled before loading it on the gel, the receptor was probably not completely denatured. Therefore, also higher protein bands were present in the gel. The identification of those proteins by mass spectrometry is discussed later on.

4.4 Identification of precipitated proteins

The precipitation experiments were performed to isolate the AChR together with associated proteins. The presence of the AChR in the precipitates had to be confirmed. When the receptor is not precipitated by the α-BT, other NMJ proteins should also be absent because they can only be precipitated by their interaction with the receptor. The presence of AChR and other NMJ proteins in the precipitated samples was controlled by immunoblotting experiments and by mass spectrometry.

4.4.1 Immunoblotting

To identify the precipitated proteins, Western blot experiments were performed. Antibodies against rapsyn, S-laminin, syntrophin, ErbB2, ErbB3 and all the subunits of the AChR were tested in different experiments. AChR subunits were detected only once in the total cell lysate before and after precipitation, as depicted in figure 11. No protein bands could ever be seen in the precipitated samples. Many explanations why no proteins could be detected were considered. First, it should be considered that the proteins were not present in the cell line from the beginning of the experiment. However, their presence was confirmed by the first immunoblotting experiment, shown in figure 4, and by the RIA experiment for the α-subunit, and thus, this can not be an explanation. It is also possible that some proteins were not solubilised in an efficient way. Although a good detergent was chosen to solubilise the AChR, different detergents may be necessary to isolate other membrane proteins. Additionally, the proteins might be degraded during the extraction process, and, therefore, they were not detected by the antibodies. Further, another possibility might be that the antibodies did not bind denatured proteins. The proteins were loaded on the gel in a denatured form, since SDS and β-mercaptoethanol were added to the loading buffer to dissolve the proteins. The antibodies against the AChR subunits obtained from Dr. SJ. Tzartos (Hellenic Pasteur Institute,

Greece) do bind denatured proteins [61]. Thus, this can not be the reason why no receptor subunits were detected. Then, it was thought that the secondary anti-mouse antibodies could not bind the primary antibodies, made in rats. However, the secondary antibodies do cross-react with immunoglobulins of rat, and thus, they did bind to the primary rat antibodies in our experiments. Additionally, the sensitivity of the Odyssey system was discussed. Possibly the sensitivity was too low to detect the immunecomplexes. The detection limit of the Odyssey system is 2 ng, and a bigger amount of protein is loaded in our experiments, as calculated in appendix 3. However, when the precipitation of the proteins did not take place in an optimal way, less protein than calculated was loaded on the gel. In a further experiment, the sensitivity of the Odyssey system will be compared to the sensitivity of another Western blot system to select the most sensitive one. Subsequently, the detergents could have had an influence on the binding capacity of the antibodies to the proteins. Immunoblotting experiments were also performed on the total cell extract without any precipitation. The cells were lysed using different detergents. When cholate was used as a detergent, the AChR subunits were seen more clear when compared to the other detergents (data not shown). As already mentioned, cholate can solubilize proteins in their native condition. However, the anti-AChR antibodies do bind denatured proteins and no differences should be seen when comparing the different detergents. Possibly some characteristics of the detergents were interfering with the binding between antibody and antigen. Then, it is also possible that the precipitated proteins formed aggregates during the experiment. Aggregates are too big to migrate into the gel, and therefore, the proteins can not be detected. Therefore, urea can be added to the loading buffer in future experiments to break the protein aggregates. The final explanation why no AChR subunits or other NMJ proteins were detected by immunoblotting experiments is that no proteins were precipitated at all. Although the band patterns visible in the precipitated samples were believed to contain the subunits of the AChR, the presence of the AChR subunits could not be confirmed yet. As mentioned before, Fuhrer *et al* were able to precipitate the AChR together with associated proteins. The presence of those proteins was confirmed using immunoblotting experiments [52]. Thus, our precipitation technique and the Western blots have to be further optimized in future experiments to isolate and detect the AChR efficiently.

4.4.2 Mass spectrometry

Mass spectrometry is beyond any doubt the key element of any proteomics project. Using this sensitive technique, known and unknown proteins can be identified. The first mass spectrometry experiment, shown in figure 12 and 13, identified desmin, vimentin, nestin, actin and keratin species in the precipitated samples. Desmin, vimentin and nestin all are intermediate filament (IF) proteins. Muscle cells contain several IF proteins, including desmin, vimentin, nestin, synemin, syncoilin, lamins and cytokeratins [66]. Desmin is the main IF protein in mature striated muscle cells and is located at the sarcolemma, the Z-lines and at the NMJ. Agbulut *et al* showed that desmin is necessary for establishing the complex folded structure in the postsynaptic apparatus of the NMJs [67]. It has also been shown that nestin is present in the junctional regions of muscle tissue [68]. Additionally, it has been shown that the TE671 cell line expresses vimentin and desmin [69]. Nazarian *et al* were able to identify among others desmin and vimentin in *Torpedo californica* by mass spectrometry analyses [3]. However, the AChR was not identified by mass spectrometry in

our precipitates. Instead, the proteins present in the gel bands corresponding to the molecular weight of the AChR were in three independent experiments identified as keratins. Since the TE671 cell line is a human cell line, the Sequest program had to search against a human protein database to identify the proteins. The keratins present in the samples can thus derive from either the cell line itself or from human skin contamination. First, it was thought that the keratins derived from human skin contaminations. Therefore, to reduce the chance on keratin contamination during the silver staining itself, the third mass spectrometry analysis was performed on gel-free digested samples. Furthermore, since about 80% of the protein content present in the silverstained gel can get lost during the analyses, a gel-free digestion can avoid this protein loss. However, still only keratins were detected in the samples. In addition, the molecular weight of the detected keratins in previous experiments corresponded to the sizes of the bands in the gel. On the contrary, keratins deriving from contaminations would be present all over the gel and would not correspond to the correct sizes. Therefore, we thought that the TE671 cell line contained many keratins that disturbed the mass spectrometry analyses. However, Quentmeier *et al* showed that the TE671 cell line is negative for keratins, which is consistent with its proposed derivation from a human rhabdomyosarcoma [69].

To reveal the source of the keratins, samples of the *Torpedo californica* were analysed together with precipitated TE671 samples. To identify proteins of the *Torpedo californica*, the Sequest computer program had to search against the protein database of *Torpedo californica* instead of the human database. The subunits of the AChR all were identified and corresponded to the expected location in the gel. The fact that the *Torpedo californica* AChR subunits were identified indicates that the silver staining and the digestion were performed in a correct way, because both samples of the *Torpedo californica* and the precipitated samples were analysed during the same experiment. Additionally, this suggests that no keratin contamination disturbed the mass spectrometry analysis. However, the presence of the AChR subunits in the precipitated samples was not confirmed. Keratins were identified in both the precipitated and the negative control samples. This suggests that the keratins present in the TE671 cell line probably bound to the beads or the α-BT unspecifically. In standard condition, protein bands in a silver stained gel that appaer as clear as our bands can easily be identified by mass spectrometry. Therefore, we presume that the bands represent only keratins instead of the AChR subunits. It can not be explained yet why differences between the precipitated and the negative control samples were seen that clear in the gel. However, the keratin species disturbed the mass spectrometry analysis and probably even the precipitation reaction strongly. In future experiments, C2C12 myotubes and rat muscle extracts can be studied in order to avoid the interactions with the keratins.

4.5 Isolation of AChR using antibodies

An alternative technique to isolate the AChR was performed, because the precipitation of AChR by α-BT could not be confirmed yet. Therefore, the AChRs were precipitated using IgG1-637 antibodies. Stassen *et al* [51] produced the human IgG1-637 anti-human AChR auto-antibody. An MG thymus-derived anti-AChR Fab637 was genetically fused to the Fc of human IgG1. The IgG1-637 was shown to specifically bind the α1-subunit of the human AChR [51]. Using this antibody to precipitate, significant differences between the precipitated sample and the negative control could

hardly be seen in figure 14. However, when blotting with antibodies against the AChR subunits, one protein band was present in the precipitated sample, whereas it was absent in the control sample. This band only had a size of 25 kDa, thus it probably represents a fragmented subunit of the AChR. Since antibodies against three subunits were added together, this band can represent either the α-, ε- or δ-subunit. In future experiments, the antibodies have to be added separately in order to study the presence of the subunits one by one. It also has to be emphasized that the TE671 cell line is expressing an abundance of partially mature α-AChR subunits [60]. These dissociated subunits can also be precipitated by the IgG1-637 antibodies. However, isolating these subunits alone will not provide information about the association of NMJ proteins with the mature AChR.
Further, many intense bands were detected by silver staining in the total cell extract after precipitation. They partly derived from the precipitating antibodies itself. Both an excess of primary and secondary antibody was added, and, therefore, the heavy and light chains were seen in the total cell extract after precipitation. This staining by immunoblotting is probably due to unspecific binding of the secondary antibody to the heavy and light chains. Additionally, since the heavy chains of the antibodies have a molecular weight of approximately 50 kDa, they were found at the same position where the AChR subunits were expected to be. This made it more difficult to detect differences between the precipitated sample and the controls. Therefore, in the second experiment no β-mercaptoethanol was added to the loading buffer. In standard conditions, the loading buffer is containing both SDS and β-mercaptoethanol to dissolve the proteins before loading them on the gel. Without β-mercaptoethanol, the heavy and light chains of the antibodies will not be separated. An intact antibody is much bigger and will not migrate into this acrylamide percentage gel. This would make it easier to detect differences between the bands of approximately 50 kDa. Despite the removal of β-mercaptoethanol no differences were seen at the height of 50 kDa, as is shown in figure 16. However, two bands of approximately 20 kDa were present in the precipitated samples, whereas they were absent in the negative controls. Although they did not have the length of one of the native AChR subunits, they probably were a part of a degraded or fragmented subunit. To confirm this, an immunoblot using anti-AChR antibodies was performed. When no receptor subunits were precipitated, they were at least expected to be present in the total cell lysate after precipitation. However, no protein bands were detected at all. The samples of these experiments were also analysed by mass spectrometry in order to identify the precipitated proteins. These experiments are not finished yet, and thus the results were not included in this report. In future experiments, the precipitation protocol using IgG1-637 antibodies has to be optimized further to isolate the AChR. Additionally, other anti-AChR antibodies can be tested to precipitate the receptor together with associated proteins.

4.6 Future experiments

The aim of this project was to study the AChR and its associated proteins of the NMJ to find a candidate for the unknown antigens in MG patients. However, many challenges have to be faced in future experiments. First of all, rat muscle extracts, and later on human muscle extracts, will be used to analyse the components of the NMJ. Muscle tissue is much more representative than a cell line to study the NMJ. In addition, muscle extracts probably do not have an abundance of keratin proteins that can interfere with the precipitations and the mass spectrometry experiments. First, a

suitable detegent has to be selected to solubilize the proteins from the muscle membrane in an efficient way. Then, the precipitation reaction will be performed on the muscle tissue, both trying the α-BT and anti-AChR antibodies. A RIA experiment will be combined with a precipitation using antibodies to measure the amount of precipitated receptor. In addition, other antibodies than the IgG1-637 will be tested. Proteins in the precipitates can be identified either by immunoblotting or by mass spectrometry. On one hand, when the precipitation technique is optimized, we can identify the AChR associated proteins that were isolated and construct a proteome map of the NMJ. On the other hand, we can use the sera of MG patients without antibodies to the AChR or MuSK to blot a 2-DE gel from muscle extracts. The detected proteins can be identified by mass spectrometry, and new candidates for the unknown antigens may be found. Later on, in addition to the precipitation experiments, also other experiments can be performed to study the components of the NMJ specifically. For example, laser capture microdissections can be performed to isolate the entire NMJ out of the muscle tissue. Furthermore, the NMJ composition in experimental autoimmune myasthenia gravis (EAMG) animals and control animals can be compared in order to find significant differences in protein expression. To achieve this, we can use the differential in-gel electrophoresis (DIGE) technique to study differential protein expression in muscle tissue. By performing DIGE, a quantitative analysis will be obtained of the protein composition of both EAMG and control animals. Then, mass spectrometry analyses will be performed to identify the differently expressed proteins. Additionally, phage display experiments can be performed. A cDNA library of the NMJ proteins can be made and the proteins will be displayed on the surface of the phage. The phage displaying the protein that specifically binds to the antibodies of the seronegative MG patients will be analysed further, and a new auto-antigen can be found. Thus, our final goal is to compose a proteome map of the human NMJ and to characterize candidates for the unknown auto-antigens in a proportion of MG patients. Discovering key players in the integrity and function of NMJs is relevant to understand neuromuscular diseases such as MG. Since the underlying disease mechanisms of MG are not completely understood, it is important to perform further research to search for new candidates that can be involved in the disease pathogenesis.

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Appendix 1: Proteins of the NMJ

Table 2: Cytoskeletal and peripheral proteins associated with the neuromuscular postsynaptic membrane

Protein	MW (kDa)	Location and comments
α-AChR	40	Transmembrane, ionotropic receptor subunit
β-AChR	50	Transmembrane, ionotropic receptor subunit
γ/ε-AChR	60	Transmembrane, ionotropic receptor subunit
δ-AChR	65	Transmembrane, ionotropic receptor subunit
Rapsyn	43	Cytoplasm, associated directly with the AChR
MuSK	97	Transmembrane, receptor for agrin
ErbB2	185	Transmembrane, activated by NRG-1
ErbB3	180	Transmembrane, activated by NRG-1
ErbB4	150	Transmembrane, activated by NRG-1
NRG-1	70	Secreted by motor nerve in synaptic cleft
Agrin	220	Released by motor nerve in synaptic cleft
Syntrophin	58	Cytoplasm, co-localized with utrophin/dystrophin
Dystrophin	430	Cytoplasm, troughs of junctional folds
Utrophin	400	Cytoplasm, crests of juntional folds
S-laminin	190	Extracellular matrix protein
α-dystroglycan	67	Extracellular, binds laminin
β-dystroglycan	46	Transmembrane, binds rapsyn and α-dystroglycan
Dok-7	53	Peripheral membrane protein, muscle-intrinsic activator of MuSK
Src-family kinase	60	Cytoplasm, phosphorylation of AChR
Desmin	53	Intermediate filament protein, cytoplasm
Vimentin	53	Intermediate filament protein, cytoplasm
Nestin	175	Intermediate filament protein, cytoplasm

Appendix 2: Raw data of RIA experiment

Table 3: Raw data of RIA experiment (part 1)

labelling day		15/01/2007
measurement day		10/04/2007
delta t		85,00
constants:		
halflife 125I		59,6
BT/AChR		2
efficiency gamma counter		0,82
s/min		60
tera		1,00E+12
milli		1,00E-03
micro		1,00E-06
n		1,00E-09
femto		1,00E-15
n*micro		1,00E-15
Bungarotoxin activity on labelling day	teraBq/mmol	6,12
Bungarotoxin activity on measurement day	teraBq/mmol	2,277
Bungarotoxin activity on measurement day	Bq/mol	2,28E+15
Bungarotoxin counts on measurement day	CPM/mol	1,12E+17
Bungarotoxin counts on measurement day	CPM/nmol	1,12E+08
AChR counts on measurement day	CPM/fmol	5,60E+01

Table 4: Raw data of RIA experiment (part 2)

	CPM1	CPM2	Average	SD
0,2% Saponina	403,8	438,9	1,50E-01	8,86E-03
	259,4	345,4	1,08E-01	2,17E-02
	303,5	345	1,16E-01	1,05E-02
0,2% NP-40	10602	11950,1	4,03E+00	3,40E-01
	5940,5	5169,2	1,98E+00	1,95E-01
	3057,1	2804,9	1,05E+00	6,37E-02
0,2% Chaps	434	394,7	1,48E-01	9,92E-03
	308,8	392,8	1,25E-01	2,12E-02
	379	360,3	1,32E-01	4,72E-03
0,2% Cholate	1979,2	2063,9	7,22E-01	2,14E-02
	1262,6	1313,2	4,60E-01	1,28E-02
	845,2	783,6	2,91E-01	1,56E-02
0,2% TritonX100	10676,3	10992,9	3,87E+00	7,99E-02
	4907,2	5400,2	1,84E+00	1,24E-01
	3110,4	2873,5	1,07E+00	5,98E-02
0,2% Digitonin	443,3	511,4	1,70E-01	1,72E-02

	468,5	451,1	1,64E-01	4,39E-03
	346,8	333,3	1,21E-01	3,41E-03
0,2% Brij-97	14665,5	14890,1	5,28E+00	5,67E-02
	7537	7124,8	2,62E+00	1,04E-01
	4113,1	3974,1	1,44E+00	3,51E-02
0,5% Saponina	466,9	383,1	1,52E-01	2,12E-02
	338,7	292,1	1,13E-01	1,18E-02
	313,9	333,5	1,16E-01	4,95E-03
0,5% NP-40	9701,9	9457,6	3,42E+00	6,17E-02
	4019,1	4543,9	1,53E+00	1,32E-01
	2707,2	2325,8	8,98E-01	9,63E-02
0,5% Chaps	289,5	351,4	1,14E-01	1,56E-02
	329	324	1,17E-01	1,26E-03
	335,6	338,4	1,20E-01	7,07E-04
0,5% Cholate	2437,5	2528,3	8,86E-01	2,29E-02
	1459,3	1367,1	5,05E-01	2,33E-02
	1018,5	924,6	3,47E-01	2,37E-02
0,5% TritonX100	8371,7	7423,5	2,82E+00	2,39E-01
	3627	3835,4	1,33E+00	5,26E-02
	2569,7	2594,1	9,22E-01	6,16E-03
0,5% Digitonin	434,7	521,2	1,71E-01	2,18E-02
	367,3	406,8	1,38E-01	9,97E-03
	396,1	381,1	1,39E-01	3,79E-03
0,5% Brij-97	10483,8	10146	3,68E+00	8,53E-02
	4607,2	3703,7	1,48E+00	2,28E-01
	2752,6	2755,9	9,83E-01	8,33E-04
1% Saponina	256,9	299,5	9,93E-02	1,08E-02
	245,4	262	9,06E-02	4,19E-03
	229,6	299,1	9,44E-02	1,75E-02
1% NP-40	8018,1	6624,6	2,61E+00	3,52E-01
	3625,1	3573	1,28E+00	1,32E-02
	1904	1748,6	6,52E-01	3,92E-02
1% Chaps	296,1	389,6	1,22E-01	2,36E-02
	302,7	249,5	9,86E-02	1,34E-02
	267,1	299,7	1,01E-01	8,23E-03
1% Cholate	2772,4	2740,9	9,84E-01	7,95E-03
	1656,5	1643,8	5,89E-01	3,21E-03
	1034,6	985,1	3,61E-01	1,25E-02
1% TritonX100	5816,2	6239,4	2,15E+00	1,07E-01
	3232,4	3263,3	1,16E+00	7,80E-03
	1829,5	1552,8	6,04E-01	6,98E-02
1% Digitonin	326,3	339,8	1,19E-01	3,41E-03
	333,6	301,6	1,13E-01	8,08E-03
	276,5	312,6	1,05E-01	9,11E-03
1% Brij-97	7389	8142,5	2,77E+00	1,90E-01
	3696	3733,9	1,33E+00	9,57E-03
	1831,8	2262	7,31E-01	1,09E-01

Appendix 3: Calculations

Table 5: Amounts of proteins used in the experiments

TE671 cells	Detergent	Amount α-AChR (RIA)	Amount α-AChR (start precipitation)	α-BT-biotin (1mg/ml)	Beads	Amount AChR in gel (Silver staining / Western blot)	Detection limit Odyssey Western blot	Detection limit silver staining
Confluent 162 cm^2 flask	NP-40	3.5 fmol/µl	3.5 pmol 140 ng	2 µl	50 µl	18 ng / 50 ng	2 ng	1-2 ng
Confluent 162 cm^2 flask	Cholate	1 fmol/µl	1 pmol 40 ng	2 µl	50 µl	5 ng / 15 ng	2 ng	1-2 ng
Confluent 162 cm^2 flask	Triton	3.2 fmol/µl	3.2 pmol 128 ng	2 µl	50 µl	16 ng / 45 ng	2 ng	1-2 ng
Confluent 162 cm^2 flask	Brij-97	4 fmol/µl	4 pmol 160 ng	2 µl	50 µl	20 ng / 55 ng	2 ng	1-2 ng

Each precipitation reaction was started using the cells of one confluent 162 cm^2 flask. Different detergents were used to solubilize the proteins out of the cell membrane. The amount of solubilised α-AChR subunit was measured by the RIA experiment. According to manufacture's recommendations, the precipitation reaction was started with 1mg of total protein. Since the deterents and protease inhibitors present in the lysis buffer were interfering with the Biorad system, used to measure the protein concentration, 1 ml of cell extract was used to start the precipitation reaction with. The molecular weight of the α-subunit was used to make the calculations. Both an excess of α-BT-biotin and beads were added in order to precipitate as much receptor as possible. Then, 60 µl of loading buffer was added to the precipitated proteins to dissolve them. 20 µl of sample was loaded on the gel to perform the Western blot, whereas 7 µl was loaded to perform the silver staining. The minimum amount of protein that can be detected by either the Western blot system or by a silver staining is described in this table.

Appendix 4: Poster

Analysis of native AChR-associated proteins in TE 671 cells

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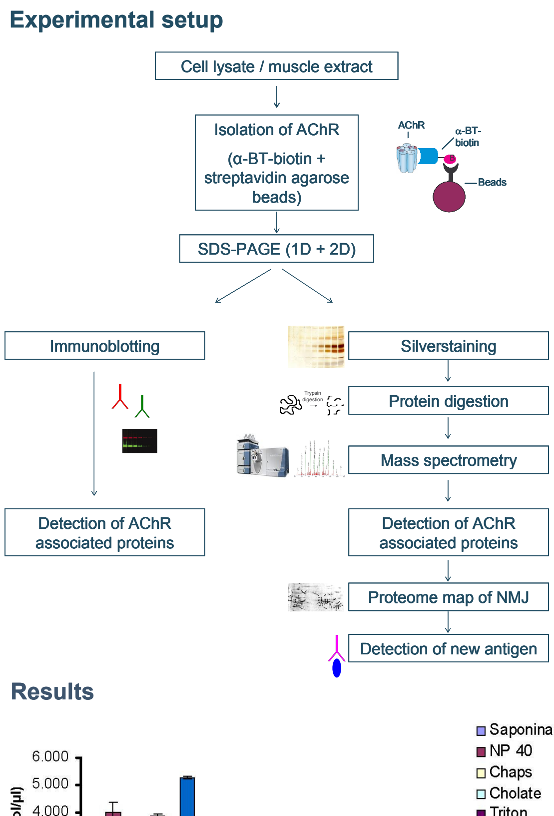
Introduction

Myasthenia gravis is an autoimmune disease characterised by auto-antibodies against proteins of the neuromuscular junction (NMJ). In approximately 85% of patients, auto-antibodies to the muscle nicotinic acetylcholine receptor (AChR) are present while in 3-70% of patients without antibodies to the AChR, pathogenic antibodies against the muscle specific kinase (MuSK) can be detected. However, a proportion of patients with myasthenia gravis neither have antibodies to the AChR, nor to MuSK. The hypothetically neuromuscular antigen of these patients is still unknown. Therefore, we will isolate AChRs together with associated known and unknown proteins that might play an important role in the NMJ. When these proteins will be characterized, they can be candidates for the missing antigen.

Material and Methods

- Radioimmunoassay (RIA)
- Precipitation of AChR using α-bungarotoxin (α-BT)
- Immunoblotting
- Silverstaining
- Mass spectrometry

Experimental setup



Results

Fig 1. Measurement of AChR by RIA using ^{125}I-labelled α-BT

Different detergents are added to the lysis buffer to solubilize the AChR out of the TE 671 cell membrane. Detergents NP-40, Triton X-100 and Brij-97 solubilize highest amounts of AChRs.

Discussion 1

The detergents NP-40, Triton X-100 and Brij-97 will be used in further experiments to solubilize the proteins out of the cell membrane. Cholate solubilizes proteins in a more functional conformation and therefore this detergent will also be used to solubilize the AChRs and associated proteins. Cells harvested in the same conditions but containing different amounts of total protein were used for each concentration of detergent (0.2, 0.5 and 1%). Therefore, different amounts of AChR can be seen when comparing the detergent concentrations.

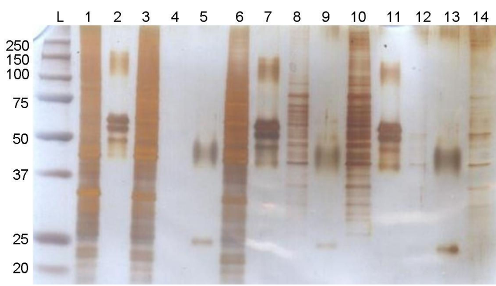


Fig 2. Silverstaining of TE 671 cell extract used for precipitation of AChRs.

TE 671 cells were lysed in a buffer containing 0.5% Brij-97. Total cell lysate before precipitation was loaded as a control (lane 1). AChRs were isolated using α-BT-biotin and streptavidin-agarose beads (lane 2, 7, 11). As controls, free uncoupled α-BT was added to the cell lysates (lane 5, 9, 13). Total cell lysates after precipitation, containing all proteins that were not binding the α-BT, were also analysed (lane 3, 6, 8, 10, 12, 14). The molecular marker is indicated by L. Samples were analyzed by SDS-PAGE and silverstaining. Differences can be seen when comparing samples precipitated with α-BT-biotin (lane 2, 7, 11) or with free α-BT (lane 5, 9, 13).

Discussion 2

The AChR subunits α, β, γ and δ have a molecular weight of 40, 50, 60 and 65 kDa respectively and the dimer of δ subunits has a weight of 130 kDa. Bands of these sizes can be seen in the precipitated samples (lane 2, 7, 11). However, we could not yet demonstrate that those bands represent the subunits of the AChR, neither by immunoblotting with anti-AChR antibodies, nor by mass spectrometry. To detect the AChR subunits by immunoblotting, antibodies recognizing denatured AChR subunits are necessary. The mass spectrometry also needs further optimization.

Future experiments

During this project, we will try to identify proteins that may play an important role in the stability and maturation of the NMJ. These proteins can be candidates for the unknown antigen in double seronegative MG patients. There are still important gaps in the pathogenesis of MG and, therefore, further research has to be performed.

Acknowledgments

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