New approaches to identify interactors of MuSK, a receptor tyrosine kinase required for the formation of the nerve-muscle synapse

INAUGURALDISSERTATION

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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aus Italien

Basel, 2006

UNI
BASEL

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
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Basel, den 28. September 2004

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Table of Abbreviations

3-AT	3-Amino-1,2,4-triazole
α-Btx	α-Bungarotoxin
α-DG	α-dystroglycan
ß-DG	ß-dystroglycan
ß-Gal	ß-galactosidase
AChR	acetylcholine receptor
AD	activation domain
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRD	cysteine rich domain
Cub	C-terminal moiety of ubiquitin
DBD	DNA binding domain
Dvl	dishevelled
ECD	extracellular domain
ErbB	receptor tyrosine kinase for neuregulins
FGF2	fibroblast growth factor 2
GGT	geranylgeranyltransferase
His	histidine
IgG	Immunoglobulin G
Leu	leucine
LG	laminin G-like domain
MASC	muscle associated specific component
MuSK	rat homologue of the muscle specific kinase (nowadays used for all species)
mYTH	membrane associated YTH system
NCAM	neural cell adhesion molecule
NFG	nerve derived growth factor
NMJ	neuromuscular junction
NRG	neuregulin
Nsk2	mouse homologue of the muscle specific kinase (old nomenclature)
NT3	neurotrophin 3
Nub	N-terminal moiety of ubiquitin
nYTH	nuclear based YTH system
PAK	P21-activated kinase
PNS	peripheral nervous system
PTB	phosphotyrosine binding domain
TF	transcription factor
TM	transmembrane domain
Trp	tryptophan
UBP	ubiquitin specific proteases
Ura	uracil
YTH	yeast two hybrid system

Summary

Efficient synaptic transmission requires a high local specialization of pre- and postsynaptic cells. At the neuromuscular junction (NMJ), these specializations include aggregates of acetylcholine receptors (AChRs). Proteins of the postsynaptic apparatus implicated in the aggregation of AChRs include utrophin, a synapse-specific homolog of dystrophin (Ohlendieck et al., 1991; Bewick et al., 1992; Tinsley et al., 1992, 1994), α- and ß-dystroglycan (Ibraghimov-Beskrovnaya et al. 1992; Fallon and Hall, 1994), and rapsyn (Frail et al., 1988; Apel et al., 1995), thought to link AChRs to the cytoskeleton. Agrin, a heparansulfate proteoglycan that is synthesized by motor neurons and deposited into the synaptic basal lamina, was shown to trigger redistribution of AChRs to form postsynaptic aggregates (McMahan, 1990; Wallace, 1996; Ruegg and Bixby, 1998). It is now clear that agrin organizes postsynaptic differentiation by stimulating MuSK, a receptor tyrosine kinase that is expressed selectively at in skeletal muscle (Jennings et al., 1993; Valenzuela et al., 1995; Glass et al., 1996). Agrin and MuSK are essential for synapse formation, as mice lacking agrin or MuSK fail to form neuromuscular synapses and consequently die at birth because of a failure to move or breathe (Gautam et al., 1996, DeChiara et al., 1996). Nevertheless, the mechanisms by which agrin activates MuSK are poorly understood. Agrin stimulates the rapid tyrosine phosphorylation of MuSK in myotubes, but, if transiently expressed in fibroblast or myoblasts, is not phosphorylated by agrin (Glass et., al 1996). These data thus indicate that activation of MuSK depends on at least one additional component expressed in myotubes but not in myoblasts. The current hypothesis predicts that this component, which was termed muscle-associated specificity component (MASC; Glass et al., 1996), together with MuSK form an agrin receptor complex.

In this thesis, we investigated of how agrin activates MuSK and of how the signal is transmitted further downstream leading to the accumulation of AChRs at the synapse. In a first part, we aimed to identify proteins that are associated with MuSK using the membrane bound split-ubiquitin system, a method that is based on the yeast two-hybrid (YTH) system. In contrast to the original YTH system, this novel method allows to screen for proteins that pass the membrane or are associated with it. In a first step, we showed that a bait and pray both containing a constitutively active form of MuSK, activates the reporter genes by self dimerization indicating that this system is a valuable method for identifying components not intact with MuSK. Moreover, we also demonstrated that the bait MuSK and a soluble form of agrin used in the YTH-screen are correctly expressed indicating that the system does not generate false-positive signals and can be used for screening. After these validation experiments, a total number of $3x10^7$ clones were screened resulting in >5'000 putative candidates. However, none of them could be could not be reconfirmed

in bait dependency tests. After several attempts to improve the method per se and to decrease the number of candidates, we were forced to drop the project as it turned out that the system had many intrinsic problems that could not be solved in a useful time window.

In a second project, we therefore concentrated to on mapping sites in agrin important for its MuSK phosphorylation and α-dystroglycan binding property. As agrin's AChR clustering activity is mediated by the most C-terminal laminin G-like domain we concentrated on this region. Moreover, one particular splice variant containing an exon of 8 amino acids in length within the B/z splice site of the LG3 domain was shown to be the most potent isoform, whereas splice variants lacking an insert in the B/z splice site are not active at all (Gesemann et al., 1996). Guided by the crystal structure of the LG3 domain derived from different splice variants (Stetefeld et al., 2004), we analyzed the contribution of single amino acids within the B/z-8 exon of agrin in activate MuSK and show that the activity resides mostly within the side chains of a three-peptide motif 'Asn-Glu-Ile', which is also highly conserved between species. In addition, we demonstrate that amino acids flanking the B/z splice site also strongly contribute to agrin's activity. Finally, we demonstrate that binding affinity to α-DG positively affects its MuSK phosphorylation activity. Based on these data we propose a model where α-DG plays an auxiliary role in capturing agrin at the muscle surface and thus efficiently presents the molecule to the agrin receptor complex.

In summary, the results reported in this thesis are a further step to elucidate the detailed mechanism of how agrin instructs the muscle to form postsynaptic structures. As similar mechanisms are also of work in the formation of synapses in the brain, these results are likely to be also important for furthering the understanding of how these structures are formed and altered during development and in process of learning and memory.

Chapter 1

Development of the neuromuscular junction: A general introduction

1.1 The synapse

First described for neurons, synapses are particularly elaborate structures where information is processed among two cells. Synapses form the cell-cell contact between two neurons or between neuron and muscle. The formation of synapses requires a series of steps including the generation of neurons and their target cells, the guidance of axons to their targets, the formation of selective connections between the developing axon and its target, the differentiation of the axon's growth cone into a nerve terminal, and the elaboration of a postsynaptic apparatus in the target cell. To ensure a precise crosstalk between the involved cells, the synapse has to be a perfect structure. It is a highly specialized and refined structure designed to ensure a rapid and efficient transmission of an action potential into depolarization of the postsynaptic target organ. A fast and precise synaptic transmission is achieved by the close spatial apposition of the presynaptic zone, containing synaptic vesicles filled with a neurotransmitter, to the postsynaptic membrane. Although the principles and mechanisms of synapse formation are likely to be similar in the peripheral nervous system (PNS) and the central nervous system (CNS), much of our understanding about the mechanisms of synapse formation arises from studies of the vertebrate neuromuscular synapse the neuromuscular junction (NMJ). The neuromuscular junction is the synapse that motor neuron make on skeletal muscle fibers. The advantages of the NMJ compared to a synapse between two neurons are striking (Fig. 1.1). Its size, its simple structure and its accessibility makes it a powerful system for the analysis of synaptic development.

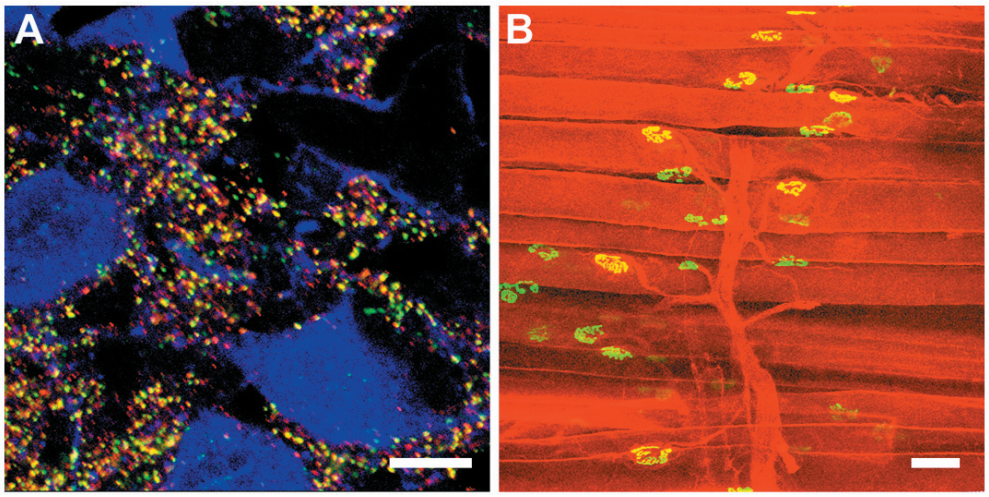


Figure 1.1 | Comparison of neuron-neuron synapses versus neuron-muscle synapse.

(a) The postsynaptic $GABA_A$-receptor $\alpha 2$ subunits (red) are clustered in dendrites of inferior olivary neurons (labelled with biocytin, blue) and colocalize (yellow) with the anchoring protein gephyrin (green). For details see (Devor et al., 2001). (b) A whole-mount view of neuromuscular synapses in rat soleus muscle. Nerve branches from presynaptic motor neurons and the postsynaptic muscle fibers were visualized with antibodies against laminin $\alpha 2$ (red). Postsynaptic AChRs (green or yellow) accumulate at the neuromuscular junctions. Note the marked difference in synapse number and size among the tissues. Scale bar in (a) 10µm in (b) 50µm. Adapted from Bezakova et al., 2003.

1.2 Development of the neuromuscular junction

The NMJ comprises portions of three cells - motor neuron, muscle fiber and Schwann cell (reviewed in Couteaux, 1973; Ogata, 1988; Engel, 1994; Sanes and Lichtmann, 1999) - which are highly specialized, containing high concentrations of organelles and molecules found at low concentrations extrasynaptically. They all originate from different precursor cells. Myoblast arise from the somite, motor axons from somata in the neural tube, and Schwann cells from the neural crest. During development all three cells travel long distances to meet at the synapse. Once they reach sites of muscles, myoblasts fuse to form myotubes, a multinucleated cell which can contain several hundred to several thousand nuclei. Just when or after myotubes form, they are approached by motor axons, followed by Schwann cells. Once the motor axon's growth cone contacts a newly formed myotube, signals are exchanged between nerve and muscle that initiate the formation and assembly of a highly differentiated presynaptic nerve terminal and a highly specialized postsynaptic apparatus (Fig. 1.2 a; Dennis, 1981; Hall and Sanes, 1993; Jennings and Burden, 1993).

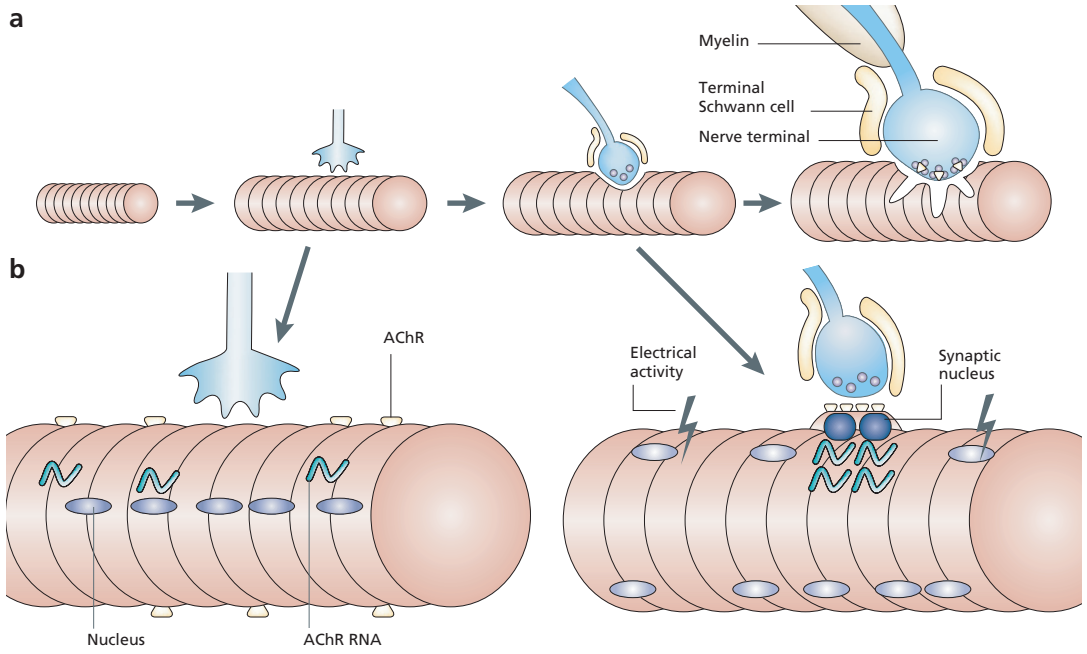


Figure 1.2 | Development of the neuromuscular junction.

(a) The motor axon approaches a newly formed myotube. At the area of contact, the axon differentiates into a motor nerve terminal that is specialized for transmitter release, Schwann cell process cap the terminal, and the muscle forms a complex postsynaptic apparatus. (b) Clusters of AChRs at the neuromuscular junction form. AChRs are initially present at moderate level throughout the muscle surface. In contrast, in adult muscle, AChRs are highly concentrated in the postsynaptic membrane and virtually absent extrasynaptically. This clustering involves both redistribution of acetylcholine receptors, and localized synaptic synthesis of AChRs. The local synthesis results from enhanced transcription of AChR genes by subsynaptic nuclei and by repression of synthesis in extrasynaptic nuclei by electrical activity. Adopted from Sanes and Lichtman, 2001.

Muscle differentiation and synapse formation occur concomitantly during development. Functional synapses form within minutes to hours after contact between developing motor nerves and myotubes (Fischbach, 1973; Anderson and Cohen, 1977). Over a period of about a week, a fully functional, albeit immature synapse forms in which both nerve and muscle are greatly transformed. In mammals it takes until several weeks after the first contacts are made to become a mature and fully differentiated synapse. The formation of a mature synapse requires further arborization of nerve terminals, withdrawal and editing of synaptic connections, an increase in the efficiency of neurotransmitter release, and additional modifications of the postsynaptic membrane. The muscle fiber will ultimately be innervated by a single motor axon that terminates and arborizes over ~0.1% of the whole cell surface.

The postsynaptic differentiation at the NMJ is characterized by three major signaling processes (Sanes and Lichtman 1999, 2001; Huh and Fuhrer 2002). First, during synaptic differentiation, proteins initially present along the entire muscle cell surface are redistributed and concentrated at the site of contact with the axon of the motor neuron. One such protein is the acetylcholine receptor (AChR). It's recruitment to sites of nerve-muscle contact represents a hallmark of inductive events of synapse formation. An extremely high concentration of AChRs is needed at the postsynaptic membrane in order to ensure a rapid and reliable response to acetylcholine, the neurotransmitter released from the overlying nerve terminal (reviewed in Salpeter and Loring, 1985). Initially the AChR receptors have a uniform density of ~1000/μm^2 over the whole plasma membrane (Bevan and Steinbach, 1977; Merlie, 1984). In mature muscle the density of AChRs reaches >10'000/μm^2 synaptically and falls to ~10/μm^2 extrasynaptically (Fig. 1.2 b; Salpeter et al., 1988). Second, the differential distribution of synaptic proteins, including AChRs, is enhanced by selective transcription of genes encoding these proteins from only those nuclei underneath the nerve terminal (Fig. 1.2 b). Third, transcription of RNAs from extrasynaptic nuclei is downregulated in response to depolarization of the myotubes caused by AChR activation. While downregulation of protein synthesis is clearly driven by electrical activity (Goldman et al., 1988; reviewed in Duclert and Changeux, 1995), aggregation of proteins at the nerve-muscle contact depends on instructive signals that are released by the motor axon.

In a pivotal series of experiments begun in the late 70s, U.J. McMahan and colleagues demonstrated that molecules stably associated with the synaptic portion of the myofiber's basal lamina sheath trigger the formation of presynaptic and postsynaptic specializations in regenerating nerve and muscle fibers. In these experiments, axons of motoneurons and myotubes were destroyed and only the basal membrane was left intact. When presynaptic and postsynaptic cells were allowed to regenerate they formed synapses at the same position where they originally were located (Sanes et al., 1978; Burden et al., 1979).

Following the demonstration that neurites organize AChR clusters at sites of nerve-muscle contact (Anderson and Cohen, 1977; Frank and Fischbach, 1979), and that basal lamina associated molecules assume instructive properties, several groups used cultured myotubes to seek clustering agents. Typically, a fluorescent derivative of α-bungarotoxin (α-Btx), a very specific quasi-irreversible ligand, was used to assay AChR distribution *in vivo* and on myotubes in culture (Lee, Tseng, and Chin, 1967). Proteins of the basal lamina were tested on their activity on cultured myotubes. It turned out that molecules that are active in this assay include fibroblast growth factor (Peng et al., 1991), HB-GAM/pleiotropin (Peng et al., 1995; Rauvala and Peng 1997), laminin (Sugiyama et al., 1997; Montanaro et al. 1998), midkine (Zhou et al., 1997), and agrin. Of these only agrin has so far been implicated in synaptogenesis in vivo.

Based on the biological activity of agrin and its tissue distribution McMahan postulated in 1990 the 'agrin hypothesis' where he proposed that agrin is the nerve derived trophic factor that is responsible for the differentiation and assembly of the postsynaptic apparatus in vivo. Thereafter, it was demonstrated that agrin is synthesized by motoneurons, transported down motor axons, and released from motor nerve terminals, and incorporated into basal lamina of the synaptic cleft (McMahan, 1990; Reist et al., 1992; Cohen and Godfrey, 1992).

1.3 Agrin

Agrin – from the Greek 'agrein' meaning 'to assemble' which was named after its ability to aggregate AChRs - was purified by McMahan and colleagues using basal lamina extracts from electric organ of the pacific electric ray *Torpedo californica*, which is a plentiful source of NMJ-like synaptic components (Godfrey et al., 1984; Nitkin at al., 1987). Agrin was then molecularly cloned from mammals and birds (Rupp et al., 1991, Tsim et al. 1992). In all of the species from which agrin was cloned, the gene encodes a protein of more than 2'000 amino acids with a predicted mass of 225 kDa. The extensive N- and O-glycosylation of the amino terminal half increases the apparent molecular mass of agrin to 400-600 kDa. At least three of the O-linked carbohydrate attachment sites function as docking sites for heparan sulfate glycosaminoglycan (HS-GAG) side chains and assign agrin to the family of heparan sulfate proteoglycans (Fig. 1.3; Tsen et al., 1995; Denzer et al., 1998). These heparan sulfate chains are clearly important for mediating the binding of some other molecules. Fibroblast growth factor 2 (FGF2), thrombospondin, and neural cell adhesion molecule (NCAM) can bind agrin via heparan sulfate chains (Cole and Halfter, 1996; Cotman et al., 1999). In addition, laminin-1 and laminin-2 bind agrin in part via heparan sulfate chains (Cotman et al., 1999). Agrin can undergo extensive alternative messenger RNA splicing,

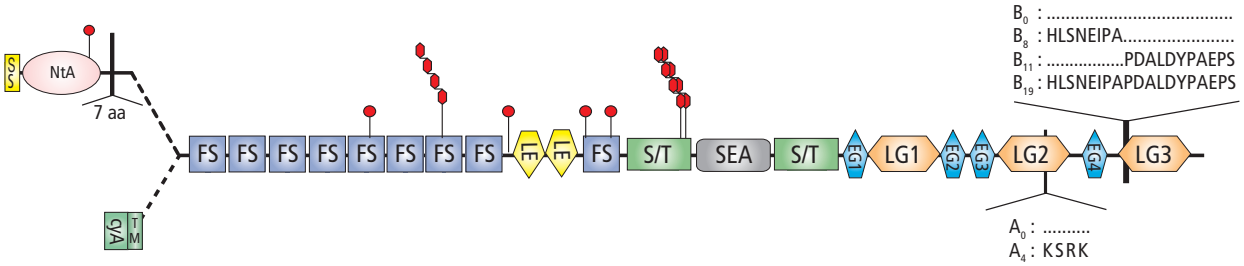


Figure 1.3 | Schematic representation of full length chick agrin.

Cartoon showing the structural domains of agrin, the sites of alternative messenger RNA splicing and the glycosylation sites. SS, signal sequence; NtA, amino terminal agrin domain; TM, transmembrane segment; FS, follistatin-like domain; LE, laminin EGF-like domain; S/T, serine/threonine rich region; SEA, sperm protein, enterokinase and agrin domain; EG, epidermal growth factor domain; LG, laminin globular like domain.

which gives rise to proteins, which differ in function and localization. Alternative splicing gives rise to two agrin isoforms with different amino terminus. One isoform encodes a cleaved signal sequence (SS) followed by the amino (N)-terminal-agrin (NtA) domain (Fig. 1.3). This form of agrin (SS-NtA-agrin) is expressed in both neural and non neural tissue, appears to account for most of the agrin in motoneurons and muscle, and is efficiently secreted and incorporated into the basal lamina (Denzer et al., 1995; Burgess et al., 2000). The other isoform encodes a shorter amino terminus with an internal, non-cleaved signal peptide, which converts the protein to a type II transmembrane (TM) protein (Burgess et al., 2000; Neumann et al., 2001). This splicing affects the association of agrin with the ECM, as only the SS-NtA-agrin isoform can bind to laminins (Denzer el al., 1995, 1997; Mascarenhas et al. 2003). In contrast TM-agrin is mostly found in the brain, which does not contain any basal lamina. The laminin binding or the transmembrane domain are followed by nine follistatin-like (FS) modules, two laminin EGF-like (LE) repeats, two serine/threonine (S/T) rich regions and a sea urchin sperm like module (SEA) (Fig. 1.3). The carboxy (C)-terminal half of agrin, containing 4 EGF-like (EG) and 3 laminin G-like (LG) modules, encodes the fragment that was originally purified by McMahan and colleagues (Nikitin at al., 1987), and is sufficient to induce AChR clustering on cultured muscle cells. This carboxy terminal part that is common to both, the SS-NtA and the TM-agrin isoforms, is subjected to alternative splicing (Fig. 1.3). These splice sites are known as A/y in LG2 domain and B/z in LG3 domain, where A and B is used to denote these sites in avians (Ruegg et al., 1992) and y and z in mammals (Ferns et al 1992). The A/y site contains a highly positively charged 4 amino acid insert (KSRK) which is essential for the binding of the inhibitor heparin to agrin, but does not affect the clustering activity of the neuron-specific isoforms of agrin (Hopf and Hoch, 1997; Gesemann et al., 1996; Campanelli et al., 1996). The best characterized splice region in the C-terminus of agrin is the B/z site, which is encoded by two exons of 24-bp and 33-bp in length. Thus, the protein either lacks the corresponding amino acids or contain 8, 11 or 19 (8+11) amino acid-long inserts (Tsim et al., 1992; Hoch et al., 1993; Wei et al., 1997). AChR aggregation assays on cultured myotubes showed

that only those splice variants containing amino acid inserts, at splice site B/z collectively called neural agrin, induce the clustering of AChR, while the variants lacking inserts, also called muscle agrin, was inactive (Ruegg et al., 1992; Ferns et al., 1993).

Three lines of evidence indicate that agrin is necessary for the clustering of AChRs at synaptic sites and for inducing postsynaptic differentiation. First, antibodies against agrin block AChR clustering at nerve-muscle synapses that form in culture (Reist et al., 1992). Second, agrin deficient mice lack differentiated NMJs including clusters of AChRs and of other postsynaptic markers (Gautam et al., 1996). Agrin also regulates the distribution of other synaptic proteins, including neuregulin (NRG), and NRG receptors (ErbBs), indicating that agrin has a central role in synaptic differentiation (McMahan, 1990; Apel et al., 1995; Rimer et al. 1998; Meier et al., 1997). Third, in a gain of function experiment, agrin complementary cDNA or recombinant agrin was microinjected ectopically into myofibers or into the muscle respectively *in vivo*. In the case of neural, but not muscle agrin isoforms, the injection causes the formation of ectopic postsynaptic specializations that contain clustered AChRs in association with many other postsynaptic proteins, deep membrane folds and adult type AChRs (Bezakova et al., 2001; Cohen et al., 1997; Meier et al., 1997; Jones et al., 1997; Rimer et al., 1997, 1998).

1.4 The agrin receptor

In efforts to understand how agrin acts, a number of proteins have been identified that interact with agrin via different domains. The N-terminal region of agrin interacts with laminins, tenascin, FGF-2, NCAM, HB-GAM/pleiotrophin, thrombospondin and ß-amyloid; whereas the C-terminal region associates with α-dystroglycan, heparin/heparan sulfates, and integrin (Bowe et al., 1994; Campanelli et al., 1994, 1996; Gee et al., 1994; Sugiyama et al., 1994; Martin and Sanes, 1995, 1997; Mook-Jung and Gordon, 1995; Storms et al, 1996; Dagget et al., 1996; Gesemann et al., 1996; O'Toole et al., 1996; Hopf and Hoch, 1996; Denzer et al., 1997; Zhou et al., 1997; Cotman et al., 1999,2000). Since the C-terminal region of agrin is sufficient to induce AChR clusters on cultured myotubes, all proteins which bind to the amino-terminus of agrin were excluded. Of all the other proteins, only α-dystroglycan was initially proposed as a functional agrin receptor that mediates downstream signaling leading to AChR aggregation (Bowe et al., 1994; Gee et al., 1994; Campanelli et al., 1994; Sugiyma et al., 1994).

1.4.1 α-dystroglycan

Dystroglycan is transcribed from a single gene and is post-translationally modified to yield two glycoproteins, namely α- and ß-dystroglycan. The N-terminal peripheral membrane protein α-dystroglycan (α-DG) links to the extracellular matrix via several ligands, whereas the transmembrane ß-dystroglycan (ß-DG) links α-DG by a tight but non-covalent link to the actin cytoskeleton via dystrophin (Ervasti and Campbell, 1991) or utrophin (Matsumura et al., 1992). The idea that α-DG could be the functional agrin receptor was supported by the fact that α-DG is a major binding protein for agrin and can bind agrin with high affinity. Moreover, agrin's binding to α-DG is Ca^{2+}-dependent as is the formation of AChRs and the binding can be inhibited by heparin. Furthermore antibodies against α-dystroglycan have been reported to perturb the formation of AChR clusters induced by agrin (Campanelli et al., 1994; Gee et al 1994). Contrarily, others have reported contemporarily the same antibodies to be ineffective in altering the response to agrin (Sugiyama et al., 1994). It was also shown that α-DG can also bind the 'inactive' agrin-B/z^- with high affinity (Gesemann et al., 1996; Hopf and Hoch, 1996; Sugiyama et al., 1994), but this form of agrin neither stimulates nor antagonizes AChR clustering by active, neural agrin (Hoch et al., 1994). In addition dystroglycan-binding sequences in agrin are not necessary for AChR clustering (Gesemann et al., 1995). Conversely, LG3-B/z^+, the minimal agrin fragment that can aggregate AChRs, does not bind to α-DG at all (Gesemann et al., 1996). Therefore, α-DG might be involved in the consolidation of postsynaptic structures but seems not to be involved in their formation, as it does not fulfill the criteria for a signaling receptor.

1.4.2 MuSK

Several groups provided contemporarily strong evidence for the involvement of another protein as putative agrin receptor, the muscle-specific transmembrane receptor tyrosine kinase, MuSK (Jennings et al., 1993; Ganju et al., 1995, Valenzuela et al., 1995).

MuSK was originally identified in an unrelated search for signaling molecules in muscle after denervation (Valenzuela et al., 1995). Its orthologue was also cloned from the electric organ of *Torpedo* (Jennings et al., 1993). At the adult NMJ, MuSK is concentrated at the postsynaptic site (Valenzuela et al., 1995; Ganju et al., 1995). Its extracellular domain (ECD) contains three IgG-like domains and 5 potential N-linked glycosylation sites, some of which are glycosylated (Fig. 1.4; Watty et al., 2002). Alternative mRNA splicing gives rise to several isoforms of MuSK that differ at two splice sites within the extracellular domain (Fig. 1.4; Valenzuela et al., 1995; Ganju et al., 1995; Hesser et al., 1999). Moreover, a Wnt-receptor cysteine-rich domain (CRD), defined by a

pattern of ten cysteine residues, was found in the ECD of MuSK (Fig. 1.4; Saldanha et al., 1998, Masiakowski and Yancopoulos, 1998). The CRD was suggested to act as binding site for Wnt proteins (Bhanot et al., 1996), but so far nothing is known about the biological function of this CRD. Intracellularly, MuSK contains a juxtamembrane (Herbst et al., 2000) domain followed by a kinase domain and a short C-terminal tail, which confers a PDZ binding domain (Zhou et al., 1999).

MuSK emerged as the best candidate agrin receptor since there are many data implicating that agrin acts as ligand for MuSK, but it turned out that MuSK alone is not sufficient. Gene targeting experiments were done to examine the role of MuSK in NMJ development. MuSK-deficient mice lack any pre- and postsynaptic specializations and die perinatally (Fig 1.5; DeChiara et al., 1996), as do agrin-deficient mice (Fig 1.5; Gautam et al., 1996). Cultured myotubes devoid of MuSK are not capable of aggregating AChRs in response to neural agrin, and the ability of myotubes to form AChR clusters recovers after MuSK expression is restored (Herbst et al., 2002). Agrin B/z$^+$, but not agrin B/z$^-$, induces MuSK phosphorylation and, as observed for AChR aggregation, the minimal active fragment comprises the LG3-B/z$^+$ domain (Gesemann et al., 1996). In cultured myotubes, agrin can be chemically crosslinked with MuSK, but agrin cannot activate MuSK expressed in myoblast or other undifferentiated cells (Glass et al., 1996). Based on this observation, it has been proposed that MuSK needs to associate with a myotube-associated specificity component, termed MASC, in order to constitute a fully functional receptor that both binds and responds to agrin (Glass et al., 1996). Despite substantial effort, the nature of MASC has remained largely elusive so far. It remains possible that a co-ligand or post translational modifications of MuSK, rather than a coreceptor, is required for agrin to stimulate MuSK.

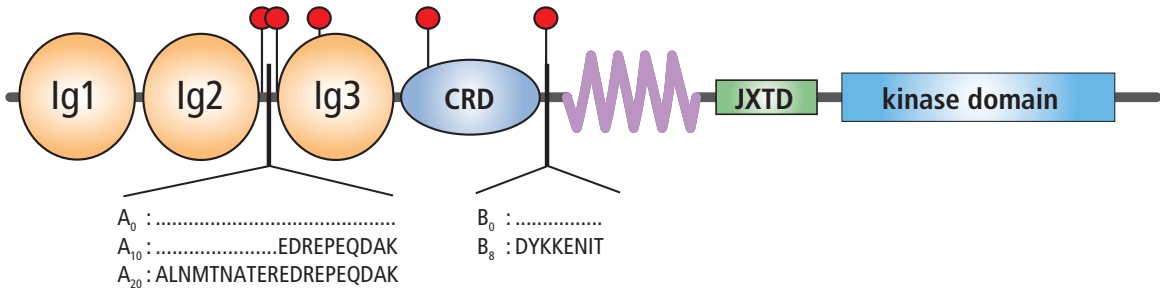


Figure 1.4 | Schematic representation of the muscle specific kinase.
Schematic drawing of MuSK showing its structural domains, the sites of alternative mRNA splicing and putative N-glycosylation sites. Ig, immunoglobulin-like domain; CRD, cysteine rich domain; JXTD, domain.

1.5 Mechanisms activated by agrin that lead to AChR clustering

Another important question is of how activated MuSK transmits a signal into the myotubes to induce organization of the postsynapse. Early experiments showed that a 43-kD protein, termed rapsyn, has an important role in agrin-mediated signaling. Rapsyn, originally identified as AChR-associated protein in *Torpedo* synaptic membranes (Sobel et al., 1978, Burden et al., 1983, Porter et al., 1983), is an amphiphatic, myristoylated peripheral membrane protein that is present at 1:1 stoichiometry with AChRs at synaptic site (Burden at al., 1983; Froehner et al., 1991; Phillips and Merlie, 1992).

Like the agrin and the MuSK knockouts, rapsyn-deficient mice have perturbed NMJs, and the animals die at birth due to failure to breathe. Presynaptic specializations still form, although the motor terminals arborize poorly and grow more extensively than in wild-type mice (Fig 1.5; Gautam et al., 1995). Importantly, normal clustering of AChRs, neuregulin receptors, utrophin and dystroglycan is lacking in rapsyn mutant mice (Gautam et al., 1995).

MuSK is the only postsynaptic protein to still be localized synaptically at mutant NMJs in rapsyn-deficient mice. In cultured myotubes from rapsyn mutant mice, neural agrin still induces rapid phosphorylation of MuSK, but the AChR ß-subunit does not become tyrosine phosphorylated. Likewise, myotubes cultured from these mutant mice do not form AChR clusters spontaneously nor in response to agrin, showing that rapsyn acts downstream of MuSK (Apel et al., 1997). This suggests that rapsyn serves as an adaptor molecule to link MuSK activation to further downstream events that are important for localization of molecules to the postsynaptic apparatus, like AChRs for instance.

These findings still leave the question open of how MuSK transduces the signal, following stimulation by agrin, further downstream. In response to agrin, MuSK undergoes rapid autophosphorylation, a response which occurs within minutes and precedes all putative downstream signaling events and AChR clustering (Glass et al., 1996). MuSK is phosphorylated on six of the nineteen cytoplasmic tyrosine residues (Watty et al., 2000). Such phosphorylated tyrosines could then act as docking sites for the binding of signal-transducing molecules, in analogy to many other RTKs. Several of MuSK's cytoplasmic tyrosine residues are indeed essential for AChR clustering in response to agrin in myotubes, as shown by mutational analysis of MuSK and transfection into $MuSK^{-/-}$ myotubes (Herbst and Burden, 2000; Zhou et al., 1999). In particular the juxtamembrane tyrosine (Y553) appears to be required for agrin-induced AChR clustering (Zhou et al., 1999; Herbst and Burden, 2000). The amino acid sequence surrounding Y553 fits a typical NPXY motif which is believed to interact with a phosphotyrosine binding (PTB) domain-

containing protein as it is the case for Shc and related proteins. However, the protein that binds to the NPXY motif remains to be identified.

Because MuSK is absolutely essential for the pre- and postsynaptic differentiation at the NMJ, extensive efforts have been invested to understand of how MuSK transduces the signal. Using the intracellular region of MuSK as bait, Dishevelled (Dvl) was identified as a MuSK binding protein. Dvl was originally discovered in Drosophila for its role in the development of coherent arrays of polarized cells (Perrimon and Mahowald, 1987). Dishevelled 1 is enriched at the NMJ (Luo et al., 2003) and inhibition of Dvl function or expression, which has no effect on AChR expression in muscle cells, attenuates agrin-induced AChR clusters and the formation of the neuromuscular synapse in culture (Luo et al., 2002). Interestingly, MuSK shares a conserved extracellular CRD domain with the Wnt receptor Frizzled (Dann et al., 2001; Masiakowski and Yancopoulos, 1998; Xu and Nusse, 1998). The finding that Dvl regulates agrin-induced AChR led to determine whether the Wnt signaling pathways are involved in this event: the canonical pathway and the planar cell polarity pathway involving activation of Rho GTPases (Habas at al., 2001). The finding that Dvl interacts with MuSK suggests that one or both pathways of Wnt may be involved in regulating or mediating AChR clustering. Indeed, it was shown that agrin stimulates Cdc42 and Rac, both of which are required for AChR clusters (Weston et al., 2000, 2003). Moreover, PAK is activated by agrin and is required for AChR clusters (Luo et al., 2002). Importantly, agrin-induced PAK activation is attenuated in muscle cell is expressing mutant Dvl, suggesting the involvement of Dvl in this event. However, the underlying mechanisms of the Dvl participation remain unclear.

Beside MuSK, agrin stimulates also tyrosine phosphorylation of AChRs ß- and ∂-subunits (Ferns et al., 1996; Fuhrer and Hall, 1996; Qu and Huganir, 1994; Wallace et al., 1991). However, the function of AChR tyrosine phosphorylation is not well understood. It was shown that it is insufficient to cluster AChRs, as AChR tyrosine phosphorylation but not AChR clustering is stimulated in muscle cells that are transfected with TrkC/MuSK chimera and treated with NT3 (Glass et al., 1997). Therefore, tyrosine phosphorylation of AChRs could regulate AChR clustering by initiating a link between AChRs and the cytoskeleton (Wallace, 1992). Consistent with this idea, a phosphotyrosine (Y393) -containing sequence in the ∂-subunit serves as docking site for Grb2 (Colledge and Froehner, 1997), raising the possibility that Grb2 serves as adaptor to link tyrosine phosphorylated ∂-subunits to additional proteins, possibly a cytoskeletal complex.

Moreover, the kinase that phosphorylates AChR appears to be distinct from MuSK (Ferns et al., 1996; Fuhrer et al., 1997; Wallace et al., 1994). Indeed, agrin causes rapid activation of Src-related kinases (Mittaud et al., 2001), but addition of staurosporine blocks agrin-stimulated tyrosine phosphorylation of AChRs without inhibiting tyrosine phosphorylation of MuSK, indicating that tyrosine kinases other than MuSK catalyze AChR tyrosine phosphorylation (Swope and Huganir,

1993; Ferns et al. 1996; Fuhrer and Hall, 1996; Fuhrer, 1999). Furthermore, agrin stimulates tyrosine phosphorylation and clustering of AChRs in mutant muscle cells lacking either Src and Fyn or Src and Yes and in muscle cells treated with inhibitors for Src-family kinases (Smith et al., 2001, Mohamed et al., 2001). Thus kinases other than Src-family kinases appear to catalyze AChR tyrosine phosphorylation and clustering.

While these studies begin to piece together signaling pathways that lead to AChR clustering, the exact pathway from MuSK to downstream molecules remains unclear.

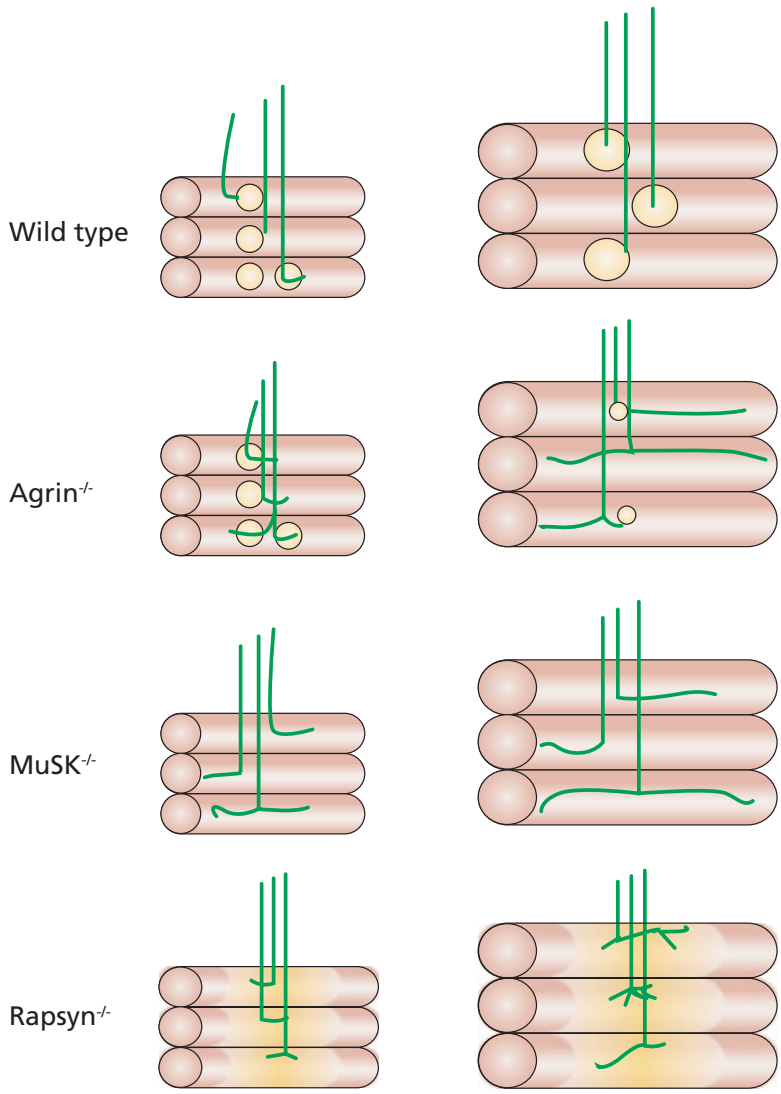


Figure 1.5 | Genetic analysis of early AChR clustering events in mice deficient for MuSK, agrin or rapsyn.

In newly innervated wild-type muscles (embryonic day (E) 14), some axons terminate on AChR-poor parts of the myotube surface and some AChR clusters are unoccupied by nerves. By E15 (E18 is shown here), the apposition of pre- and postsynaptic specializations is markedly improved. AChR clusters form normally in mutant lacking B/z^{+} agrin or all forms of agrin. Later, clusters become smaller, dimmer and fewer in agrin mutants. No AChR clusters form in mice lacking muscle-specific kinase or rapsyn, but localized AChR transcription persists (yellow region) in the absence of rapsyn. Adapted from Sanes and Lichtman, 2001.

1.6 Glycosylation at the synapse

Differential glycosylation of MuSK and other synaptic molecules could be an alternative way to explain the difficulty in identifying MASC. The possibility that cellular interactions within the vertebrate nervous system are mediated by cell surface carbohydrates has been considered on numerous occasions. The initial suggestions that carbohydrate structures might mediate neural cell adhesion were based on the expression of complex oligosaccharides, in particular gangliosides, by neural cells, and the detection of cell surface glycosyltransferases that were proposed to function non-enzymatically as receptors for cell surface oligosaccharides (Roseman, 1970; Roth et al., 1971; Marchase, 1977; Suhr and Roth, 1975). Subsequent progress in elucidating the function of cell surface carbohydrates in the nervous system has, however, been slow, in part because of the difficulties (i) in purifying and characterizing complex oligosaccharides that are expressed on small subsets of neural cells and (ii) in generating these structures synthetically. In addition, the identification of cell surface molecules such as NCAM, N-cadherin, and integrins (Edelman, 1986a, 1986b; Takeichi, 1988; Ruoslathi and Pierschbacher, 1987; Rutishauser and Jessel, 1988) has focused attention on mechanisms of neural cell adhesion that involve direct protein-protein interactions.

While it is clear that protein-protein associations are important for the aggregation of molecular complexes at the neuromuscular synapse, the role of carbohydrates in this process is largely unknown. Given that very high concentrations of carbohydrates are present at synapses, it is likely that their structure contributes to the organization of synaptic molecules. In fact, most of the molecules found in the extracellular matrix, which are important during development, regeneration and synaptic plasticity, are highly glycosylated with carbohydrate structures. Moreover, it was shown that synaptic molecules of the NMJ have unique carbohydrate structures with respect to the extrasynaptic membrane (Martin and Sanes, 1995; Martin et al., 1999). Therefore, carbohydrates have probably more than just a passive role at the synapse, they may also affect synaptic adhesion, signaling, and transmission (Martin, 2002).

Given that many different carbohydrate structures can be synthesized by a single cell, the potential complexity of carbohydrate structures, on a molecule per molecule basis, exceeds that of proteins. For example, combining the eight common mammalian carbohydrates into a chain seven carbohydrates long in all possible configurations and forms would yield a number of structures that exceeds the number of neurons in the brain. The tremendous structural diversity of glycan chains allows for immense combinatorial possibilities that might underlie the fine-tuning of cell-cell and cell-matrix interactions as well as signaling processes (reviewed in Martin, 2003; Kleene and Schachner, 2004).

1.6.1 Glycosylation important for agrin signalling?

One half of the apparent molecular weight of agrin on SDS-PAGE is based on protein mass and the other half is due to carbohydrates. Several groups therefore suggested that carbohydrates might also be important for agrin signaling. Initial experiments showed that heparin and heparan sulfates inhibit agrin signaling in muscle cells (Wallace, 1990) and agrin binding to α-dystroglycan (Campanelli et al., 1996, Gesemann et al., 1996). Moreover, muscle cells that are deficient in heparan sulfate biosynthesis are defective in agrin-induced AChR clustering (Gordon at al., 1993; Sugiyama et al., 1994; Bowen et al., 1996).

The involvement of carbohydrate chains of agrin in the binding of agrin to its physical receptor can immediately be excluded, since the smallest active G3-B/z^+ fragment has neither N- nor O-glycosylation sites. Conversely, it has been shown that the three LG-like domains of agrin can mediate binding to carbohydrates (Xia and Martin, 2002). Thus, agrin may bind to other proteins via their carbohydrate chains.

The selective expression of certain glycosyltransferases at synaptic sites in skeletal muscle, raises the possibility that carbohydrate modifications of MuSK, expressed selectively in myotubes, may be essential for agrin to bind and activate MuSK. In experiments done by Watty and Burden (2002), *N*-linked glycosylation sites on MuSK were analyzed with regard to agrin activation. By mutating these sites and expressing mutant proteins in MuSK-deficient myotubes, they found that agrin activation of MuSK did not require the *N*-linked glycosylation sites on MuSK protein. These mutants, however, did posses a higher level of spontaneous activation, suggesting that *N*-linked glycosylation serves to alter MuSK structure or function in some still unknown way. These results are consistent with observations made for TrkA, the receptor tyrosine kinase for nerve derived growth factor (NGF), where non glycosylated TrkA receptors become constitutively active (Watson et al., 1999). Conversely, adding sialic acid or sialyl-*N*-acetylglycosamine, which 'cap' the end of the carbohydrate chains, to the culture medium of myotubes reduces agrin-induced AChR clustering (Grow and Gordon, 2000; Xia and Martin, 2002). Finally, a particular *N*-acetylglycosamine transferase that attaches specific *ß*-linked *N*-acetylglycosamine moieties to carbohydrate side chains is enriched at the NMJ and, when overexpressed throughout skeletal muscle, it causes the formation of ectopic AChR clusters and affects postsynaptic differentiation at the NMJ (Xia and Martin, 2002).

Table 1.1 | Potential roles of carbohydrates in synaptic protein function (reviewed in Martin 2003)

Protein	Type of glycosylation	Function of glycosylation
Dystroglycan	O-linked (via Mannose) CT antigen (O-linked)	Laminin, agrin, perlecan, neurexin binding Altered matrix/utrophin binding
Agrin	GAG (Heparan sulfate) O-linked (via GalNAc)	NCAM, laminin, FGF2 binding FGF2 signaling Musk signaling
MuSK	N-linked	Spontaneaus activity
NCAM	Polysialyc acid (N-linked)	Agrin binding Homophilic binding
V-gated Na Channel	Sialic acid (N-linked)	Voltage gating
Biglycan	GAG (Chondroitin sulfate)	Dystroglycan binding
Perlecan	GAG (Heparan sulfate)	Acetylcholinesterase binding
Acetylcholinesterase	GalNac (linkage unknown)	Unknown
Laminin	N-linked	Galectin, integrin binding
AChR	N-linked	Bungarotoxin binding

Abbreviations: GAG, glycosaminoglycan; NCAM, neural cell adhesion molecule; FGF-2, basic fibroblast growth factor 2; MuSK muscle-specific kinase; V-gated Na channel, Voltage-gated sodium channel; AChR, acetylcholine receptor.

1.7 Alternative splicing

Alternative splicing is a common mechanism for regulating gene expression in mammalian cells. Protein structure and activity is often altered by the variable inclusion of coding exons or portions of exons into an mRNA. Alternative splicing of pre-mRNAs is therefore a powerful and versatile regulatory mechanism that is used to enhance the information contained within a gene. An example for the highly complex splicing events is illustrated in the case of the 'Down syndrome cell adhesion molecule' (Dscam) of *Drosophila melanogaster*, where one single gene can give rise to over 38'000 different alternatively spliced mRNA isoforms, which is about 2-3 times the number of predicted genes in the entire organism. This issue would become even more complex, if we would consider also other RNA processing events, such as RNA editing for instance (reviewed in Graveley, 2001).

Some alternative splicing events appear to be constitutive, with mRNA variants coexisting at constant ratios in the same cell, whereas others are regulated in response to developmental or physiological cues. The consequences range from switching expression of a protein on and off (e.g. by including or excluding stop codons as in Sxl (Bopp et al., 1991)), to structural and functional diversification of protein products (by including or excluding elements as small as a single amino acid, for example in Pax-3 and Pax-7 (Vogan et al., 1996)). Hence, alternative splicing is a versatile process that can be integrated with other regulatory mechanisms to generate complex genetic switches, to modulate cellular responses to developmental and physiological signals, to

fine tune the function of regulatory factors, and to diversify the biochemical inventory within and between cells.

The mechanism of alternative splicing, is extensively used in the nervous system. Indeed, splicing events in the nervous system are known for a vast category of proteins, which include receptors for signaling molecules (e.g. MuSK, neurexin, alpha and beta subunit of integrins, fibroblast growth factor receptor (Ganju et al., 1995; Valenzuela et al., 1995; Missler and Südhof, 1998; Fornaro and Languino, 1997; Ornitz et al., 1996; Shi et al., 1993)), signaling molecules (e.g. neurexins, neuregulins (reviewed in Missler and Südhof, 1998; Graveley, 2001; Lemke, 1996)), and extracellular matrix and cell adhesion molecules (e.g. agrin, laminin, fibronectin, neuroligin (Ruegg et al., 1992; Rupp et al., 1992; Thomas et al., 1993; Kornblihtt et al., 1996; Sherman et al., 1998; Scheiffele et al., 2000).

The importance of alternative splicing is nicely illustrated also in processes which lead to the induction of the formation of the postsynaptic apparatus. In the early 90' McMahan and colleagues could show that agrin is modulated by such events, and that the activity of agrin relies on one single exon, which is only 24 nt in length. Interestingly, agrin is expressed almost in the whole body, like brain, spinal cord, kidney, lung, blood capillaries, muscles and motor neurons for instance (Stone and Nikolics, 1995). But depending on its localization and the task agrin has to fulfill it is differently spliced.

MuSK is thought to be a part of the agrin receptor complex, but does not bind agrin itself (Glass et al., 1996). Moreover the putative coreceptor MASC has not been identified yet. Alternative splicing therefore raises the possibility that MuSK expresses a splice variant which confers direct binding to agrin. Unfortunately, in contrast to agrin, splice variants of MuSK have poorly been characterized. So far there are reports about three different splice sites in the extracellular part and one in the intracellular part of MuSK (Ganju et al., 1995; Valenzuela et al., 1995; Hesser et al., 1999). Their function and distribution is totally unknown and has still to be elucidated.

1.8 Aim of the thesis

In this thesis I aimed in elucidating of how agrin induces postsynaptic differentiation of the neuromuscular junction. In a first approach, I tried to identify the agrin receptor and other proteins associated with the muscle specific kinase in order to understand of how agrin activates the signaling pathway leading to AChR clustering. In a second project, the importance of the B/z splice insert in activating the MuSK pathway was analyzed. Moreover, I found a correlation between the ability of agrin to bind to α-DG and its MuSK phosphorylating activity, showing that α-DG plays an important auxiliary role in activation of the agrin receptor complex.

Chapter 2

Application of the split-ubiquitin membrane yeast two-hybrid system to investigate MuSK interactors

2.1 Summary

Since the postulation of the agrin-hypothesis by McMahan in 1990, many groups were trying to unmask the identity of the agrin receptor. First, α-DG was thought to be the physical receptor as it was found to be the main binding protein of agrin on the muscle surface (Bowie et al., 1994; Gee et al., 1994), but very soon this hypothesis was disproved. Right after MuSK was proposed to be the agrin receptor, as the release of agrin by the nerve leads to rapid activation of MuSK to mediate AChR clustering (Glass et al., 1996). Furthermore, it was shown that both agrin and MuSK are essential for synapse formation, as mice deficient for agrin or MuSK lack any pre- and postsynaptic specialization (Fig. 1.5; DeChiara et al., 1996; Gautam et al., 1996). Importantly, postsynaptic clusters of AChR are completely absent in MuSK-deficient mice (DeChiara et al., 1996). It is now clear that MuSK is the signaling component activated by neural agrin. However, MuSK does not bind directly to neural agrin indicating that activation of MuSK depends on a muscle-associated specific component (MASC; Glass et al., 1996). Many groups tried to identify the nature of MASC using many different approaches unsuccessfully. We now use a new approach based on the method of the yeast two-hybrid system to identify the so long sought agrin receptor and other MuSK associated proteins.

2.2 Introduction

The neuromuscular junction is a synapse between motor neurons and skeletal muscle fibers that exhibits the high degree of subcellular specialization characteristic of chemical synapses (Hall and Sanes, 1993; Sanes and Lichtman, 1999, 2001). Due to its relative simplicity and accessibility, the NMJ has been studied extensively as a model of synaptogenesis. A hallmark of the NMJ is the high concentration of synaptic proteins including AChRs in the postsynaptic membrane that guarantees fast and accurate neurotransmission (Burden, 1998; Colledge and Froehner, 1998; Fallon and Hall, 1994, Sanes and Lichtman, 2001). In the absence of innervation, the muscle seems to be prepatterned (Ferns and Carbonetto, 2001; Lin et al., 2001; Yang et al., 2001, 2000). Small AChRs clusters aggregate in the central region of the muscle, forming a central band that appears to be wider and more poorly defined than in innervated muscle. Upon innervation, AChR clusters are restricted to a narrow central band. Studies of regenerating synapses demonstrate that the synaptic basal lamina contains a signal to cause redistribution of AChRs (McMahan et al., 1992). This signal is believed to be agrin, a 225 kD polypeptide that is expressed in motoneurons, transported to presynaptic nerve terminals, and deposited in the basal lamina in the synaptic cleft. Neural agrin induces postsynaptic differentiation in cultured muscle cells (Campanelli et al., 1991, Tsim et al., 1992, Wallace 1989). Studies of mutant mice convincingly demonstrate that agrin is essential for AChR clustering in the membrane opposite the presynaptic terminals (Gautam et al., 1996, Ruegg and Bixby, 1998).

MuSK, a receptor tyrosine kinase, was discovered because of its abundance in the synaptic-rich Torpedo electric organ, whose principal cells are modified muscle cells that are innervated by cholinergic synapses (Jennings et al., 1993). Studies of the MuSK mouse homologue reveal that MuSK is specifically expressed in skeletal muscle and is colocalized with AChRs in the postsynaptic membrane of the NMJ (Valenzuela et al., 1995). Agrin activates MuSK, and expression of a dominant-negative form of MuSK inhibits agrin-induced cluster formation in cultured myotubes (Glass et al., 1996). The extracellular domain of MuSK inhibits agrin-induced AChR clustering in C2C12 muscle cells (Glass et al., 1996). Moreover, $MuSK^{-/-}$ mice fail to form the NMJ (DeChiara et al., 1996; Lin et al., 2001; Yang et al., 2001). Agrin was unable to induce AChR clustering in muscle cells of MuSK mutant mice (Glass et al., 1996), but agrin sensitivity was restored by introduction of wild-type MuSK into $MuSK^{-/-}$ myotubes (Herbst and Burden, 2000; Zhou et al., 1999). These results demonstrate that MuSK is essential for agrin-induced AChR clustering. Additionally, it was also demonstrated that agrin failed to induce MuSK phosphorylation in myoblasts or if expressed of in heterologous cells, like fibroblasts (Glass et al., 1996). This shows that to activate MuSK a muscle specific component is needed, which was termed MASC. MuSK is therefore be-

lieved not to be the physical receptor itself but rather composes the transducing part of the receptor complex (Glass et al., 1996). In response to agrin, MuSK undergoes tyrosine phosphorylation (Glass et al., 1996), which may create docking sites for signaling molecules or contribute to proper folding of the kinase. For example, the juxtamembrane tyrosine (Y553) is required for agrin-induced AChR clustering (Herbst and Burden, 2000; Zhou et al., 1999). Agrin stimulates tyrosine phosphorylation of AChRs, probably via Src kinase, leading to an increase of AChR clusters (Willimann and Fuhrer, 2002). In addition, activities of intracellular enzymes increase in the muscle in response to agrin. For example, Rho GTPases are activated by agrin, and inhibition of Cdc42 or Rac blocks agrin-induced AChR clustering (Weston et al., 2000, 2003). Downstream of the small GTPases may be p21-activated kinase (PAK), a cytoplasmic kinase involved in cytoskeleton regulation (Luo et al., 2002). PAK is activated by agrin in a manner dependent on Cdc42 or Rac1 and is required for agrin/MuSK-mediated AChR clustering (Luo et al., 2002). Moreover PAK interacts with disheveled (dvl1) which itself is associated with MuSK. Overexpression of Dvl mutants incapable of interacting with MuSK or PAK inhibited agrin-induced PAK activity (Luo et al., 2002). This complex might help to recruit PAK into the subsynaptic compartment for efficient PAK activation. These observations point out a signaling pathway involving Rho GTPases and PAK in regulating AChR clustering. An important but unsolved question is the identity of the mechanism immediately downstream of MuSK that is essential to activate GTPases and/or trigger AChR clustering.

To gain insights into molecular mechanisms that lead to activation of MuSK by agrin and to downstream signaling, which lead to AChR clustering upon agrin induction, we searched for proteins that interact with MuSK.

2.2.1 The yeast two-hybrid system

The analysis of membrane protein interactions is difficult because of the hydrophobic nature of these proteins, which often renders conventional biochemical and genetic assays fruitless. Traditionally, biochemical methods such as co-immunoprecipitation, crosslinking, and co-purification by chromatography have been used to investigate the composition of the MuSK receptor complex, hence without success. Biochemical methods often require harsh treatments for cell disruption and therefore may not preserve weak and/or transient interactions. To address technical difficulties associated with the biochemical characterization of physical protein-protein interactions, alternative genetic methods have been developed. A very powerful genetic method for the study of protein-protein interaction is the yeast two hybrid system (YTH), which is based on reconsti-

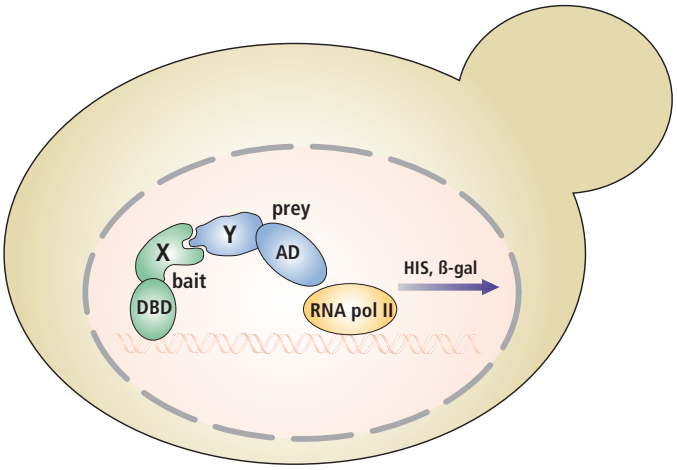


Figure 2.1 | The principle of the yeast two-hybrid system.
ProteinX is expressed as a fusion to the DNA-binding domain (DBD) and constitutes the bait. The DBD-X fusion protein is bound to the operator sites in the promoter region but does not activate transcription of the downstream reporter gene because it lacks an activation domain (AD). The interaction of DBD-X with its partner Y fused to an AD recruits the AD-Y fusion protein (prey) to the promoter where it forms a functional transcriptional activator. Consequently, transcription of the reporter gene is switched on.

tution of functional transcription factor through a defined protein-protein interaction (Fig. 2.1; Fields and Song, 1989; Chien et al., 1991; Hollenberg et al., 1995).

The assay is conducted in *Saccharomyces cerevisiae*, the bakers yeast, and uses transcription of yeast reporter genes to measure the protein interaction, which takes place in the nucleus. One of two proteins is expressed as a fusion to a DNA-binding domain from a transcription factor (TF), and the other is expressed as a fusion to a transcription activation domain (AD). If the fusion proteins interact, they activate transcription of specially designed reporter genes that carry binding sites for the DNA binding partner (Fig 2.1; reviewed in Brent and Finley, 1997).

Despite significant progress in development of the YTH system, the analysis of interactions between membrane proteins remained a significant challenge because of the transmembrane domain of these proteins which render them insoluble (Auerbach et al., 2002; Stagljar and Fields, 2002). In addition, integral and membrane associated proteins often undergo posttranslational modifications or oligomerize via interactions between their transmembrane domains, all of which is unfavorable for a traditional YTH assay (reviewed in Fields and Sternglanz, 1994).

To overcome these problems, Stagljar and colleagues have adapted the YTH system for membrane proteins. In recent experiments they have shown that the modified split-ubiquitin system can be used as a genetic assay for the *in vivo* detection of interactions between two transmembrane proteins, nicely shown with the example of the essential subunits of the yeast oligosaccharyl transferase membrane protein complex, Ost1 and Wbp1 (Stagljar et al., 1998; Stagljar and te Heesen, 2000).

This so-called split-ubiquitin membrane YTH (mYTH) system takes advantage of the split ubiquitin approach first described by Johnsson and Varshavsky (1994) (Fig. 2.2). It is based on the ability of the N- and C-terminal halves of ubiquitin, Nub and Cub, to reassemble into a quasi-native ubiquitin (split-ubiquitin). Ubiquitin-specific proteases (UBPs), present in the cytosol and nucleus of all eucaryotic cells, recognize such reconstituted ubiquitin but not its halves and cleaves

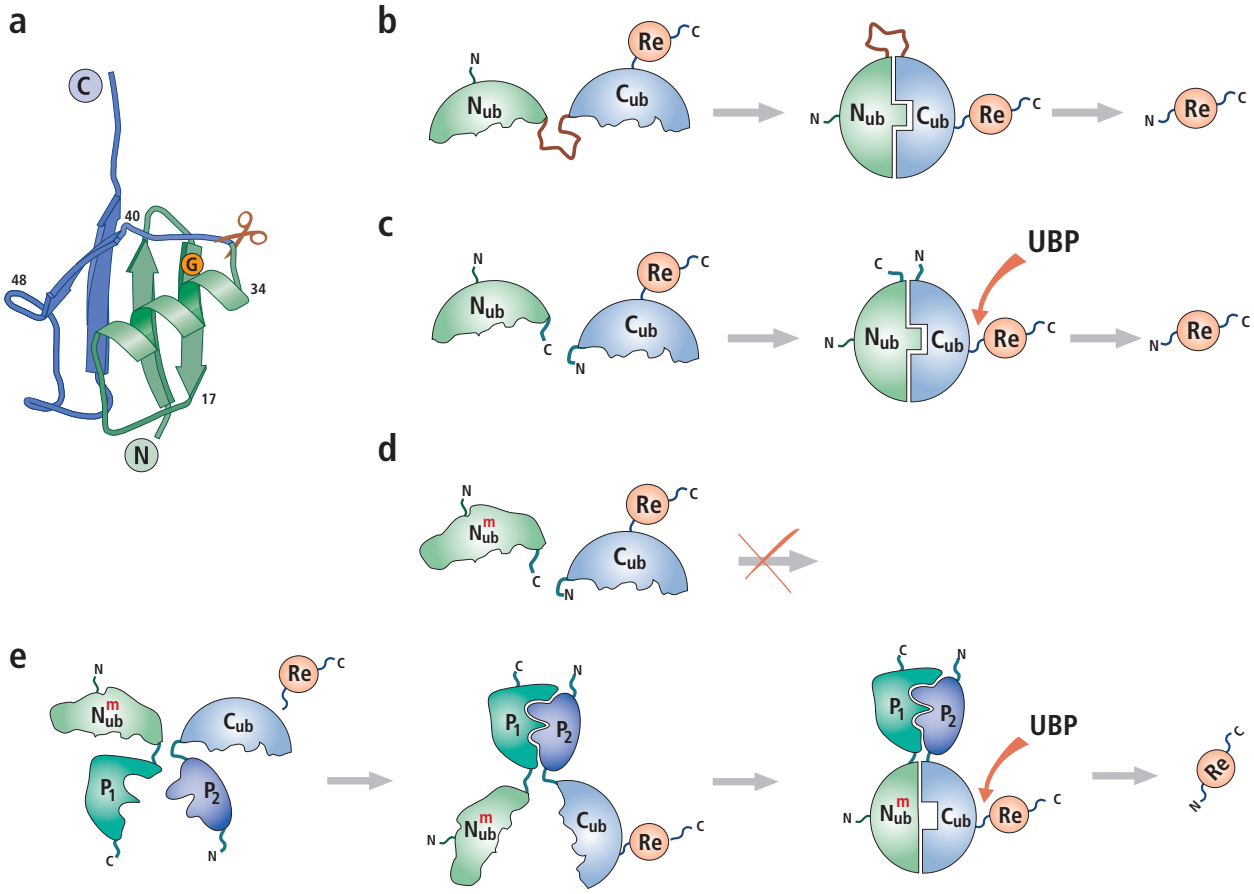


Figure 2.2 | Principle of the split-ubiquitin system.

(a) A ribbon diagram of Ubiquitin (Ub; Vijay-Kumar et al., 1987; Kraulis, 1991), with its two subdomains colored blue and green (as in b-e). The scissor denotes the site of either a 68-residue insertion or a cut between the subdomains. Some of the residue numbers are indicated. Ile-13 in the second strand of the ß-sheet is mutated to Gly, whereas it normally interacts with the opposed hydrophobic face of the α-helix. (b) A newly formed Ub moiety bearing an insertion (wavy brown line) between its N-terminal (Nub; green) and C-terminal (Cub; blue) subdomains are linked to a reporter protein (Re; orange). This insertion does not detectably interfere with the Ub folding, which is required for the in vivo cleavage of the fusion by Ub-specific preoteases (UBPs), yielding the free reporter. (c) When Nub and Cub are coexpressed as separate fragments, with Cub still linked to the reporter, Ub is reconstituted *in vivo* to give quasi-native Ub recognizable by UBPs. (d) *In vivo* reconstitution of Ub from its separate, coexpressed fragments did not occur with a mutant Nub fragment, denoted as Nub^{m}, that contains a single-residue replacement at position 13. Conformational destabilization of Nubm relative to its wild-type counterpart Nub is indicated by the altered shape of the Nub^{m} subdomain. (e) The mutated Nub^{m} supports reconstitution if the two Ub fragments are linked to polypeptide P1 and P2 (green and blue, respectively) that interact in vivo. Reduced conformational stability of Ub that has been reconstituted with Nub^{m} instead of Nub is denoted by a gap between the Ub subdomains. Adapted from Johnsson and Varshavsky,1994.

off a reporter protein that is linked to the C-terminus of Cub. The assay is designed in such way that the association of Nub and Cub is only efficient if the ubiquitin halves are linked to two proteins that interact in vivo (Fig2.2 d, e). This is achieved by the introduction of a point mutation into the N-terminal moiety of ubiquitin, which converts Isoleucine at position 13 to a Glycine (NubG), resulting in a decreased affinity between Cub and NubG (Fig 2.2 a, d).

In the split-ubiquitin mYTH system, one protein of interest, the bait X, is fused the Cub domain, followed by a reporter protein which is an artificial transcription factor (TF) composed of LexA, the DNA binding domain from the bacterial repressor LexA (Brent and Ptashne, 1985), and VP16 the activation domain (Sadowski et al., 1988; Stern Tanaka and Herr, 1989; Triezenberg 1988a, 1988b), and the other, the prey Y, is fused to NubG. To detect a potential interaction be-

tween these two proteins X and Y, the two plasmids are introduced into a suitable yeast reporter strain. Interaction of both proteins results in the assembly of split-ubiquitin and the proteolytic release of the TF by UBP's, allowing the TF to enter the nucleus, leading to activation of the yeast reporter genes (Fig 2.3; Stagljar et al., 1998; Thaminy and Stagljar, 2004).

We used the mYTH system to address the question what proteins interact with MuSK, with the main goal to identify the agrin receptor. In order to stabilize the formation of the complex, the host strain was designed in such a way that agrin is constitutively synthesized and secreted out of the cell, where it is enabled to interact with the receptor complex (Fig 2.3).

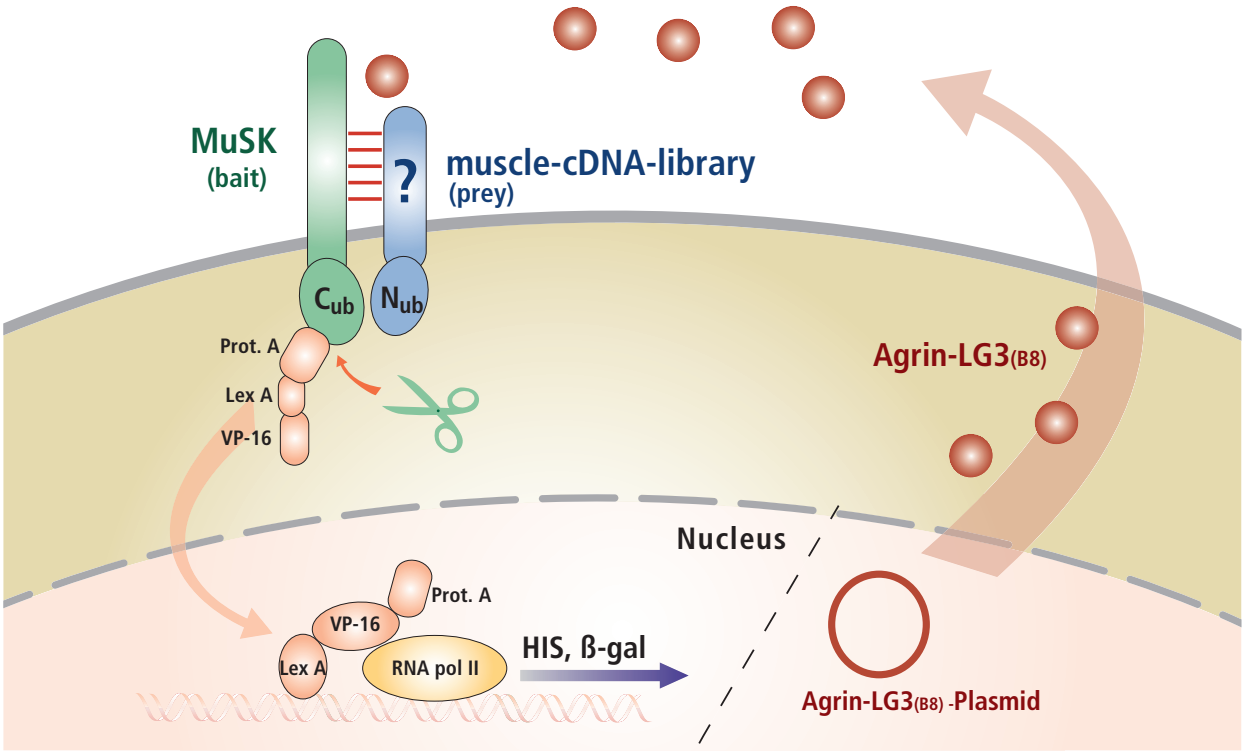


Figure 2.3 | Principle of membrane-based split-ubiquitin system used to screen for MuSK interactors.

MuSK protein is fused to the Cub domain followed by the reporter composed of protein A and the artificial transactivator LexA-VP16. Since the transactivator is immobilized at the membrane, it can not reach the nucleus and is unable to activate the reporter genes. Co-expression of an interacting protein Y derived from a muscle cDNA library fused to NubG, triggers the re-association of split-ubiquitin, followed by cleavage and release of the transactivator. Upon diffusion to the nucleus, the transactivator activates the reporter genes, conferring the cells the capability to grow on selective medium lacking histidine and to become blue in a b-galactosidase assay. The system also expresses the LG3B8 domain of agrin to ensure an interaction of MuSK and the unknown agrin co-receptor.

2.3 Material and Methods

Construction of plasmids

Plasmid sequences and detailed construction schemes of all constructs used for this work are available on request. All plasmids were verified by sequencing, and the expression of all constructs was checked by western blot analysis or immunehistochemistry using suitable antibodies.

In brief, **pNsk2-Cub-PLV** was first constructed in bluescript pKS+ by opening the vector with EcoRI/BamHI and ligating with the 5' fragment of Nsk2 amplified by PCR and subsequently digested the same way. The PCR fragment was generated with sense oligo Eco_Nde_Nsk2s46 (AACATAGAATTCCATATGAGAGAGCTTGTCAACATT) and antisense oligo Nsk2_as685 (ATTCAGGAGCACGCAGGAT) using pNsk2$_0$CP (previously described in thesis of Dominik Hauser) as a template. The 3' fragment of Nsk2 was then inserted by amplification with oligos Nsk2_504s (CTGGATCAAGGGGGACAA) and as2640Nsk2_Xba (ACTAGTCTAGAGA-CACCCACCGTTCCCTCT) using pNsk2$_0$CP as template. The PCR fragment was then cleaved with BamHI/XbaI and ligated into the vector previously constructed plasmid digested with BamHI/XbaI. pNsk2 in Bluescript was then digested with endonucleases EcoRI/XbaI and moved to pY-Cub-PLV vector previously opened with EcoRI/XbaI. **pNsk2-NubG** was constructed in analogy to pNsk2-Cub-PLV other than for the amplification of the 5' fragment of Nsk2 the antisense oligo as2640Nsk2_SmaI (ACTATCCCGGGACACCCACCGTTCCCTCT) was used. Subsequently the whole Nsk2 fragment was moved to the pX-NubG plasmid by cleaving with endonucleases Nde and SmaI.

pNsk2neuTNsk2-Cub-PLV was generated by inserting the transmembrane region of neuT by cleaving MuSKneuTMuSK construct (Jones et al., 1999) with endonucleases HindIII and BlpI. This fragment was purified and inserted into the pNsk2-Cub-PLV construct opened with HindIII/ BlpI. The generation of **pNsk2neuTNsk2-NubG** was done by a triple ligation. The vector pNsk2-Cub-PLV was opened with MscI and HindIII. One insert was generated by digesting pNsk2-Cub-PLV with endonucleases MscI and BlpI, the second fragment containing the transmembrane domain of neuT was generated by cleavage of the MuSKneuTMuSK construct with HindIII and BlpI endonucleases. The generation of **pCI^{neo}-Nsk2-NubG** and **pCI^{neo}-Nsk2-Cub-PLV** constructs were accomplished by moving the inserts from the bait and the prey plasmids into pCIneo vector respectively. The construction of **pSS-MFα1-C21B8** was performed by amplification of signal sequence of MFα1 using oligos s_MF-alpha1 (CATAAGCTTATGAGATTTCCTTCAATTTTTACTGC) and as_MF-alpha1 (ACATGGATCCAGCTAATGCGGAGGATGCTG) and pDA6300 (Dulic et al., 1991; Tan et al., 1993) as a template. C21B8 fragment of agrin was generated by cleaving with

a previously described (Alexandrescu et al., 2001) agrin construct with endonucleases BamHI and EcoRI. The vector was opened with HindIII and EcoRI and the MFα1 fragment was digested HindIII and BamHI. After agarose gel purification the 3 DNA pieces were ligated to give the final construct. **pOst1-NubG** and **pWbp1-Cub-PLV** were described previously (Stagljar et al., 1998). **pGAD** was a gift from Igor Stagljar.

Yeast strains, media

Standard yeast media and techniques were used (Sherman, 1979,1991). For selection of plasmids, dropout media containing all except the specified amino acids were used. All plasmids were transformed into yeast by lithium acetate transfomation (Ito et al., 1983, Schiestl and Gietz, 1989).

All constructs were expressed in Saccharomyces cerevisiae modified L40 (Vojtek et al., 1993) or a derivative TAT7 (Sternglanz).

L40: *(MATa trp1 leu2 his3 LYS2::lexA:HIS3 URA3::lexA-lacZ)*

TAT7: *(MATa trp1 leu2 his3 ura3 LYS2::lexA:HIS3 URA3::lexA-lacZ)*

MCY001: Background L40 *(MATa trp1 leu2 his3 ura3 LYS2::lexA-HIS3, URA3::lexA-lacZ, LEU2::pNsk2-Cub-PLV[LEU2], URA3Δ::kanMX6[G418], pAgrinC21B8[URA3])*

MCY003: Background YG0673 (Stagljar et al., 1998) *(MATa trp1 leu2 his3 ura3 LYS2:: lexA-HIS3, URA3::lexA-lacZ, wbp1::pRS305(Δwbp1-Cub-PLV), URA3Δ::kanMX6[G418], pAgrinC21B8[URA3])*

MCY006: Background TAT7 *(MATa trp1 leu2 his3 ura3 LYS2::lexA-HIS3, URA3::lexA-lacZ, LEU2::pNsk2-Cub-PLV[LEU2], pAgrinC21B8[URA3])*

MCY009: Background TAT7 *(MATa trp1 leu2 his3 ura3 LYS2::lexA-HIS3, URA3::lexA-lacZ, LEU2::pNsk2-Cub-PLV, WBP1::pRS305-Wbp1-CubPLV[LEU2], pAgrinC21B8[URA3])*

Yeast knockout

In order to knock out the URA3 gene, PCR-based gene disruption was performed as previously described by Wach et al. (1994, 1996). A 1.5 kb PCR fragment was generated by using pFA6-KanMX6 plasmid (Wach et al 1994) as template and two primers (s_URA_Kan6 AACCAACTG-CACAGAACAAAAACCTGCAGGAAACGAAGATAAATCCGTACGCTGCAGGTCGAC and as_URA_Kan6 CCACACCGTGTGCATTCGTAATGTCTGCCCATTCTGCTATTCTGTATC-GATGAATTCGAGTCTG) with 18-19 nucleotide homology to the multiple cloning site of pFA6-kanMX6. These primers have additional nucleotides which were either homologous to the region immediately upstream or downstream of the URA3 gene. The PCR product was purified and transformed into the L40 strain. Transformed cells were resuspended in 1 ml YPAD and preincubated for 3-4 hours at 30°C. Subsequently cells were collected by centrifugation, resuspended in 200µl deionized water and plated on YPAD-G418 (200mg/ml) containing agarose-plates.

Protein extraction and western blot analysis

Cells were grown at 30°C to OD_{600} of 0.8-1.0 in appropriate liquid dropout medium. Proteins were extracted essentially according to Horvath and Riezman (1994). Cells were pelleted washed once with sterile water and then resuspended in 200µl breaking buffer (50mM Tris-HCl pH 8, 100mM NaCl, 1mM EDTA, 2% SDS, 1µg/ml each of Antipain, Leupeptin, Pepstatin A, Aprotinin and Chymostatin) and the halve amount of 1000µm glass beads (Sigma) was added. The samples were vortexed 3 times for 20s at full speed using the fast-prep machine from bio101. Subsequently the samples were centrifuged at full speed in a benchtop centrifuge (Eppendorf) for 2 min. 250µl supernatant were transferred into a new tube and centrifuged again for 2min at full speed. 200µl of the supernatant were then re-transferred into a fresh tube where 200µl of 2x reducing SDS-samplebuffer was added to the cell-lysate and subsequently boiled for 5 min at 95°C. Samples were then centrifuged again and 50µl were for separation by 7.5% SDS-PAGE (Laemmli, 1970), and transferred to nitrocellulose (Towbin et al., 1979). Membranes were probed with 194T anti-MuSK antibody at 1:5000, with anti-HA 12CA5 antibody at 1:2000 or with anti LexA antibody at 1:2000. The appropriate horse radish peroxidase-conjugated secondary antibody was used at a dilution of 1:5,000 (Jackson Laboratories). Immunoreactive protein bands were visualized by the ECL detection method (Pierce).

Analytical PCR

A small amount of yeast colony material was resuspended in 5µl of nanopure water in a 200µl PCR tube. The yeast cells were boiled for 5 min at 95°C. Subsequently 20µl PCR mastermix (2.5 µl 10x Taq PCR buffer (Roche), 2.5µl 2mM dNTPs, 0.25µl 100µM mmUb1a_fw primer [5'-ATGCAGATCTTYGTGAARACC-3'], 0.125µl 100µM mmUb2a_rv primer [5'-TTGTAGTCTGACAGGGTGCGG-3'], 0.125µl 100µM mmUb2b_rv primer [5'-TTGTAATCAGAGAGRGTGCGG-3'], 0.1µl 5U/µl Taq DNA Pol and 14.4µl nanopure H_2O) were added and cycled as following: 95°C hotstart, 35 cycles [95°C 30s, 60°C 30s, 72°C 30s]. 15µl of the reaction mixture were then analyzed on a 2% agarose gel. The expected fragment size for ubiquitin containing clones was 179bp.

Library screen

Cells were grown at 30°C to OD_{600} = 1.0 in 600ml liquid dropout medium lacking Trp and Ura and supplied with Ade. Cells were centrifuged and washed with sterile water. Then the cells were centrifuged again and resuspended in 10ml 100mM LiAc and incubated for 30 min. The cells were then collected by centrifugation and the following reagents were added strictly in the subsequent order, 24ml 50% PEG 4000, 3.6 ml 1M LiAc, 1ml single stranded salmon sperm carrier

DNA (10mg/ml, GibcoBRL), 50µg library plasmid DNA (X) and finally 7.4ml – Xml library DNA sterile H_2O. The transformation mix is then vigorously vortexed until the cells are homogeneously resuspended, and thereafter aliquoted in 100 sterile eppendorf tubes. The aliquots were incubated at 30°C for 30 min and then heat shocked for 35min at 42°C. Subsequently cells were collected by centrifugation, resuspended in 200µl sterile water and plated on agarose-plates lacking Trp, Ura, Leu, His and supplied with 10mM 3-Amino-1,2,4-triazole (3-AT).

β-Galactosidase Activity Tests

Cells were grown for two days at 30°C on dropout agar plates lacking leucine, tryptophan and uracil. Then the colonies were lifted onto a Whatman filter. Subsequently the cells were permeabilized by dipping the filters into liquid nitrogen for 1 min. After thawing, the filters with colonies up, were overlaid with 0.5% agarose in TBE containing 0.1 mg/ml 5-bromo-4-chloro-ß-D-galactopyranoside (X-Gal), and incubated at RT for 1-24 h.

Construction of a cDNA library

As the split-ubiquitin YTH method allows to screen for integral membrane proteins, two cDNA libraries need to be constructed, one for type I and another for type II transmembrane proteins. This is due because the proteins expressed by the library are linked in frame to the NubG reporter at the intracellular part of the protein. But depending on the type of transmembrane protein, they have their C-terminus either inside or outside the cell. Therefore to cover all types of proteins two different libraries have to be constructed. Subsequently, typeI-library is used to call the library for typeI transmembrane proteins and typeII-library for typeII transmembrane proteins.

Total RNA was isolated from six 15cm dishes of cultured C2C12 myotubes, using TRI-Reagent (MRC, Molecular Research Center Inc.). Total RNA was subsequently purified in two steps. First RNA was isopropanol precipitated and subsequently rinsed with ethanol. Total RNA was then resuspended in binding buffer (10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.3M NaCl, 0.1% (w/v) SDS). Thereafter Poly(A+)-RNA was isolated using an Oligo(dT) Cellulose Column (GibcoBRL, Life Technologies) strictly according the manufacturers protocol. For the preparation of cDNA, the 'SuperScript™ Choice System for cDNA Synthesis' (GibcoBRL, Life Technologies) was used. For the experimental procedure the manufacturers protocol (Cat. Series 18090) was strictly followed. According to the protocol, in following variable steps subsequent decisions were taken: (a) for the first strand synthesis 5µg of poly(A+)-RNA was primed with 150ng random hexamers, and (b) in the purification step to remove the excess of EcoRI-adapters from cDNA by column chromatography, fractions 6-12 were collected. The fractions were pooled and re-purified twice

over two different size exclusion columns (Clontech). In a first step chromaspin-1000 and in a second step chromaspin-400 was used. This additional step was done to totally deplete the cDNA from adaptor-molecules, as their presence lower tremendously the ligation efficiency with the library-plasmid. Prior ligation, 5 µg of each of the two library plasmids, pX-NubG and pNubG-X (I. Stagljar), were linearized using EcoRI (NEB) and dephosphorylated using calf intestinal phosphatase (Roche), and the cDNA was concentrated by ethanol precipitation. To ensure a maximum ligation efficiency the vector to insert ratio was determined by doing some test ligations. Ligation was then performed overnight at 4°C using 5x concentrated ligase (Roche). Moreover, to guarantee a maximum and reliable transformation efficiency, commercial electrocompetent bacteria DH10B (GibcoBRL, Life Technologies) were used to electroporate the previously precipitated ligation mix. The electroporation was carried out using following settings, 1.8kV, 25µF and 200Ω. Immediately after electroporation pure LB was added to the cells and incubated for recovery for 30 min at 37°C. Then each library was distributed on 125 15cm LB-agarose plates containing 100µg/ml ampicillin (Sigma). The number of plates was previously determined and chosen the way that every plate contained about 40'000 single colonies. The calculated complexity resulted in $5x10^6$ independent clones for each library. The colonies were washed off with pure LB and pooled. One part was used to isolate the plasmids with a maxiprep kit (Qiagen) and the rest was supplied with glycerol and frozen down at –70°C. To check the quality of the library, a part of the isolated plasmids of each library was retransformed back into XL1-blue (Bullok et al., 1987). From 12 single colonies the plasmids were digested and the dimension of the inserts was determined. About 60% of all plasmids contained an insert, with an average size of 1.3 kb (Fig. 2.4).

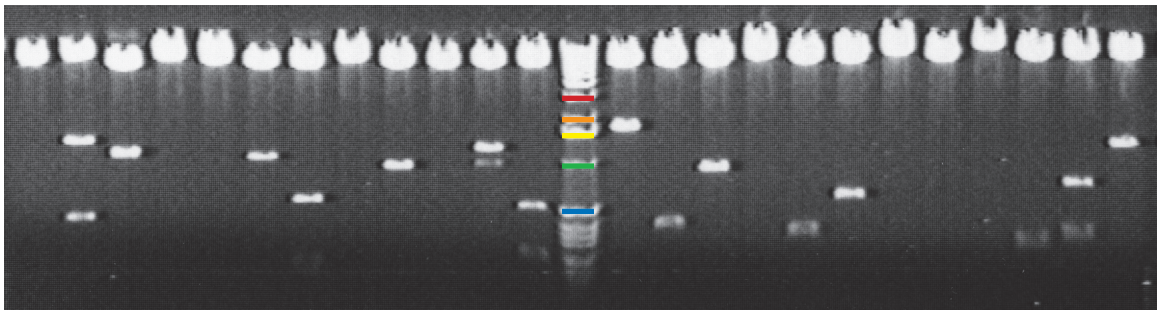


Figure 2.4 | Percentage of plasmids containing an insert and insert size of the cDNA libraries.

Isolated plasmids from type I- and type II-libraries were back-transformed into bacteria and streaked onto selective plates. 12 clones were randomly picked for each library and the purified plasmids subjected to restriction digestion. The digested clones were run on a 1% agarose gel to identify the insert size and the number of clones that contain an insert. ~60% of plasmids of both libraries contained an insert with an average size of ~1200 bp. The DNA-ladder: red, 3000bp; orange, 2000bp; yellow, 1600bp; green 1000bp; blue, 500bp.

2.4 Results

2.4.1 MuSK and agrin are expressed in yeast cells

Even though yeast cells are also eucaryotic cells, the protein synthesis and export machinery is similar but not the same as in mammalian cells. Therefore, not all mammalian proteins are suitable for expression in yeast. Proteins might not be folded correctly, lack some post-translational modifications or be mistargeted due to a signal sequence which is not recognized by the yeast cell (Lee and Lee, 2004). Additionally, mammalian proteins can also become toxic to yeast since usually strong promoters are used for protein expression and enormous amounts of protein is present in the yeast cell.

Prior to expressing MuSK in yeast, the MuSK bait and prey constructs were subcloned into a mammalian expression plasmid and analyzed for correct expression in COS-7 cells. As shown in Fig 2.5, MuSK is correctly expressed at the expected size as bait (pMuSK-Cub-PLV) and as prey (pMuSK-NubG) protein (Fig. 2.5 a), and is additionally phosphorylated upon overexpression (Fig 2.5 b). Thereafter, pMuSK-Cub-PLV and pMuSK-NubG were expressed in yeast. Their correct expression was also confirmed by western blot analysis (Fig 2.5 c). In contrast, agrin expression and functionality was analyzed by measuring its clustering activity on cultured C2C12 myotubes.

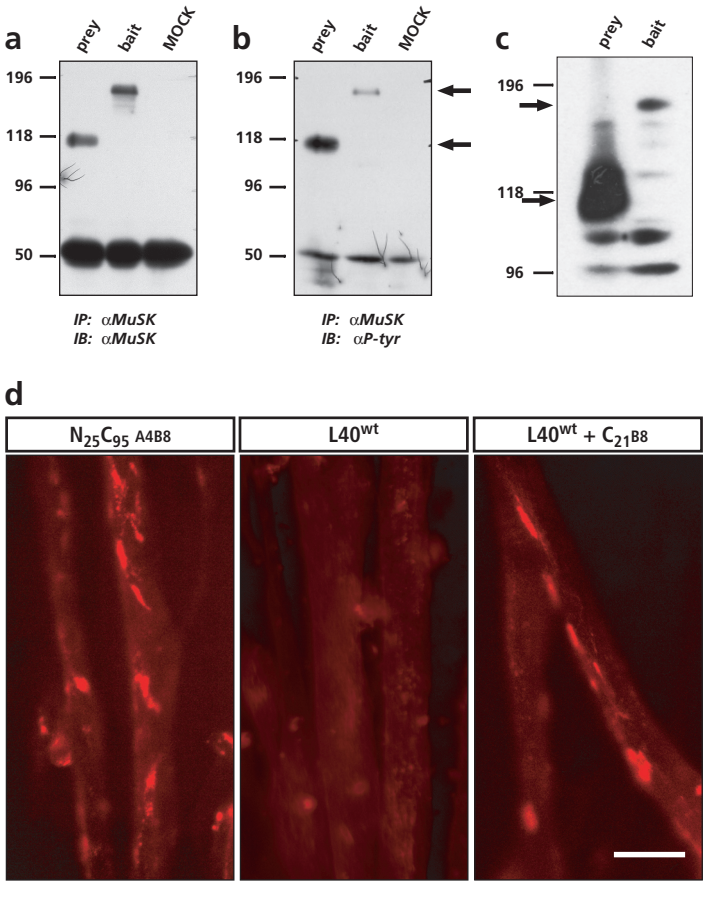


Figure 2.5 | Analysis of chimeric MuSK proteins expressed in COS-7 and yeast cells.

COS-7 cells were transiently transfected with pMuSK-NubG (prey), pMuSK-Cub-PLV (bait) and mock. MuSK containing fusion proteins were immunoprecipitated using the 194T anti-MuSK antisera and run on a 3-12% PAGE. Western blots were probed with the same antibodies against MuSK (a) and with antibodies against phosphotyrosine (b). (c) The same constructs were analyzed after transformation into yeast. Total yeast extract was loaded on a 3-12% PAGE. The Western blot was probed with 194T antibody. (d) The L40 reporter strain was transformed with $LG3_{B8}$ agrin and grown in liquid media. The cells were broken up without detergent and the supernatant was concentrated. The concentrated supernatant and the cell extracts were given to cultured myotubes. After 16h, AChR clusters were visualized with rhodamine-conjugated a-bungarotoxin (red). As negative control the wild-type L40 ($L40^{wt}$) strain and as positive control purified $N25C95_{A4B8}$ agrin was used. Scale bar 50µm.

Yeast extract and concentrated supernatant yeast medium, from agrin expressing and wildtype L40^{wt} cells, was mixed with differentiation medium for C2C12 cells in a ratio 1:1. The extracts were given to differentiated C2C12 cells and incubated for 16h. As shown in figure 2.5 d, yeast extract that was previously transformed with the agrin construct shows similar clustering activity as 1nM purified N25C95A4B8 agrin. In contrast extract from L40^{wt} showed only some rare spontaneous AChR clusters. Thus, we can conclude that MuSK and agrin are correctly expressed in yeast cells.

2.4.2 Proof of concept

In a first step it was important to show that the bait itself would not activate the system. Therefore L40^{wt} was transformed with the MuSK-bait construct and two different control preys containing either MuSK or Wbp1. As depicted in table 2.1, nor the bait alone, nor coexpressed with pMuSK-NubG or the other control prey pWbp1-NubG, could activate the reporter genes. These results show that the bait does not interact unspecifically with the control preys. Furthermore this is an indication that the bait is correctly targeted to the membrane, otherwise some degradation products, since not correctly targeted proteins which are in part in the cytosol, can activate the reporter genes by diffusion to the nucleus (personal communication with I. Stagljar).

After demonstrating that the bait and agrin are correctly synthesized, we next performed experiments to show that the specific interaction of the bait with some other protein can activate the reporter genes. Unfortunately, until the timepoint when the project was started, no physical interactor of MuSK was known. Therefore we took advantage of the knowledge about RTKs. An

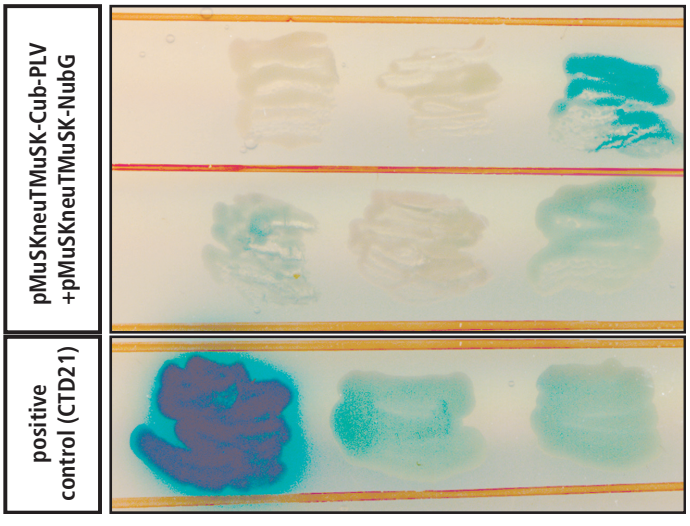


Figure 2.6 | MuSKneuTMuSK-Cub-PLV self dimerizes with MuSKneuTMuSK-NubG.

The L40 reporter strain was transformed with pMuSKneuTMuSK-Cub-PLV and pMuSKneuTMuSK-NubG. To analyze the interaction between the control bait and prey, 6 clones were randomly picked and streaked on plates containing X-Gal and lacking leucine, tryptophane and histidine (-LTH). All clones grew on –LTH plates and three of them turned blue. As positive control CTD 21 was used, a clone that growths on –LTH and shows b-Gal activity (gift from I. Stagljar)

overall accepted model for most of the RTKs is, that ligand induced dimerization of monomeric RTK leads to transphosphorylation and activation (reviewed in Schlessinger, 2000). Furthermore, it was also shown that chimeric MuSK receptors, containing the transmembrane domain of neuT, an oncogenic variant of the neu/erbB2 receptor, resulted in a constitutively active form of MuSK which leads to spontaneous homodimerization of the receptor (Jones et al., 1999). Based on this knowledge we constructed a chimeric bait and prey where the transmembrane domain of MuSK was replaced by the one neuT. These constructs were then transformed into L40^{wt} strain and analyzed on selective plates. Only those clones which contained the constitutively active form of MuSK as bait and prey did grow on –leucine, tryptophane, histidine (-LTH) selective plates and were positive for ß-Galactosidase (details in table 2.1, Fig 2.6).

These experiments show that the system is tight and can be used for screening.

Table 2.1 | Summary of positive controls done with the L40^{wt} reporter strain. Different prays were tested with several baits and their growth was analyzed on selective plates. Interactions was additionally tested for ß-Gal activity.

bait	prey	-L	-LT	-LTH	β-Gal	comment
pMuSK-Cub-PLV (linearized)		++			--	the bait is not selfactivating (an integration vector has to be linearized prior transformation in order to allow homologous recombination).
pMuSK-Cub-PLV (non linearized)		--			--	the non-linearized bait will not integrate and therefore doesn't grow
pX-Cub-PLV (linearized empty vector)		++			++	The vector alone is not targeted to the membrane. Thus the reporter is soluble and can activate the reporter genes.
pGAD (CEN/ARS)		++			--	Positive control for growth
pWbp1pp-Cub-PLV		++			--	Control bait (Stagljar et al., 1998)
pMuSK-Cub-PLV (linearized)	pMuSK-NubG	++	++	--	--	By overexpression alone the reporter is not cleaved off
pMuSK-Cub-PLV (linearized)	pOst1-NubG	++	++	--	--	Control prey does not interact with bait
pWbp1p-Cub-PLV	pMuSK-NubG	++	++	--	--	Control bait does not interact with MuSK
pWbp1p-Cub-PLV	pOst1-NubG	++	++	++	++	Control bait interacts with control prey (Stagljar et al., 1998)
pMuSKneuTMuSK-Cub-PLV		++			--	Constitutively active form of MuSK is not selfactivating
pMuSKneuTMuSK-Cub-PLV	pMuSK-NubG	++	++	--	--	Constitutively acting form is not interacting with wilde-type MuSK
pMuSKneuTMuSK-Cub-PLV	pMuSKneuTMuSK-NubG	++	++	++	++	Constitutively active form of MuSK is needed as prey in order to activate the reporter

Abbreviations: -L: SD-plates -leucine; SD-plates-LT: -leucine, tryptophan; SD-plates -LTH: -leucine, tryptophyne, histidine; ß-Gal: ß-galactosidase.

2.4.3 Screening

Finally, after all controls were done successfully, a screen for type I transmembrane proteins was carried out under the supervision of I. Stagljar and the help of S. Thaminy.

Prior to a large scale screen was started the optimal conditions were established. A general problem of the L40 strain is the leakiness of the HIS3 marker. It can happen for some reporter strains that the HIS3 gene is transcribed at a very low level even without activation of the reporter genes, thus some colonies can appear on selective plates lacking histidine increasing the number of false-positives. To circumvent the problem of a high background, 3-Amino-1,2,4-triazole (3-AT) can be added to the selective plates. As competitive inhibitor of the HIS3 protein (His3p), 3-AT is a commonly used reagent supplied to selective plates in YTH screens, in order to inhibit low levels of His3p expressed in a leaky manner in some reporter strains (Bartel et al., 1993; Durfee et al., 1993). The concentration of 3-AT was determined for each library (typeI and typeII) by titrating the concentration from 40mM down to 0mM. The best concentration was elicited at 5mM for both libraries. In a second step the transformation efficiency was determined ($5x10^4$-10^5 /µg library DNA). Then we decided in a first attempt, to screen only the library for type I transmembrane proteins since the library for tape II transmembrane proteins gave an enormous amount of unspecific interactors (personal communication with I. Stagljar). Finally $6x10^6$ colonies

Table 2.2 | Sequence analysis of the candidates that were found to be positive in the 1st screen

Clone number	Blast hit, description
A004	Mus musculus RIKEN cDNA 5830471E12 gene
A007	Mus musculus platelet derived growth factor receptor (PDGFR)
A032	Mus musculus E1B 19K/Bcl-2-binding protein
A040	No hit, unknown
A043	Mus musculus nuclear receptor co-repressor 1
A044	Mus musculus mRNA for beta-sarcoglycan
A056	Mus musculus eukaryotic translation elongation factor
A069	Mus musculus glycosylphosphatidylinositol-l...
A092	Mus musculus RIKEN cDNA 2810411G23 gene
A116	Mus musculus expressed sequence C81457 (C81457)
A120	Mus musculus EAAT3 glutamate transporter regulator
A139	Mus musculus DnaJ (Hsp40) homolog, subfamily A,...
A165	Mus musculus, Similar to cytochrome c oxi
A178	Mus musculus fibronectin 1 (Fn1), mRNA
A184	Human c-erb-B-2 mRNA
A185	Human DNA sequence from clone RP11-472K8 on chro...
A187	Mus musculus similar to glycogenin-interacting
A201	Mus musculus actin, alpha 1, skeletal muscle
A202	Mus musculus mel-13a transcript
A220	Mus musculus muscle glycogen phosphorylase
A221	Mus musculus actin, alpha 1, skeletal muscle
A228	Mus musculus feminization 1 homolog a
A232	Homo sapiens cDNA FLJ14006 fis, clone Y79AA10023
A238	Mus musculus plakophilin 1 (Pkp1), mRNA

from the typeI library were screened. 238 colonies survived on selective plates (-LTUH) supplied with 5mM 3-AT and also turned blue in the test for ß-Galactosidase (ß-Gal) activity.

In order to get to fast results, the library plasmids were recovered from each positive colony and sequenced. 211 of all clones turned out to be false positives. Most of them (90%) encoded for ubiquitin and 10% for cytochrome C. Only 24 clones or 11% of all sequenced clones, shown in table 2.2, ended up to be putative candidates as MuSK interactors.

2.4.4 Analysis of the candidates

Closer analysis of the candidates showed that some of them were transmembrane (TM) proteins and some were specifically expressed at the NMJ (Table 2.2). To confirm the hits, we next performed bait-dependency tests. The bait-dependency test demonstrates the dependence of the activation of the reporter system from a given bait. In addition, the sequence has to be analyzed to make sure the coding sequence of the candidate is in-frame with the NubG reporter. In a first bait dependency test, none of the candidates resulted to be positive. Therefore, another screening round was performed for typeI as well as for typeII transmembrane proteins. For type I transmembrane protein 10^7 colonies and for type II transmembrane proteins $3x10^6$ colonies were screened.

The type I library screen resulted in 1042 colonies growing on selective medium that were picked and patched again on selective plates. 19% were false positives and did not regrow. All the 844 remaining clones showed ß-Gal activity, and were considered as putative candidate. In the first screen we experienced a high amount of false positives to be ubiquitin. Hence, an analytical PCR was designed in order to specifically discard all false positive clones containing ubiquitin. Thus from the remaining 844 clones another 81% could be eliminated. Moreover, the remaining 164 clones were subjected to restriction digestion analysis. Finally, from all 164 plasmids only 63 carried an insert. Thus, almost 95% of all clones were false positives and could be discarded, resulting in 63 candidates, which were then subjected to bait dependency tests.

With 1011 picked colonies, almost the same number of positive clones were patched for the typeII library screen. But in contrast to the type I screen, only the large and the medium sized colonies, which were the minority of all colonies, were taken for re-growth and for analysis for ß-Gal activity. Unexpectedly, all colonies re-grew on selective medium in this case. While a consistent difference in ß-Gal activity is usually observed, all clones showed an overall similar behavior and turned blue within 2h. Compared to the type I screen where most of the false positive clones were found to be ubiquitin, sequence analysis of 20 randomly picked positive clones of type II screen gave a heterogeneous population of candidates. Since there is no big population of false positives,

as it was the case with ubiquitin in type I screen, false positives could not be easily discarded by application of an analytical PCR. Furthermore the large number of clones, more than 3000 (including the small colonies) led us finally to discard all these candidates without further analysis.

Already during the first experiments a peculiar behavior of the L40^{wt} reporter strain was noted. These observations were confirmed after the bait dependency test for the first 25 candidates was done. Beside that none of the candidates passed the bait dependency test, not even the control prey Ost1, which was shown to interact with Wbp1 (Stagljar 1998) activated the reporter genes. Together with the observations that L40^{wt} strain (a) grows very slowly in liquid media and on plates, (b) has a long recovery time after entering stationary phase, (c) has heterogeneous colony size on plates, (d) possesses a high variation in transformation efficiency, and (e) aggregates in liquid culture, we concluded that the L40wt reporter strain could be genetically instable or could carry second-site mutations that affect its performance. One possibility for this instability is that further expression of the strain led to mutations.

Subsequently, to ensure accurate results, we therefore purchased the original strains from Rolf Sternglanz's group. The reporter strains containing the bait and control bait were reconstructed. The newly constructed strains, MCY006 (TAT7 + pMuSK-Cub-PLV + C21B8-agrin) and MCY009 (TAT7 + pWbp1p + C21B8-agrin), were subjected to tests for growth, tightness of the auxotrophy markers, transformation efficiency, and activation of the reporter genes by the control bait and prey. The new yeast strains showed a much improved reliability in all aspects and, most importantly, the controls for the bait dependency tests gave the expected results.

Finally, the obtained candidates could be tested for bait dependency in the new strains. However, the new strains gave the same response as the old strains as depicted in table 2.3. Except one, none of the tested candidates, 25 from the 1st screen and 63 from the 2nd screen for typeI TM-proteins, activated the reporter genes. Moreover, the only positive clone could not be confirmed by repeating the bait dependency test. Since the screen was carried out with the old strain and the reporter gene assays in the new strain, we decided to repeat the screen for the third time using the new strain. In a first attempt the screen was performed only for the typeI library. Only 55 clones grew out of 4x10^6 screened, which is compared to the number of candidates obtained until now a reasonable amount of clones. To confirm this promising result, the clones were subsequently analyzed on ubiquitin inserts by analytical PCR. Surprisingly, none of the clones contained ubiquitin. Since 55 clones can easily be handled, the plasmids were immediately recovered and tested on bait dependency. However again, all of the putative candidates failed to be responsive to the reporter genes.

Table 2.3 | Bait dependency test of candidates that came up in screen I & II of type I library

	ß-gal test		survival test	
colony#	MCY006	MCY009	MCY006	MCY009
A 004	-	-	0	3
A 007	-	-	0	0
A 032	-	-	0	0
A 040	-	-	0	3
A 043	-	-	0	0
A 044	-	-	0	3
A 056	-	-	0	0
A 069	-	-	0	0
A 092	-	-	0	0
A 116	-	-	0	0
A 117	-	-	xx	xx
A 120	-	-	0	0
A 134	-	-	0	3
A 139	-	-	0	0
A 165	-	-	0	0
A 178	-	-	0	3
A 184	-	-	0	0
A 185	-	-	0	0
A 187	-	-	0	0
A 201	-	-	0	0
A 202	-	-	0	0
A 220	-	-	0	0
A 221	-	-	0	0
A 228	-	-	0	0
A 232	-	-	0	0
A 238	-	-	0	3
controls				
A114 (only 2 patches)			0	2
OST 1 (only 2 patches)			0	2
pX_HA_NubG			0	0
D 001	0	0	0	0
D 002	2	2	2*	2
D 005	0	0	0	0
D 008	0	1	0	0
D 010	1	0	2	0
D 011	0	0	0	0
D 014	0	0	0	0
D 015	2	2	2*	2
D 017	2	2	2*	2
D 018	0	0	0	0
D 019	2	2	2*	2
D 022	0	0	0	0
D 025	0	0	0	0
D 026	2	2	2*	2
D 029	0	0	0	0
D 032	1	2	2*	2
D 033	0	1	0	0
D 035	1	0-1	0	0
D 036	0	0	0	0
D 037	1	1	0	0
D 040	2	2	2*	2

	ß-gal test		survival test	
colony#	MCY006	MCY009	MCY006	MCY009
D 042	2	2	2*	2
D 044	0	0	0	0
D 046	0	0	0	0
D 047	0	1	0	0
D 049	0	1	0	0
D 050	0	1	0	0
D 051	1	2	0	2
D 052	0	1	0	0
D 053	0	1	0	0
D 062	2	2	2*	2
D 064	2	2	2*	2
D 068	1	2	1*	2
D 070	1	2	0	2
D 074	0	0	0	0
D 076	1	2	0	2
D 078	0	0	0	0
D 082	2	2	2*	2
D 084	0	0	0	0
D 087	0	0	2	2
D 093	0	1	0	2*
D 094	2	2	2	2*
D 095	2	2	0	2
D 097	0	1	2	2
D 099	0	1	2	2
D 102	1	1	0	0
D 105	1	1	0	0
D 106	0	0	0	0
D 111	1	1	0	0
D 112	0	1	0	0
D 119	1	0-1	0	0
D 122	2	2	0	2
D 123	1	1	0	0
D 124	1	1	0	0
D 125	2	2	2*	2
D 127	1	2	0	2
D 133	2	2	0	2
D 137	1	2	0	2
D 138	2	2	1*	2
D 139	0	2	0	2
D 146	0	1	0	0
D 151	0	0	2	2
D 153	1	1	2	2
controls				
sss1	2	2	0**	2
MuSK	0	1	0	0
pX-HA-NubG	0	2	0	2**

All clones numbered with A are candidates from the 1st screen and clones from the 2nd screen were labeled with D. The ß-Gal test was performed only for candidates of the 2nd screen. Three patches of each clone were tested for ß-Gal activity, whereas the colonies were left to react for ~6h hours. Code for ß-Gal test: 0: no color; 1: light blue color; 2: all colonies have strong blue color. The survival test was performed on three randomly picked colonies, which were grown on plates containing histidine (His) and immediately re-streaked on selective plates lacking His. Plates were analyzed for growth after 2-3 days. Code for survival test: 0: no growth; 1: different growth of the 3 clones; 2: normal growth of all three clones; *: weak growth; **: revertants came up. Abbreviations: MCY006: L40 strain expressing pMuSK-Cub-PLV and LG3B8-agrin - test bait; MCY009: L40 reporter strain expressing pWbp1 and LG3B8-agrin - control bait; A114: clone found in the first screen, containing ubiquitin - positive control; OST1: known interaction partner of Wbp1 - positive control; MuSK: MuSK containing prey - negative control; sss1: known interaction partner of Wbp1 - positive control.

2.4.5 New controls to verify the mYTH system

It was shown that Src and fyn, two tyrosine kinases belonging to the family of Src-kinases are associated with MuSK (Mohamed et al., 2001). Furthermore, while the screening was on its way the group of Lin Mei published one paper where they describe Dvl1 to be a novel interacting partner of MuSK (Luo et al 2002). In the meantime, the same group identified an additional MuSK interactor, geranylgeranyltransferase I (GGT; Luo et al., 2003). Both, Dvl1 and GGT were identified using a nuclear YTH-system with the MuSK-intracellular domain as bait.

Since the mYTH-system is designed to also trap intracellular interactors, we thought to test our system using these interactors. This was done by using a prey containing dvl1 and a fragment of dvl1, dvl1ΔDIX that was found by Luo and colleagues (2002) to strongly interact with MuSK. To avoid the reporter to obstruct dvl1 in the interaction with MuSK, two constructs were generated for each of dvl1 and dvl1ΔDIX where NubG was placed either at the C-terminus or the N-terminus. Subsequently, the new preys were tested for activation in the MuSK-bait expressing reporter strain. Again, dishevelled was not able to activate the reporter genes, even though we could show by western blot analysis that dvl1 constructs are abundantly and correctly expressed in yeast.

Because, none of the candidates could be confirmed and because the Dvl1-prey failed to activate MuSK, we initiated a new series of experiments to analyze again the expression level of the bait pMuSK-Cub-PLV. Thus, the question arose why pMuSK-Cub-PLV could not be detected despite pMuSK-NubG was always shown to give a huge expression signal, even though the expression level between Wbp1 and truncated ADH promoter is only about 10 times higher. How can an interaction take place, if the bait cannot be detected? A solution to this problem would be to enhance the expression of the bait, but since changing the weak Wpb1 promoter to a stronger one would lead to self-activation we landed in a dead end. Luckily, Stagljar and colleagues experienced the same problems, whereas we got a set of new plasmids they have constructed, containing a stronger expression level (pCMBV, pAMBV, pTMBV). But in order to avoid selfactivation a mutation was introduced in the DNA binding domain LexA. This mutation resulted in a lower binding affinity of LexA to its operon, hence the expression level could be increased without activating the reporter genes.

All the new vectors were non-integration vectors but single copy episomal CEN/ARS-vectors. In the new vectors the protein A tag was replaced by an HA-Tag which has the advantage that it is much smaller than protein A. Additionally, these plasmids contained either CYC1, ADH or TEF-promoter, that are approximately 2-, 4- or 10-fold stronger that Wbp1 respectively. Thereafter, a new bait under the control of the TEF promoter was constructed. But also with the strongest available promoter, no protein could be seen by Western blot analysis. Contradictory, Wbp1, which

was also cloned into the bait plasmid containing the TEF promoter showed a significant lower expression level compared to the previously used control Wbp1-bait under the Wbp1 promoter. Even after these results we did some more efforts in improving expression of MuSK in the bait plasmid, but without success. This was the point where we decided to stall the project, because we were not able to get reproducible results to show the expression of the bait.

2.5 Discussion

The fact that not only dvl1 did not activate the reporter genes but that also none of the initially positive candidates could never be confirmed in the bait dependency test, raised even more doubts about the reliability of the system. Irreproducible results found in investigating expression of the bait were even more puzzling. By systematically analyzing the system several reasons were found that could explain the failing of the mYTH-system, (a) possible toxicity of the bait, (b) localization of the bait, (c) expression level of the bait and (d) spatial arrangement of the two halves of ubiquitin.

2.5.1 Toxicity

In previous experiments we have demonstrated that overexpression of MuSK in HEK 293 cell leads to autophosphorylation of the kinase (Fig 2.5 b). Receptor tyrosine kinases at the cell surface are best known to act as mediators for downstream signalling pathways, a process which is initiated by transphorylation of the involved kinase. Autophosphorylation of MuSK could therefore erroneously lead to switch on some unrelated downstream events that result toxic to the yeast cell. Another possibility would be that MuSK is overexpressed in such a way that the yeast cell cannot handle this large amount of produced protein. Nevertheless, if this would be the case, transformation efficiency of MuSK would expected to be much lower, and general growth of yeast should be slow as well

2.5.2 Protein localization

Although proper localization of the MuSK-bait could not been demonstrated by imunofluorescence microscopy directly, several observation suggest that MuSK is correctly targeted to the membrane. For example expression of the bait alone does not result in any selfactivation. A wrong localization and therefore also a degradation of the bait would lead to the release of 'PLV', the reporter fused to MuSK. The released reporter would diffuse into the nucleus and activate the reporter gene for ß-Galactosidase. As this is not the case, it is highly likely that the MuSK-bait is localized to membranes in the yeast strain.

2.5.3 Expression level

The expression level of the prey can immediately be excluded as reason for the failure of the screens, since all tested prey constructs showed a very strong expression, without causing any visible toxicity.

In contrast, the bait is a very critical parameter in the membrane-based split-ubiquitin YTH system. A very strong expression of the bait can lead to self activation of the system. However, if the expression is too low, the reporter system might not respond to its activation. Therefore an appropriate promoter has to be chosen. There was the choice between Wbp1, which is a weak promoter, or the stronger, truncated ADH promoter. In control experiments we could observe that pMuSK-Cub-PLV gives significant self-activation if expressed under the control of the truncated ADH promoter. Therefore, the choice fell on the weaker Wbp1 promoter. Consequently, it was difficult to extract the bait and to show its expression by Western blot analysis, a fact, which persisted throughout all our experiments. We invested a lot of effort changing the expression level of the bait without causing self-activation of the reporter genes. Nevertheless, different expression systems were tested no considerable improve in expression was achieved. Since the improvement of the system per se was beyond our objectives, we finally decided to stall the project and wait until the system is matured to reach our goal.

2.5.4 Reconstitution of ubiquitin

Another disadvantage of the split-ubiquitin based YTH system is the probability of reassembly of the carboxy- and the amino terminal halves of ubiquitin, since reconstitution of ubiquitin through an interaction between the bait and the prey is essential. The mYTH system is therefore much more susceptible to changes in three dimensional conformation of the interacting proteins compared to the nuclear YTH (nYTH). In the nYTH once the bait and the prey associate, the RNA polymerase starts transcription, irrespective of the spatial arrangement between bait and prey (reviewed in Brent and Finley, 1997). However, in the mYTH the place of interaction of bait and prey is very critical. If the interaction of the prey with the bait, which is always associated to the membrane, is for example very close to it or is at a place where the two halves of ubiquitin can never be assembled because of the rigid conformation of the involved proteins, ubiquitin can never be reconstituted. This hypothesis is supported by the fact that only the positive control gave an activation of the reporter genes, where constitutive active form of MuSK was used as bait and prey. Since the intracellular domains of the bait and the prey protein were the same in size and conformation, reassembly of the carboxy- and amino-terminal halves of ubiquitin was more likely. Thus, the addition of a long and flexible linker between the bait/prey and the two halves of ubiquitin respectively may be a substantial improvement of the system.

2.6 Conclusions

The yeast two hybrid system is a powerful method for the identification of interaction partners for cytosolic proteins. Stagljar and colleagues successfully adapted the nuclear YTH system to a more sophisticated method which enables to study the interaction among membrane-associated proteins or membrane-associated and cytosolic proteins. Nevertheless, when we started the project, the system was in a rather immature stage. From controls, protein expression to the construction of the cDNA library, many hurdles had to be taken during the whole work. Even though, many aspects could be improved, we finally came to a point, where we had to decide to stop the project and wait until the system has become more reliable.

Nevertheless, once this interesting technique becomes reliable it will be a powerful tool for large scale-screening of interactors of integral membrane proteins. The subsequent application of this novel method in the identification of interactions between postsynaptic proteins could lead to decisive findings to finally elucidate the mechanisms of postsynaptic assembly during development.

2.7 Acknowledgements

I am very are grateful to Dr. Igor Stagljar and Dualsystems for providing all tools for the application of the mYTH system and Safia Thaminy for her assistance during the first screen. Moreover, I would like to thank Dr. Florian Schärer for his precious contribution to this work. Furthermore, I would like to thank Dr. Lin Mei for providing us the full length and the ΔDIX clone of Dvl1, originally found by their YTH screen. Finally, I would like to thank also Dr. Rolf Sternglanz to provide us the L40 and TAT7 reporter strains.

Chapter 3

Site-directed mutagenesis of agrin splice inserts reveals amino acids involved in postsynaptic differentiation at the neuromuscular junction

3.1 Summary

Agrin, a basal lamina protein that is released from motor neurons, is required and sufficient for the formation of a stable neuromuscular junction. This activity resides in the carboxy-terminal portion of the protein and is strongly modulated by alternative mRNA splicing. In particular, amino acid inserts are required at the B/z-splice site in the most C-terminal, laminin G-like (LG) domain. One of the essential components that mediates agrin's effect is the muscle-specific receptor tyrosine kinase MuSK. Indicative of MuSK activation, addition of agrin splice variants carrying amino acid inserts at the B/z- site, induce phosphorylation of MuSK within minutes.

Guided by the crystal structure of the LG domain derived from different agrin splice variants (Stetefeld et al., 2004), we set out to determine the contribution of single amino acids of the B/z site to agrin's synaptogenic activity. Here we show that the amino acid motif Asn-Glu-Ile within the B/z splice site is conserved across all species and dispensable for activating MuSK. Significantly, the moving of some amino acids flanking the B/z exon to a more exposed site strongly contribute to agrin's activity. Furthermore, we could show that α-dystroglycan (α-DG) has a role as auxiliary factor to concentrate agrin at the muscle surface and that the binding to α-DG is adjusted by inserts at A/y and B/z splice site of agrin. Finally, we propose a model where the splice inserts play a role in modulating the binding of agrin to α-DG and consequently affect the capability of agrin to activate MuSK.

3.2 Introduction

Agrin is a heparansulfate proteoglycan best known for its function to induce and maintain postsynaptic specializations at the neuromuscular junction (NMJ). Agrin-deficient mice are incapable of forming functional NMJs and lack postsynaptic specializations at birth (Gautam et al., 1996; Lin et al., 2001). Such mice also display aberrant presynaptic nerve terminals suggesting that proper formation of postsynaptic structures is requisite for presynaptic differentiation. As a consequence, agrin-deficient mice die at birth due to respiratory failure. Agrin alone is also sufficient to induce postsynaptic structures when expressed in non-synaptic regions in adult, fully innervated muscle *in vivo* (Jones et al., 1997; Cohen et al., 1997; Meier et al., 1997). These data are strong evidence that agrin is both, required and sufficient to generate fully functional postsynaptic structures in skeletal muscle (reviews see Ruegg, 1998; Sanes, 2001). More recent evidence also suggests a role of agrin in the formation of the immunological synapse, synapses in the central nervous system (CNS) and the treatment of particular forms of muscular dystrophy (reviewed in Bezakova and Ruegg, 2003).

The agrin gene undergoes alternative mRNA splicing at several locations giving rise to protein isoforms that differ in expression and function (Fig. 1 A). Alternative usage of exons at the 5' end of the gene results in two different ways of attachment to cellular structures. The secreted form binds into basement membranes necessary to allow the formation of postsynapses in muscle (Burgess et al., 2000). The second form encodes an internal, non-cleaved signal peptide that results in agrin being incorporated into plasma membranes as a type II transmembrane protein (Burgess et al., 2000; Neumann et al., 2001). The large distance (> 7 kb) between the exons encoding these two forms strongly suggests that they may also use different promoters. Support of this hypothesis is given by the fact that the expression of the two forms is cell specific. The secreted form is mainly expressed in non-neuronal cells and motor neurons of the spinal cord. Neurons in the brain appear to express the transmembrane version of agrin (Burgess et al., 2000; Neumann et al., 2001).

Two additional splice inserts are located in the carboxy-terminal half of agrin and they are both localized within laminin G-like domains (LG). The insert within LG2, called A in chick and y in rodents, is encoded by a 12-bp long exon that encodes a heparin-binding site (Campanelli et al., 1996; Gesemann et al., 1996; Hopf et al., 1996). This insert is included in agrin transcripts expressed in neurons and glial cells while non-neuronal cells in the periphery, such as muscle, Schwann cells or kidney, lack this insert (Ruegg et al., 1992; Hoch et al., 1993; Stone et al., 1995). The second splice site, called B in chick and z in rodents, is encoded by two separate exons of 24 bp and 33 bp length, respectively. Alternative splicing of these exons results in protein isoforms having 0, 8, 11 or 19 (8 + 11) amino acids inserts at the B/z-site. Splicing at the B/z-site influences

agrin's function to induce postsynaptic structures at the developing NMJ. Like mice deficient in the entire agrin protein, mice in which the two exons encoding the B/z-site were deleted and thus express only agrin isoforms that lack any amino acid inserts at this site fail to form functional NMJs (Burgess et al., 1999).

The activity of agrin to induce postsynaptic specializations at the NMJ can be reproduced *in vitro* using cultured myotubes. In this assay, the presence of amino acid inserts at the B/z-site is also necessary to induce the aggregation of acetylcholine receptors (AChRs; Ruegg et al., 1992; Ferns et al., 1993; Gesemann et al., 1995). The AChR-aggregating activity of agrin requires the muscle-specific receptor tyrosine kinase MuSK. MuSK-deficient mice show the same phenotype as agrin mutants (DeChiara et al., 1996) although recombinant MuSK does not directly bind to agrin (Glass et al., 1996). Nevertheless, its phosphorylation correlates well with the AChR-aggregating activity of agrin isoforms. For example, agrin B/z-0 isoforms, which lack AChR-aggregating activity do not induce MuSK phosphorylation, while agrin isoforms with high AChR-aggregating activity are able to activate MuSK at low concentrations (Glass et al., 1996). These data thus indicate that MuSK is an essential component in an agrin receptor complex and that phosphorylation directly correlates with AChR aggregation activity of agrin isoforms.

Another function that involves the LG domains of agrin is the binding to α-dystroglycan, a peripheral membrane protein expressed in many tissues including muscle. α-DG arises from the precursor protein dystroglycan by posttranslational cleavage (Ibraghimov-Beskrovnaya et al., 1992). Besides agrin, α-DG binds to the LG domains of several laminin isoforms, perlecan and neurexins (reviewed in Winder, 2001). Although initially postulated, binding of agrin to α-DG is not necessary for its AChR-aggregating activity (Gesemann et al., 1996). The binding of agrin to α-DG is also regulated by alternative mRNA splicing at sites A/y and B/z because a carboxy-terminal fragment that includes amino acid inserts at both sites (agrin A/y-4, B/z-8) binds to a-dystroglycan with several fold lower affinity than the corresponding fragment lacking amino acid inserts (i.e. agrin A/y-0, B/z-0). In summary, these results indicate that splicing at sites A/y and B/z influence the biological activity of agrin strongly.

Recent structural analysis of different variants of LG3 has led to new insights of how agrin may exert its AChR-aggregating activity and its binding to α-DG. The main results were that (1) the overall fold of the LG3 domain is very similar to that of LG domains in neurexin (Rudenko et al., 1999), laminin-1 (Hohenester et al., 1999) and Gas6 (Sasaki et al., 2002), (2) the B/z splice insert is highly flexible indicative of an "induced-fit" mechanism of docking to its putative receptor. (3) The structure of different splice forms of the LG3 domain diverge already 7 amino acids before and 6 amino acids after the inserts. (4) Depletion of Ca^{2+} from LG3B/z-8 induces structural changes at the same amino acids where the different protein isoforms diverge.

Here, we studied the influence of individual amino acids on agrin's function. We find that mutations in B/z-NB8A and B/z-EB8A of the B/z-8 site in LG3 attenuates the capability to induce phosphorylation by ~100 fold. Surprisingly, mutations of all eight amino acids to alanine did not further lower its activity and MuSK phosphorylation was still much higher than that of the agrin splice variant lacking any amino acid inserts at the B/z-site. Together with the result that, in contrast to the insert at the A/y-site, mutations in the B/z-site do not affect α-dystroglycan binding, we present a model whereby amino acids outside of the B/z-site strongly contribute to agrin's function in activating a MuSK receptor complex.

3.3 Material and Methods

Antibodies

Polyclonal antisera 194T against the extracellular domain of MuSK (MuSK-ECD) was raised in rabbits by injecting a His-tagged MuSK-ECD, purified from supernatants of transfected HEK293 EBNA cells. For the first injection ~60µg of protein in complete Freund's adjuvant was used. Specificity of anti-MuSK-ECD IgG was determined by staining cryostat-sections of embryonic day 18 mouse soleus muscle and Western blot analysis.

Cell Culture and Transfections

Myotubes from the mouse cell line C2C12 were prepared as previously described (Gesemann et al., 1995). Human embryonic kidney cells, which constitutively express the EBNA-1 protein from Epstein-Barr virus (293 EBNA, Invitrogen) were cultured in DMEM supplemented with 10% fetal calf serum (PAA), 10 mM sodium pyruvate, 100 U/ml penicillin and 100µg/ml streptomycin. 293 EBNA cells were transiently transfected with the agrin expression constructs using the JetPei transfection reagent (QBiogene). After 2-7 days the conditioned medium was collected and the concentration of the recombinant agrin protein was determined as described below. Additionally, the proteins were visualized by Western blot analysis (supplementary Fig. 1).

AChR Aggregation Assay

Approximately 4x 10^5 C2C12 cells were seeded on gelatin-coated, 35mm tissue culture dishes (Falcon) and after one day, medium was replaced with DMEM supplemented with 5% horse serum, 10 mM sodium pyruvate, 100 U/ml penicillin and 100µg/ml streptomycin. After 4-6 d, cells had fused and AChR aggregation assays were performed. The myotubes were incubated with recombinant agrin proteins for ~16 h at 37°C. Thereafter the cells were incubated in the same medium containing 1µg/ml rhodamine-α-bugarotoxin (Molecular Probes, Eugene, OR) for 45-60 min at 37°C. Cells were washed three times with prewarmed PBS, fixed for 10 min in 2% PFA. After washing the cells were mounted on glass coverslips with cityfluor (Plano) and examined with a microscope equipped with epifluorescence (Leica, Nussloch, Germany).

Expression Constructs

wt-proteins: The constructs pc95A4B8, pc95A0B0 (Gesemann et al., 1995) and phumanγ1 (F. Oliveri) were used as PCR-templates to generate the cDNA construct encoding C45A4B8-agrin, C45A0B0-agrin fused to a human γ1 Fc tag. Primers used to amplify the fragments for C45A4B8-agrin and C45A0B0–agrin were s4655_NheI, 5' CGAAGCTCAGTGAGCAGGAC-CACACCATG 3' and as6227_BamHI, 5' CACGAGGATCCCTTTGGCTGATCAGTGTAATA 3' and primers used for the amplification of the humanγ1-Fc-tag were shγ1_BamHI, 5' TGAG-GATCCTAGAGCCCAAATCTTCTGAC 3' and ashγ1_XhoI, 5' GCTGACTCGAGTCATT-TACCCGGAGACAGGGAGAG 3'. The PCR products were cloned in-frame to the BM-40 signal peptide (Kohfeldt et al., 1997) into the expression vector pCEP-Pu opened with XhoI and NheI. This vector included the BM-40 UTR (including a Kozak sequence), the BM-40 signal peptide for proper secretion into the culture medium. The C45A4B0-humanγ1-Fc construct was generated by digesting C45A4B8-humanγ1-Fc with HindIII/XhoI and ligating the fragment containing the A4-insert into C45A0B0-humanγ1-Fc opened with HindIII/XhoI. Similarly the C45A0B8-humanγ1-Fc construct was generated by digesting C45A0B0-humanγ1-Fc with BamHI/XhoI and ligating the fragment containing the B0-insert into C45A4B8-humanγ1-Fc opened with BamHI/XhoI.

Mutant proteins were generated by site-directed mutagenesis using pbluescript KS+ vector (Stratagene) containing the C45A4B8-agrin fragment as template. Mutations were introduced by designing forward and reverse primers with overlapping 5' ends and encoding sequences on either side of the mutation (primer sequences are available on request). PCR was performed by amplifying the whole plasmid with long expand polymerase kit (Roche Diagnostics). Thereafter, the template was removed by DpnI digestion and the new plasmid containing the mutation was transformed into DH10B chemically competent cells. After confirmation of the sequences by dye terminator cycle sequencing ready reaction kit (ABI), the mutated C45A4B8-agrin fragments were subcloned into BamHI/EcoRI opened pCEP-Pu vector containing BM-40 and humanγ1-Fc tag.

Quantification of Agrin-Protein

To quantify recombinant agrin proteins, 100µl goat anti-humanγ1 IgG (1µg/ml, Novocastra) in 50 mM sodium carbonate buffer (pH 9.6) was immobilized on a 96-well high-binding microtiter plate (Costar) followed by incubation of agrin proteins at a concentration between 0.1 and 100 ng/ml. As a secondary antibody, a HRP-conjugated goat anti-human γ1 IgG was used in a 1:10,000 dilution. As internal standard, affinity-purified recombinant C45A4B8 agrin of known concentration was used. Immunoreactivity was measured by using McEvans solution, ABTS and H_2O_2 as an HRP-substrate. The absorbance was measured on an ELISA-reader at 405 nm.

Solid Phase MuSK Phosphorylation Assay

C2C12 myoblast were grown on gelatin-coated 96 well Microtest plates (falcon) as previously described (Gesemann et al., 1995). The differentiated C2C12 myotubes were stimulated 30min with different dilutions of agrin wild-type- and mutant-proteins in phosphate buffer, pH 7.4 supplemented with 5.6 mM Glucose, 137 mM NaCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 25mM HEPES and 1mg/ml BSA. The stimulated proteins were extracted in lysis buffer (20mM HEPES, 1mM EDTA, 1mM EGTA, 150 mM NaCl, 1% Nonidet P40, phosphatase inhibitors and protease inhibitors) for 30 min at 4°C. The extracted proteins were transferred and incubated for 5h at 4°C to a 96-well high-binding microtiter plate (Costar), which was previously coated overnight at 4°C, with 100µl/well of anti-phosphotyrosine antibody 4G10 (1µg/ml, Upstate) in 50 mM sodium carbonate buffer pH 9.6 and blocked in PBS containing 0.5% Tween-20 and Top Block (Juro). The extracts were removed and the microtiter plate was washed with PBS containing 0.1% Tween 20 (PBST). Thereafter the microtiter plate was incubated overnight at 4°C with 100µl/well of affinity purified 194T primary antibody against MuSK (1:5000) in PBS containing 0.1% Tween 20 and 0.1% Top Block (incubation buffer). The plate was rinsed 4 times with PBST and incubated 4h at 4°C with 100µl/well of an HRP-conjugated donkey anti-rabbit IgG secondary antibody in incubation buffer. Subsequently, the plate was washed with PBST four times and once with PBS. For detection of the immunoreactivity the HRP substrate solution QuantaBlue (Pierce) was mixed according to the manufacturer and 100µl were added into each well. After incubation at RT for 30 min the reaction was stopped by adding 100µl/well of QuantaBlue stop solution. The fluorescence was measured on an ELISA reader (Gemini) at 420nm after excitation at a wavelength of 325 nm.

Purification of α-Dystroglycan

10g of frozen chicken breast were homogenized in 40ml of 50mM Tris/HCl pH 7.4 containing 0.2 M NaCl (buffer A). After centrifugation (12'000g) the supernatant was filtered using a Whatman filter paper. The solution was incubated with 10ml of DEAE-Sephacel beads (Pharmacia-Amersham) equilibrated with buffer A, at 4°C. After extensive washing with buffer A, bound proteins were eluted in 50mM Tris/HCl pH 7.4 and 0.5 M NaCl (buffer B). The eluate was subsequently incubated with 5ml in buffer B equilibrated WGA-sepharose. After washing the resin 5 times with buffer B elution from WGA-sepharose was achieved by adding 300mM N-acetylglucosamine. Eluted proteins were then dialyzed against 150mM NaCl, 10mM Tris-HCl pH 7.4. The purity of α-dystroglycan was verified on transfer overlay binding assay as subsequently described.

Solid Phase α-dystroglycan Binding Assay

Enzyme-linked binding assays were performed with 100µl of α-dystroglycan (5µg/ml) in 50 mM sodium carbonate buffer pH 9.6, immobilized on a 96-well high-binding microtiter plate (Costar) by absorption overnight at 4°C. After blocking with PBS containing 0.05% Tween-20, 1mM $CaCl_2$, 1mM $MgCl_2$ and 3% BSA (blocking buffer) wells were incubated with different dilutions of agrin wild-type- and mutant-constructs in blocking buffer. The wells were washed with blocking buffer and incubated with a HRP-conjugated goat anti-human-γ1 IgG in a 1:10,000 dilution. The color was developed using McEvans solution and ABTS/H_2O_2 as substrate. The absorbance was measured on an ELISA-reader at 405nm. To avoid saturation of the signal, measurements were performed every 5min.

Transfer Overlay Binding Assay

Purified chicken skeletal muscle α-dystroglycan was treated with reducing SDS sample buffer, separated by 3-12% gradient SDS-PAGE (Laemmli, 1970), and transferred to nitrocellulose (Towbin et al., 1979). Blots were blocked for 2h with PBS containing 0.05% Tween-20, 1mM $CaCl_2$, 1mM $MgCl_2$ and 5% dry milk powder (blocking buffer). The membrane was cut in strips and subsequently incubated overnight at 4° with different recombinant agrin proteins in blocking buffer. Thereafter the nitrocellulose membrane strips were washed 3 times with blocking buffer and agrin immunoreactivity was detected by incubating the blots for 2h with a horse radish peroxidase-conjugated goat anti-human-γ1 IgG (1µg/ml, Novocastra) at a dilution of 1:10,000. Finally the membrane strips were washed three times with blocking buffer and once with PBS containing 0.05% Tween-20, 1mM $CaCl_2$ and 1mM $MgCl_2$. Immunoreactive protein bands were then visualized by the ECL detection method (Pierce).

3.4 Results

3.4.1 Constructs and cellular assays

As previously shown, binding to α-dystroglycan and AChR aggregation can be separated by generating a deletion construct that comprises only the LG3 domain (Gesemann et al., 1996; Cornish et al., 1999). This single LG domain does not bind to α-dystroglycan but is still active in AChR aggregation assays although with ~100-fold lower potency. To allow direct comparison of α-dystroglycan binding and AChR-aggregating activity of recombinant agrin, we used fragments encompassing the two carboxy-terminal LG modules separated by one epidermal growth factor (EGF)-like repeat (Fig. 3.1B). For detection and purification of the recombinant proteins, the γ1 chain of human IgG was fused in-frame to the carboxy-terminal end. Recombinant protein was generated as described elsewhere (Eusebio et al., 2003) using HEK293 EBNA cells. As a readout for agrin's activity to induce postsynaptic differentiation, we additionally established a two-side enzyme-linked immunosorbent assay (ELISA; Fig. 3.1 C) that allows measuring MuSK phosphorylation induced in cultured C2C12 myotubes by the use of an antiserum raised against the extracellular region of mouse MuSK. As shown in Figure 3.1 D, the assay was able to detect MuSK phosphorylation upon stimulation with neural agrin, while no signal above background could be detected in non-treated myotubes and myoblasts treated with neural agrin. The background signal originated mainly from binding of the anti-MuSK antiserum to the coated 4G10 antibody (data not shown) which might be due to tyrosine phosphorylation of some of the IgG. Together, these results confirm the specificity of the two-side ELISA to measure phosphorylation of MuSK in cultured C2C12 cells, therefore we decided to use this assay in evaluating the contribution of individual amino acids for agrin's MuSK phosphorylation activity.

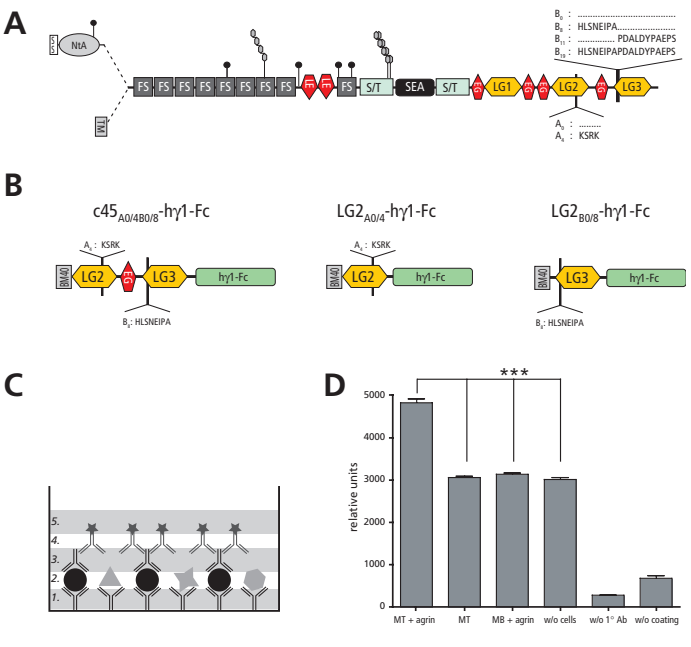


Figure 3.1 | Structure of chick agrin, used constructs and proof of principle of the phospho-ELISA

(A) Schematic representation of full length chick agrin showing its structural domains, sites for potential N-glycosylation and glycosaminoglycan side chain attachment, and location of splice sites A and B including amino acid sequence. SS, signal sequence; NtA, amino terminal agrin domain; TM, transmembrane segment; FS, follistatin-like domain; LE, laminin EGF-like domain; S/T, serine/threonine rich region; SEA, sperm protein, enterokinase and agrin domain; EG, epidermal growth factor domain; LG, laminin globular like domain. (B) C45A0/4B0/8-hγ1-Fc construct is composed of the last two LG domains separated by an EG domain. It is preceeded by the signal sequence from BM40 and followed by Fc region of hγ1. LG2-hγ1-Fc and LG3-hγ1-Fc were constructed analogously. BM40: signal sequence; hγ1-Fc: Fc part of hγ1 IgG. (C) Cartoon showing the principle of the solid phase phospho-ELISA in one well, to measure the phosphorylation state of MuSK in cultured C2C12 cells in 96-well plate format. The tyrosine phosphorylation assay is divided in 5 steps: 1. Coating with Mouse anti-phospho-tyrosine IgG 2. C2C12-cell extract 3. Rabbit anti-MuSK IgG (194T) 4. Donkey anti-rabbit HRP-conjugated IgG 5. HRP - fluorescent substrate (D) MuSK phosphorylation measured from myotubes (MT) activated by agrin (MT + agrin), myotubes without agrin (MT), and myoblasts incubated with agrin (MB + agrin). As negative control phosphorylation was measured without cell extract, without addition of primary antibody (194T) and without coating (n>5, ***P<0.0001 in two-tailed t test. Bars +/- SEM).

3.4.2 Amino acids within the B/z site important for agrin-MuSK phosphorylation

The previous results have shown that amino-acid inserts at the B/z site are essential for agrin-induced AChR clustering (Gesemann et al., 1995; Ferns et al., 1992) and MuSK phosphorylation (Glass et al., 1996; Hopf and Hoch, 1998). As shown in Figure 3.2 A, 5 out of the 8 amino acids B/z are identical between species and amino acid sequence flanking the B/z site is also highly conserved. In a first series of experiments, we compared MuSK phosphorylation activity of agrin splice variants lacking amino acids at the B/z site with a construct where all 8 amino acids were mutated to alanine (construct C45$_{B8\text{-}A8}$). While the neural form of agrin (construct C45$_{A4B8}$) exerted high MuSK phosphorylation activity, both isoforms lacking amino acids at the B/z site (C45$_{A4B0}$, C45$_{A0B0}$) were inactive in this assay. Mutation of all 8 amino acids (B8-8A) shifted the potency by approximately 100-fold (Fig. 3.2 C; Table 3.1). Interestingly, however, this mutant was still more active than the agrin isoforms lacking any amino acids at the B/z site. Thus, beside amino-acid side chains within the B/z site, there must be additional residues outside of this splice site that contribute to MuSK phosphorylation activity of agrin. Consistent with this, peptides com-

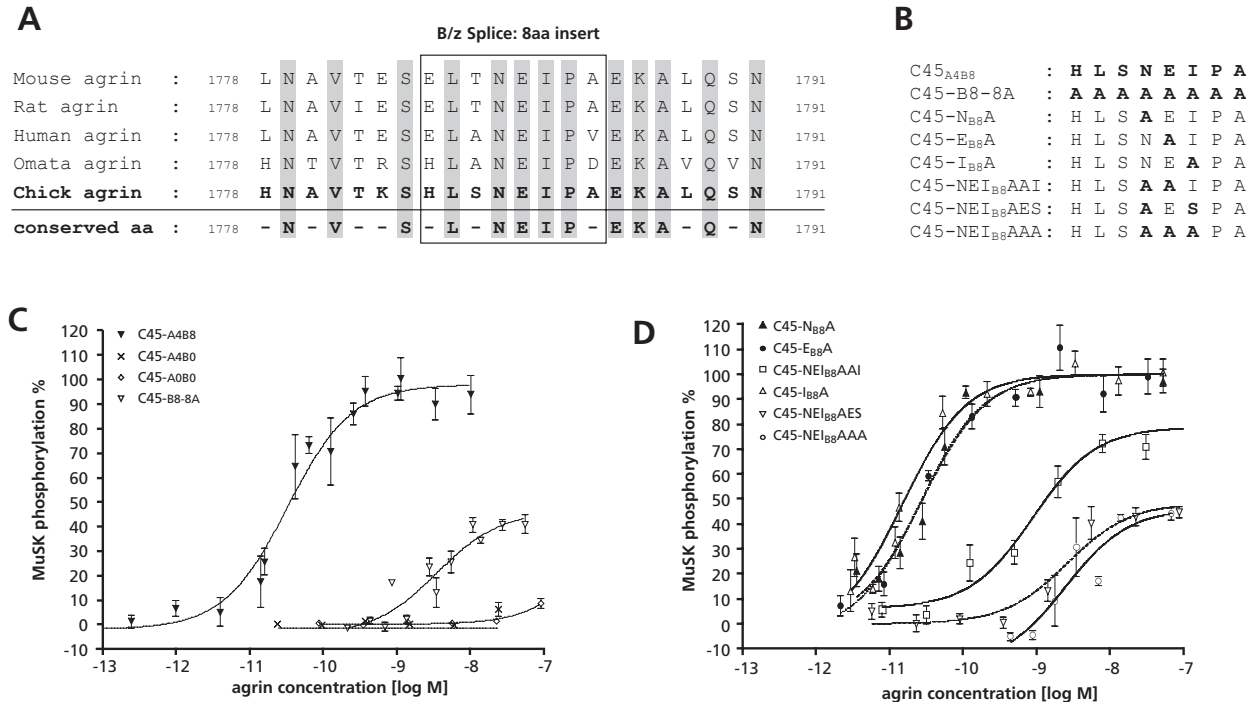


Figure 3.2 | Sequence comparison of agrin within the B/z exon and dose response curves of MuSK phosphorylation induced by recombinant agrin carrying mutations within the B/z splice site.

(A) Comparison of agrin sequence between species reveals 5 conserved amino acids within the B/z splice site, 3 immediately before and 5 just after the B/z exon. (B) Mutations generated within the 8 amino acids of the B/z exon of C45$_{A4B8}$ construct. (C) Comparison of MuSK phosphorylation between isoforms of muscle agrin (C45$_{A0B0}$, C45$_{A4B0}$) and neural agrin (C45$_{A4B8}$). Dose response curves show that insertion of the 8 amino acids long exon within B/z splice site is indispensable to phosphorylate MuSK. Mutation of all 8 amino acids within the B/z splice site show significant 100-fold reduction of potency. (D) Single mutants within the B/z splice site show no significant effect on MuSK activation. Double mutation C45$_{NEIB8AAI}$ within the NEI motif shows a shift of $EC50_{MuSK}$ from 32pM to 874pM. The triple mutation C45$_{NEIB8AAI}$ show a decrease in potency similar to C45$_{B8\text{-}A8}$. 100% was set for maximum phophorylation level of C45$_{A4B8}$ agrin. Each data point represents mean +/- SEM of at least 4 replicates.

prising the 8 aa insert or a multimer of such peptide does not induce nor inhibit MuSK phosphorylation (data not shown).

In a next step, we aimed at determining the critical amino acids within the B/z site. To this end, each conserved amino-acid within the B/z site (see Fig. 3.2 A and B) was mutated to alanine and MuSK phosphorylation was measured. All of these single mutants had the same potency as C45$_{A4B8}$ (not all mutants are shown, Fig. 3.2 D; Table 3.1). We therefore made double and triple mutants and evaluated their activity. As shown in Figure 3.2D, the triple mutant C45$_{NEIB8AAA}$ and the double mutant C45$_{NEIB8AES}$ exerted a similarly low potency as the C45$_{B8\text{-}8A}$ mutant. The double mutant C45$_{NEIB8AAI}$ had an activity that was between C45$_{A4B8}$ and the C45$_{B8\text{-}8A}$ mutant. Concomitant with the loss in potency, we also observed a decrease in efficacy (Fig. 3.2 D). Moreover, the construct C45$_{HLSNEIPAB8AAANEIAA}$, where only the NEI motif is maintained, shared a similar activity as C45$_{A4B8}$ (Fig 3.2 D). Thus, we suggest that side chains derived from the NEI tripeptide within the B/z-8 exon are the only determinants of agrin's activity.

Table 3.1 | Summary of EC50s for MuSK phosphorylations measured upon activation with wild-type and mutant agrin

	EC50 [pM]		SEM [pM]
C45-$_{A4B8}$	32.17	+/-	4.4
C45-$_{A0B0}$	n/d		
C45-$_{A4B0}$	n/d		
C45-$_{A0B8}$	8.72	+/-	1.1
C45-B8-8A	3432.50	+/-	746.8
C45-H$_{B8}$A	14.80	+/-	3.9
C45-N$_{B8}$A	27.06	+/-	4.5
C45-E$_{B8}$A	24.66	+/-	3.6
C45-I$_{B8}$A	14.58	+/-	2.2
C45-NEI$_{B8}$AAI	874.17	+/-	146.7
C45-NEI$_{B8}$AES	2598.20	+/-	498.5
C45-NEI$_{B8}$AAA	2430.20	+/-	1024.4
C45-V$_{1781}$A	19.28	+/-	2.8
C45-N$_{1779}$A	0.53	+/-	0.01
C45-N$_{1791}$A	38.06	+/-	3.8
C45-N$_{1779}$A/N$_{1791}$A	0.55	+/-	0.26
C45-E$_{1785}$A	22.87	+/-	3.9
C45-K$_{1786}$A	55.48	+/-	8.8
C45-H$_{1778}$A	15.29	+/-	3.0

3.4.3 Amino acids surrounding the B/z splice site do not show significant change in MuSK activation

Because mutation of all amino acids within the B/z site did not result in a complete loss of activity, we decided to mutate amino acid residues in the region flanking the B/z site.

Beside comparison of amino acid sequence across different species the residues to mutate were selected based on the 3 dimensional structure of LG3 domain of agrin (Stetefeld et al. 2004). The main difference between the structures of the inactive B/z-0 form and the active B/z-8/11 neural isoforms extend from His1778 to Ser1790 (Fig 3.2 A), which encompass the splice site. This led us to speculate that amino acid at His1778, Asn1779 and Asn1790, which reside exactly at the point of divergence of the two structures may contribute to agrin's activities. The same region was also reported to show the largest change by binding of Ca^{2+} (Stetefeld et al., 2004).

Furthermore, residue Glu1785 is demonstrated to be involved in indirect coordination of Ca^{2+} through water bridges (Stetefeld et al., 2004). Moreover, Ca^{2+} coordination by Glu1785 is further stabilized by the neighboring amino acid Leu1786. According to this data and previous studies

where it was shown that clustering activity of agrin is Ca^{2+} dependent (Campanelli et al., 1996; Borges et al., 2002; Megeath and Fallon, 1998), we additionally mutated Glu1785 and Lys1786 to Ala (Fig. 3.3 A). None of these single mutants affected agrin's MuSK phosphorylation activity (Fig. 3.3 B). Thus, coordination of Ca^{2+} seemed to be compensated by other residues since neither Glu1785- nor Lys1786-mutant did affect agrin's activity. The only exceptions were mutation of Asn1779 to Gly and the double mutant Asn1779Gly/Asn1791Gly, which resulted in approximately 50-fold increase in potency (Fig. 3.3 B; Table 3.1). In contrast, mutation of Asn1791 alone did not affect agrins activity at all, although this amino acid also lies at the site where the structures of agrin-B/z-8 and agrin B/z-0 isoforms deviate (Stetefeld et al., 2004). In addition, to being more active, constructs carrying a mutation in Asn1779 were expressed at considerably lower levels than any other construct (see supplementary figure 1 B). This gain in potency might be due to increase in flexibility of the B/z loop caused by the loss of the side chain of Asn, which might as well destabilize the 3-dimentional structure of the LG domain through its carboxyl- or amino-group.

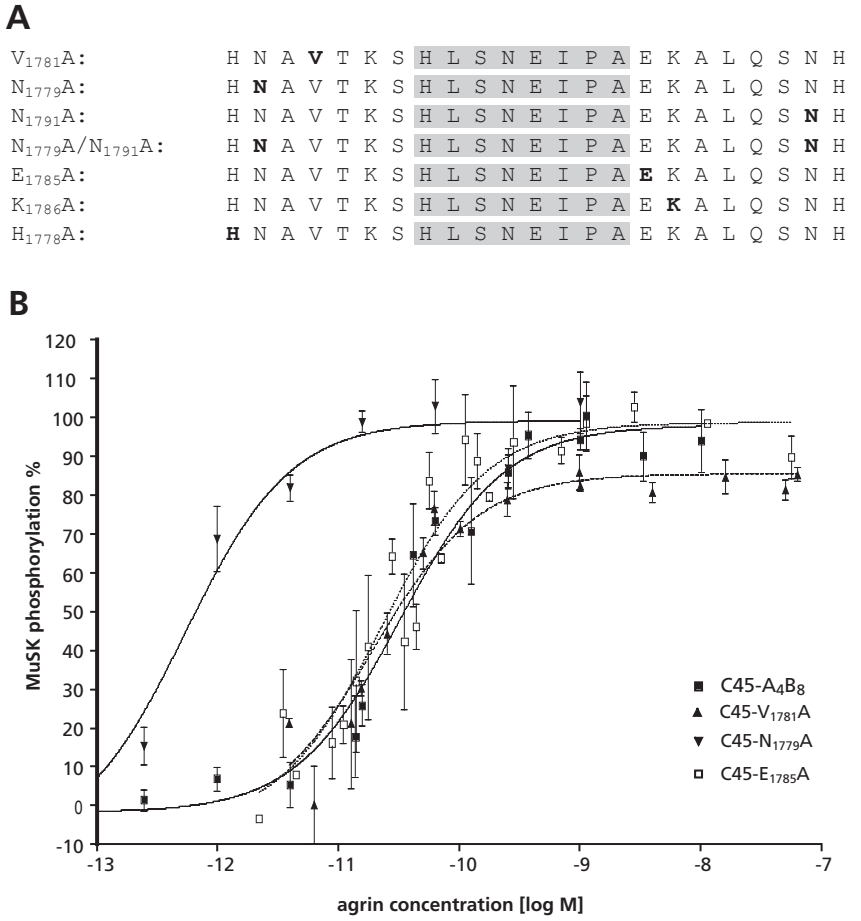


Figure 3.3 | Effects on MuSK phoshorylation of agrin proteins containing mutations within amino acids flanking the B/z splice site.

(A) Mutations introduced in $C45_{A4B8}$ agrin construct immediately before and after the B/z exon. (B) Except mutation $C45_{N1779A}$, which showed a 60-fold increase in potency to an $EC50_{MuSK}$ of 0.5pM, for no other mutation flanking the B/z region any considerable effect in inducing MuSK phosphorylation was observed. 100% was set for maximum phophorylation level of C45A4B8 agrin. Each data point represents mean +/- SEM of at least 4 replicates.

3.4.4 Agrin binding to α-dystroglycan is modulated by the presence of an insert in its A/y and B/z splice sites

It was previously demonstrated that muscle agrin binds to α-DG with a 10-fold higher affinity than neural agrin (Gesemann et al., 1996; Sugiyama et al., 1994). Therefore, we decided to measure α-DG binding of all our mutation constructs to see whether the binding to α-DG inversely correlates with potency to induce MuSK phosphorylation. In a first approach, the binding of agrin to α-DG was analyzed on an overlay transfer assay. Purified α-DG was separated by gel electrophoresis, transferred to nitrocellulose and probed with the agrin mutants. Bound agrin was subsequently detected with an antibody directed against human γ1. In this assay neither the native C45A4B8 neural agrin (Fig 3.4 B) nor recombinant agrin mutated within the B/z-8 insert showed a binding to α-DG (not shown), however C45A0B0, C45A4B0 and C45A0B8 agrin bound strongly (Fig 3.4 B). Thus, we decided to measure α-DG binding with the more sensitive solid phase binding assay. Bound agrin was detected with an antibody directed against human γ1, and the signal was quantified using an ELISA reader. In agreement with the transfer overlay assay C45A4B8 agrin had the same binding affinity as all the mutants (data not shown) and was significantly lower than affinity of all other tested agrin constructs (Fig 3.4 B, C, D, and E; Table 3.2). Therefore, mutations of amino acids within or surrounding the B/z splice site do not influence the binding to α-DG even though some of them showed a significant reduction in potency to activate MuSK, demonstrating that the binding to α-DG is mediated by other sites within the LG domain.

Surprisingly, C45A0B8 agrin showed a higher binding affinity to α-DG than C45A4B0 agrin (Fig. 3.4 E and F; Table 3.2). Concomitantly, with high binding affinity to α-DG, C45A0B8 has a 4-fold lower $EC50_{MuSK}$ compared to C45A4B8 agrin (Fig. 3.4 A; Table 3.1), indicating that binding to α-DG might play an auxiliary role for activation of MuSK. Further, we were asking if dimerization of two single LG domains would bind to α-DG. To address this question we generated four constructs analogously to the C45-agrin containing the constant region of human γ1 IgG, which force-dimerizes two proteins. The constructs contained either the 2nd or the 3rd LG domain of agrin, with or without exon in the respective splice site. α-DG binding of these constructs was then tested in solid phase binding assay. Interestingly, only LG2A0 and LG3B0 lacking the exon in their respective splice sites bound weakly to α-DG. In contrast LG2A4 and LG3B8 did not bind to α-DG at all (data not shown). Based on these results we can conclude that exons within the LG2 or LG3 domain interfere with α-DG binding-sites of agrin. Moreover, the $EC50_{MuSK}$ of LG3B8 domain of agrin is ~4nM (data not shown) which is similar to the EC50 ~13nM previously measured for AChR clustering (Gesemann et al., 1995), in contrast LG3B0 and LG2A4/0 did not activate MuSK at all.

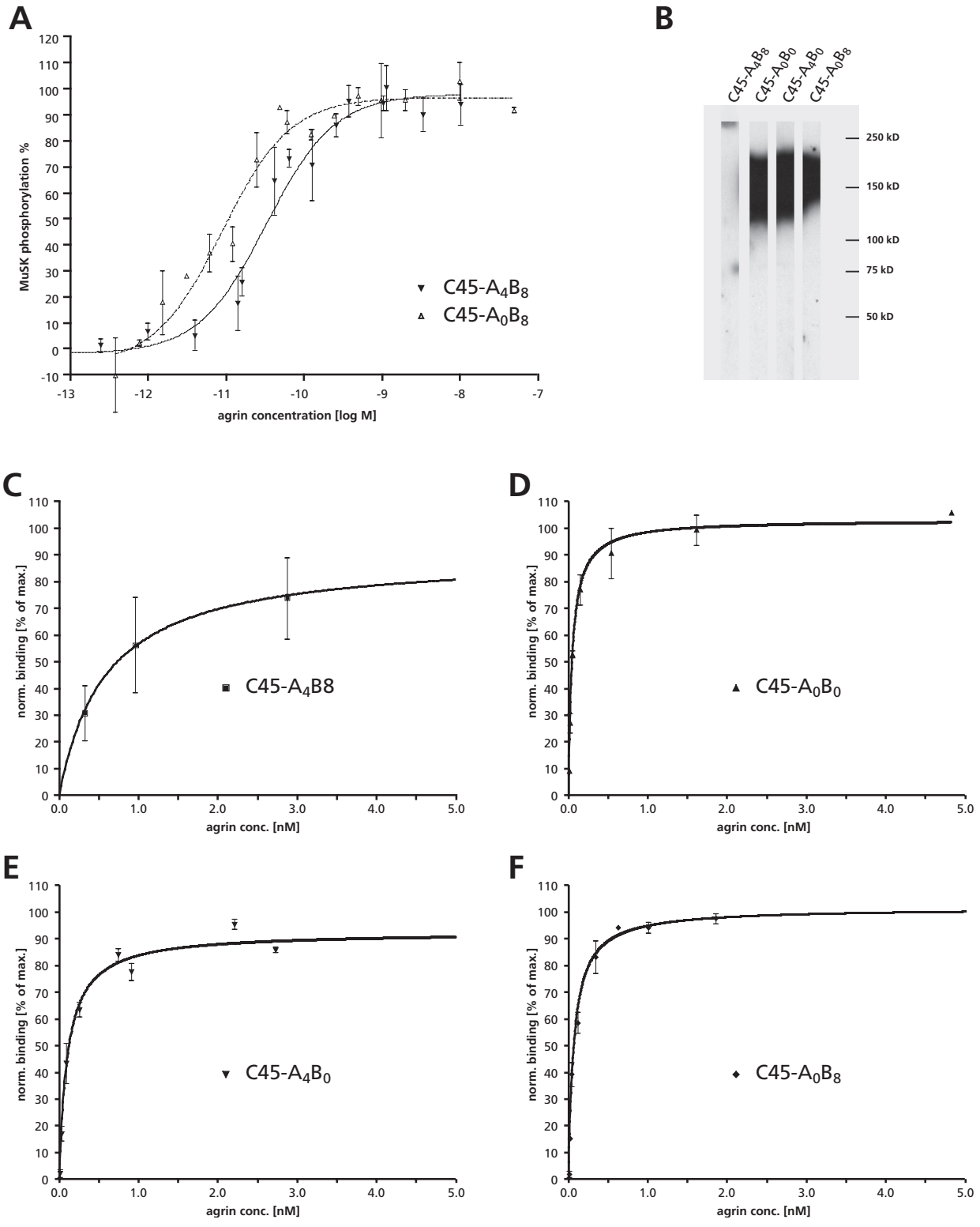


Figure 3.4 | Phosphorylation of MuSK is increased in parallel to an increase in binding affinity to α-dystroglycan but alterations in its potency do not affect α-dystroglycan binding.

(A) C45$_{A0B8}$ agrin lacking an insert at its A/y splice site has an increased potency to phosphorylate MuSK compared to C45$_{A4B8}$. (B) purified a-dystroglycan was separated on a 7.5% SDS-PAGE, blotted to nitrocelluloce and overlaid either with C45$_{A4B8}$, C45$_{A0B0}$, C45$_{A4B0}$ and C45$_{A0B8}$ agrin, whereas only binding of C45$_{A4B8}$ agrin is not detectable (C, D, E, F) Analysis of α-dystroglycan binding of the same constructs in a solid phase binding assay showed different affinities for the tested constructs demonstrating that Kd$_{C45\text{-}A0B0}$ > Kd$_{C45\text{-}A0B8}$ > Kd$_{C45\text{-}A4B8}$ > Kd$_{C45\text{-}A4B8}$. To allow comparison of the binding curves for different agrin constructs, data were normalized to the fitted value for absorbance at total saturation. Each value represents mean +/- SEM of 3 replicates.

3.4.5 Agrin's activity to phosphorylate MuSK is shifted 140-fold to lower potency after blocking it's binding to α-DG

It was shown that α-DG is not the agrin receptor (Meier et al., 1996; Jacobson et al., 1998) and that postsynaptic specializations like AChR clustering for instance can also form in absence of α-DG in cultured myotubes (Jacobson et al., 1998) and *in vivo* (Cote et al., 1999). Here we provide data, where we suggest that α-DG supports agrin in activating MuSK phosphorylation, by capturing agrin at the muscle surface and hence to induce AChR aggregation. To support this hypothesis, the binding of C45 agrin to α-DG was either blocked with heparin (Gesemann et al., 1996) or competed with excess of N25C95A0B0 muscle agrin, which strongly binds to the muscle surface having additionally a laminin binding domain. Heparin inhibition of C45A4B8 and C45A0B8 binding to α-DG shows a 20- and 10-fold shift to lower $EC50_{MuSK}$ respectively (Fig. 3.5 C and D; Table 3.3). The activity to phosphorylate MuSK of C45A4B8 versus C45A0B8 agrin is stronger inhibited by heparin due to the presence of the KSRK motif within the A/y splice site of C45A4B8, which was shown to strongly bind heparin (Gesemann et al., 1996). Furthermore, competition of binding of C45 agrin isoforms to α-DG with 100-fold excess of N25C95A0B0, endures a 140-fold shift of the $EC50_{MuSK}$ for C45A4B8 and a ~60-fold shift for C45A0B8 to lower potency (Fig. 5A and B; Table 3.3). Interestingly, after inhibition of agrin's binding to α-DG, the measured $EC50_{MuSK}$ for C45A4B8 is very similar to the $EC50_{MuSK}$ measured for LG3B8, a truncated form of agrin, which does not bind to α-DG at all. These experiments strengthen the hypothesis that the binding of agrin to α-DG is necessary to increase agrin's MuSK phosphorylating activity. Analogously, it was shown that AChR clustering by N25C21B8 agrin (Meier et al., 1998) is strongly increased compared to soluble C21B8 (LG3B8). The higher clustering activity was attributed to the N-terminus, which anchors the protein to laminin and hence is more concentrated in the basal lamina compared to the soluble LG3B8-agrin.

Table 3.2 | exon insert in agrins A/y and/or B/z splice site determines α-DG binding

	kD [pM]	SEM [pM]
C45-A_4B_8	590.4	+/- 102
C45-A_0B_0	47.4	+/- 6
C45-A_4B_0	104.9	+/- 14
C45-A_0B_8	69.0	+/- 7

Table 3.3 | Inhibition of agrin binding to α-dystroglycan results in a shift in potency

	EC50 [pM]	SEM [pM]
C45-A_4B_8	32.1	+/- 4.4
C45-A_0B_8	8.7	+/- 1.1
C45-A_4B_8 + N25C95-A0B0	4559.0	+/- 2460
C45-A_0B_8 + N25C95-A0B0	557.4	+/- 93
C45-A_4B_8 + Heparin	756.5	+/- 162
C45-A_0B_8 + Heparin	107.7	+/- 48

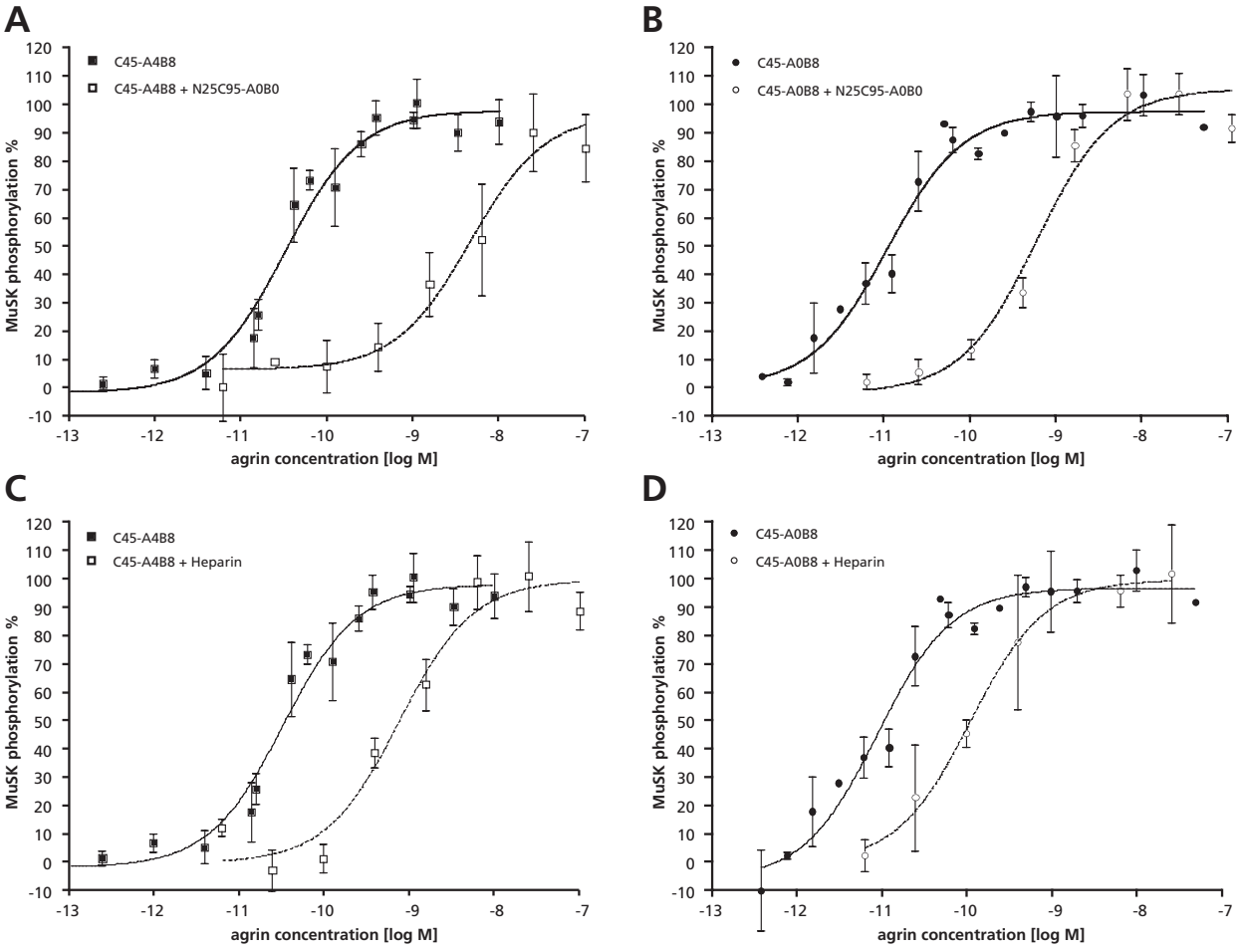
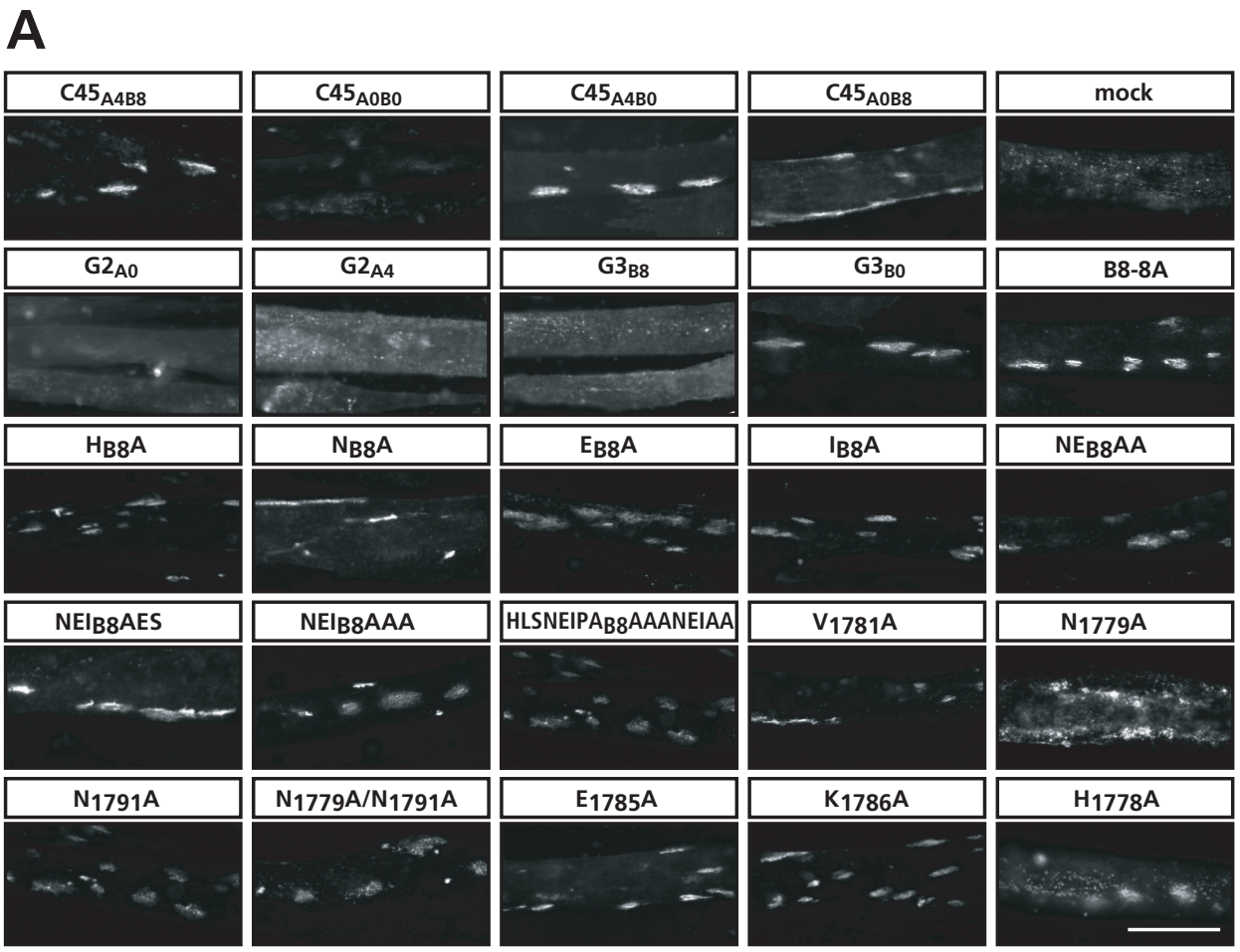
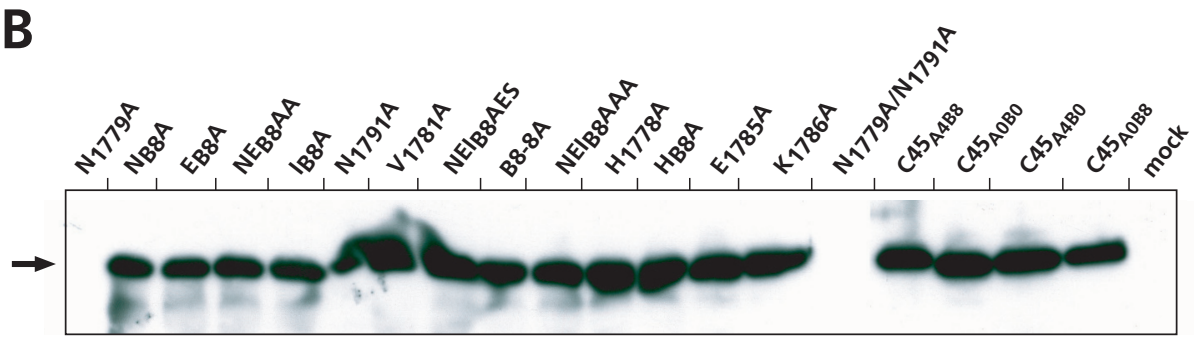


Figure 3.5 | MuSK phosphorylation can be inhibited with heparin and an excess of muscle agrin.

(A, B) Activation of MuSK was competed with N25C95$_{A0B0}$ agrin. A shift of MuSK phosphorylation to lower potency is observed for C45$_{A4B8}$ and C45$_{A0B8}$ agrin (A, B). A similar, but less prominent shift is seen by competition of MuSK activation with heparin (C, D). 100% was set for maximum phophorylation level of C45$_{A4B8}$ agrin. Each data point represents mean +/- SEM of at least 4 replicates.





Supplementary Figure 1 | Expression and AChR clustering activity of all recombinant agrin constructs.

(A) Western blot analysis of all agrin proteins expressed from HEK 293-EBNA cells. With exception of mutants $C45_{N1779A}$ and $C45_{N1779A/N1791A}$ that show a very poor expression level all other recombinant agrin proteins are expressed at the expected size of 75 kD. (B) AChRs are visualized by staining with α-Btx. All recombinant isoforms of agrin that showed MuSK phosphorylation also show AChR clustering activity. $C45_{A4B0}$ surprisingly showed to cluster AChRs without bearing any MuSK phosphorylation activity.

3.5 Discussion

The mechanism of mRNA splicing is coming up more frequently in processes where the interaction between two proteins is fine tuned in order to increase the ligand-receptor affinity leading to specific signaling events during development (reviewed in Graveley, 2001). In keeping with this model, also development of the neuromuscular junction underlies such mechanisms, as agrin was shown to undergo extensive alternative splicing (reviewed in Bezakova and Ruegg, 2003). Agrin's most potent AChR inducing activity was mapped to the three C-terminal LG-domains including an exon of 8 amino acids in length in the B/z splice site (Gesemann et al., 1995), which is essential for activating MuSK and to induce AChR clustering. Using the newly developed two-side phosphorylation ELISA, we now examined the B/z exon and mapped amino acids important for inductive events, which lead to phosphorylation of MuSK and additionally determined their contribution to the binding of α-DG.

In initial studies, we compared MuSK phosphorylation induced by muscle agrin (C45A0B0, C45A4B0), C45A4B8 neural agrin and a mutated form of neural agrin where all eight amino acids of the B/z inserts were mutated to alanines, where we could show that the contribution of the amino acid side-chains are essential for agrin's activity. In fact, we measured a 100-fold loss in potency for C45B8-8A agrin mutant, which surprisingly still showed a higher activity than muscle agrin that was not active at all. Moreover, by introducing single, double and triple mutations into the B/z exon we were able to define agrin's activity to the Asn-Glu-Ile motif, three highly conserved amino acids across all species used for comparison. This finding was confirmed by the C45HLSNEIPAB8AAANEIAA mutant, where only the NEI triplet was preserved and all other amino acids were mutated to alanine, showing almost the same activity as C45A4B8 agrin.

These findings led to two conclusions about the importance of the inclusion of an exon at the B/z splice site. First, the activity of the B/z-8 insert is exclusively given by the tripeptide NEI, which is also highly conserved between all species. Second, all other inserted amino acids function as a spacer in order to move the side chains of flanking amino acids to a more exposed place in the structure of the LG3 domain where they are enabled to additionally influence agrins activity. As shown by the crystal structure of LG3 domain of agrin, the accommodation of the B/z-8 exon into the LG3-domain does not perturb the overall structure of the LG3 domain but only shifts 6-7 amino acids in proximity to the B/z insert to a site out of the globular structure of the LG3 domain (Stetefeld et al 2004).

Further analysis of amino acids flanking the B/z splice site did not reveal any new indications about the contribution of their side chains. The initial hypothesis that the Glu1785 and/or Lys1786 may contribute to the coordination of Ca^{2+} (Stetefeld et al., 2004) and therefore also stabilize the

B/z loop was not confirmed. Interestingly however, mutation of Asp1779 resulted in a 60-fold increase in potency (Table 3.1). One interpretation of this result is that Asp1779 strongly stabilizes the structure, and therefore the lack of this side chain has destabilizing effects on the structure, increasing the flexibility of the B/z loop resulting in a higher activity. These observations fit with the model proposed by Stetefeld et al (2004), where they suggest that the plasticity of the interaction interface optimizes the selectivity through induced fit binding. Furthermore, the low expression level of the constructs containing the Asp1779 mutation strengthens this hypothesis.

Next, we attempted to determine the contribution of the B/z splice insert in the binding to α-DG, suggesting that agrin mutation with lower potency in activating MuSK may result in increase of the binding to α-DG, as it was shown for muscle and neural agrin that an insert at both splice sites decreases about 10-fold the binding affinity to α-DG (Gesemann et al., 1996; Sugiyama et al., 1994). This hypothesis could not be verified, as all mutations within and outside the B/z splice site had exactly the same α-DG binding affinities as the wild-type C45A4B8 agrin. Moreover, binding studies of different muscle and neural agrin isoforms showed that the strongest binding agrin isoform lacked both inserts at its A/y and B/z site and the weakest binding agrin isoform included an exon at both splice sites. In contrast agrin isoforms lacking either one exon at its A/y or B/z splice site did bind stronger than C45A4B8 but weaker than C45A0B0 agrin. Interestingly, the stronger binding of C45A0B8 to α-DG was concomitant with the activity of this agrin isoform, which also showed a 4-fold increase of the $EC50_{MuSK}$ compared to C45A4B8 agrin. From these results we get two further insights. First, increased binding of agrin to the muscle surface by interacting with α-DG leads to a higher activity of agrin and loss in activity by changing amino acid residues does not increase the binding to α-DG. Second, the binding to agrin is mediated by a domain lying close to the A/y- or B/z-splice sites and that the insert significantly disturbs the interaction of agrin to α-DG. Furthermore, this hypothesis is supported by experiments showing that dimerization either of LG2 or LG3 including an exon do not bind to α-DG whereas the same domains lacking an insert show weak binding. Based on these findings we propose a model for the binding of agrin to α-DG, suggesting that the binding interface of agrin to α-DG needs the exposure of two LG domains, whereas the interacting sites are in close vicinity to the splice inserts A/y and B/z, as an insert in these sites can disturb the binding (Fig. 3.6).

In a next series of experiments we analyzed whether the binding of agrin to α-DG per se is implicated in agrins activity. Inhibition of agrin to bind to α-DG by heparin or by competing with N25C95A0B0 agrin showed a 100- and 150-fold decrease in potency for C45A0B8 and C45A4B8 respectively. In contrast, further experiments showed that MuSK phosphorylation by the single LG3B8 domain could not be inhibited neither by heparin nor by muscle agrin. Interestingly, $EC50_{MuSK}$ for C45A4B8 where the binding to α-DG was inhibited showed the same potency as the

EC50MuSK measured for LG3B8, which does not bind to α-DG at all. According to these results agrin needs to bind to the muscle surface in order to efficiently activate MuSK phosphorylation. Analogously, it was reported that the LG3B8 domain is much more efficient in inducing postsynaptic specializations, if provided with the N-terminal domain, which confers its binding to laminin (Meier et al., 1998). Therefore, we can conclude that α-DG is used as an auxiliary factor in order to capture agrin to the muscle surface, enabling agrin to act in much lower doses.

In summary, our data provide strong evidence that activity of agrin resides mostly in the NEI tripeptide motif within the B/z splice site and is additionally supported by amino acids immediately flanking the B/z insert. Further, our data also show that α-DG may impinge on agrins activity by concentrating the molecule at the muscle surface where it is needed to induce postsynaptic differentiation by interacting with its still unknown receptor. This further characterization of the active domain of agrin combined with structural information of agrin might be a useful tool to identify the receptor for agrin by computational analysis. In this aspect, the binding of nidogen to laminin, which is mediated by a similar motif DPNAV might be a model to consider (Pöschl et al., 1996).

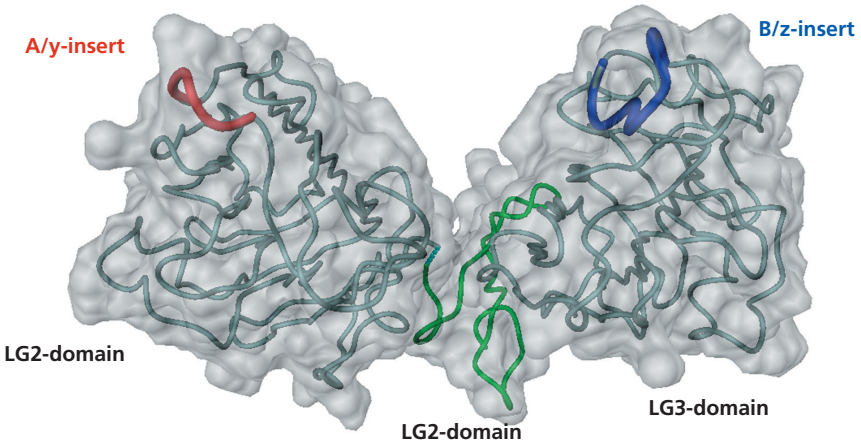


Figure 3.6 | Tandem model of LG2A4-EGF-LG3B8.
The backbone of both LG-domains is drawn in steelblue and the EGF domain in green. Splice inserts at the A- and B-site are shown in red and blue, respectively. A surface presentation is underlined semi-transparent.

3.6 Acknowledgements

I am very are grateful to Dr. Joerg Stetefeld, who provided the basis of the whole work in contributing with his knowledge about agrins crystal structure, he recently resolved. Further, I would like to thank Dorothee Bleckmann and to Dr. Thomas Meier for establishing the phospho-ELISA and to let me use the 'GEMINI' whenever I needed. Finally, I would like to thank also Mike Stebler for his contribution to this work.

Chapter 4

General Discussion

4.1 The right approach to find the agrin receptor

During embryonic development motor axons induce the formation of the motor endplate, a small region of the myotube membrane rich in AChRs. The heparan sulfate proteoglycan agrin, which is secreted by the growing axon, is the key organizer of postsynaptic specializations such as clustering of AChRs (McMahan, 1990). The release of agrin by the nerve leads to rapid activation of MuSK to mediate AChR clustering (Glass et al., 1996). It is now clear that MuSK is the signaling component activated by neural agrin. However, MuSK does not bind directly to neural agrin indicating that activation of MuSK depends on a muscle-specific co-receptor (Glass et al., 1996). Even though a number of key players involved in NMJ development are known, neither the mode of how agrin activates MuSK nor of how MuSK activates downstream signaling, which leads to AChR clusters is known.

The search for the agrin co-receptor has remained elusive so far. Many research groups, including our lab, have invested big effort using a variety of techniques to identify the agrin binding protein, but traditional methods that were applied successfully in other cases failed. The possibility that the co-receptor might be a membrane-associated protein could cause decisive difficulties in interaction studies because of its hydrophobic nature, therefore the choice of the appropriate approach has to be well considered. We chose the yeast-based membrane associated split-ubiquitin system to seek for the agrin receptor (Stagljar et al., 1998). Based on the powerful technique as the YTH system (Fields et al., 1989), this novel method was adapted to search for proteins that encompass the membrane or are linked to it. The advantage of the mYTH system is that not only membrane-associated proteins that interact with MuSK can be obtained but also intracellular MuSK-interactors. Another general advantage in yeast-based approaches is that the interaction is taking place in vivo, that the organism is easy to handle and allows the screening of a large number of proteins compared to mammalian cell based systems, like fluorescence or bioluminescence energy transfer system (FRET, BRET; reviewed in Boute et al., 2002). In contrast, the big disadvantage of yeast is that mammalian proteins might miss important posttranslational modifications or might be mis-targeted in the cell. Nevertheless the mYTH was the system with the most promising success rate.

Since MuSK is believed to be associated with the co-receptor, it was used as bait in the mYTH screen. Moreover, in order to have all components of the receptor complex present in the yeast cell, and thus to ensure a possible interaction between MuSK and the candidate co-receptor, we further modified the mYTH to express also the ligand to result in a 'three hybrid system'. Therefore, the yeast reporter strain was constructed in a way that additionally to MuSK it expresses also the most C-terminal LG3B8 fragment of agrin, which is the smallest still active form of agrin that

can induce MuSK phosphorylation and AChR clustering (Gesemann et al., 1996; Cornish et al., 1999).

Taking together, after a promising start we ended up with thousands of candidates that could never be confirmed. The failure of the project was probably due to technical limitations of this novel system and not because of the biological nature of the interaction between the co-receptor, agrin and MuSK.

The failure to seek the identity of the agrin co-receptor up to now, indicates that the co-receptor necessary for agrin-MuSK signaling is either 'just' a not identified modification of MuSK, which might confirm technical limitations of available systems, or might be a receptor of higher order comprising more than one additional component like it was shown for e.g. the ciliary neurotrophic factor (CNTF) receptor that was found to be a ternary complex formed by the membrane-anchored CNTF receptor and two receptor tyrosine kinases gp 130 and LIFRß (Davis et al., 1993; Stahl and Yancopoulos, 1994). Beside this hypothesis, there are additional possibilities that could underlie agrin signaling. These are: (*i*) a transmembrane or a membrane anchored protein that binds agrin as bona fide agrin receptor, (*ii*) a soluble co-ligand that acts with agrin to activate MuSK, (*iii*) a post-translational modification or (*iv*) alternative mRNA splicing of MuSK that allows agrin to bind directly to MuSK. In five out of the six listed possibilities also the mYTH would have failed.

As it was previously shown that dimerization of MuSK can lead to a fully developed postsynaptic apparatus in vivo (Hopf and Hoch, 1998; Jones et al., 1999), the presence of another kinase like in the complex mechanism of CNTF signaling is none likely. Moreover, since biochemical and genetic methods failed to find a co-receptor, a fascinating idea would be if MuSK itself would be the agrin receptor where its specificity is acquired by posttranslational modifications or by alternative mRNA splicing. Models where posttranslational modifications, such as glycosylations, can serve as cofactors to recruit ligands to their cognate receptors to ensure efficient interactions is becoming more and more considered (Bernfield et al., 1999; Perrimon and Bernfield, 2000). Especially, the importance of differential sulfations and epimerization of polysaccharide chains, which leads to the more complex heparan sulfate proteoglycans (HSPG), has been well illustrated in animal model systems, which demonstrated that genetic removal of enzymes involved in heparan sulfate (HS) polymerization reactions causes severe and early developmental patterning defects of a variety of signaling pathways (Bülow and Hobert, 2004; Herman and Horvitz, 1999; Inatani et al., 2003; Morio et al., 2003; Perrimon and Bernfield et al., 2000). Moreover, recent studies showed that overexpression of the glycosyltransferase LARGE can restore the binding of α-dystroglycan to laminin in cells derived from patients suffering from congenital muscular dystrophy, where mutations in glycosyltransferases have been found to be responsible for the disease (Grewal and

Hewitt, 2002; Barresi et al., 2004; Kanagawa et al., 2004).

Even though it was shown that certain glycosyltransferases are expressed selectively at synaptic sites in skeletal muscle (Scott et al., 1990), resulting in concentration of certain carbohydrate epitopes attached to proteins at neuromuscular synapses (Scott et al., 1988), glycosylation has not been considered in agrin-MuSK interaction. Up to now, there is just one report where glycosylation sites in MuSK were analyzed but without any significant result (Watty and Burden, 2002).

Closer analysis of putative glycosylation sites in MuSK showed that MuSK contains five putative glycosylation sites (Fig 1.4) and not three as reported by Watty and Burden (2002). Interestingly, these two additional glycosylation sites reside in only an alternatively spliced exon encoding 10 amino acids. This finally raises an additional hypothesis where MuSK-specificity can be additionally modulated by alternative mRNA splicing as it is known for agrin.

4.2 Amino acids within and outside the B/z splice site contribute to agrin's inductive activity, which lead to MuSK phosphorylation

McMahan formulated the 'agrin hypothesis' in 1990 postulating that agrin, which is released from the motor-nerve terminal, binds to a receptor on the muscle-cell surface inducing postsynaptic differentiation. Depending on the time point and the tissue where agrin is expressed, different exons are combined by alternative splicing to result in agrin proteins with different characteristics. While the amino-terminal domain of agrin is responsible for anchoring the protein to the membrane or its binding to laminin (Denzer at al., 1995, 1997; Mascarenhas et al., 2003; Neumann et al., 2001; Burgess et al., 2000) the carboxy-terminus is responsible for binding to α-dystroglycan and to the agrin receptor. Whereas for the binding to α-dystroglycan, the first two LG domains are needed, the binding to the receptor is confined to the last LG domain (Gesemann et al. 1996; Hopf and Hoch, 1996). It was also shown that only agrin isoforms containing an insert at its B/z splice site are capable of inducing AChR clustering and phosphorylation of MuSK, whereas the isoform containing the shortest exon of 8 amino acids is the most active one (Gesemann et al., 1995). In our work we aimed at identifying single amino acids responsible for agrin's capability to activate MuSK for two reasons. First, the identification of a precise map of the 'active' amino acid side-chains could give new insights in the mechanism of action of agrin with its non-identified co-receptor. Further, together with 3-dimensional structure of the LG3-domain of agrin and today's protein databases one could aim to identify the agrin co-receptor based on computational models. Second, the exact mapping of the amino acids responsible for MuSK activation could give

the fundaments for designing new drugs by 'peptido mimetica' that are able to activate the MuSK pathway, which would lead to the stabilization of the NMJ's and therefore to a delay of dystrophic symptoms in e.g. hospitalized patients.

In summary, we identified the NEI tripeptide motif within the B/z-8 splice variant, which provide the side-chains mainly responsible for agrins capability to induce MuSK phosporylation. In spite of this, the NEI motif is not totally responsible for agrins activity. In fact, our results show that also amino acids flanking the B/z insert critically contribute to agrins activity. However, investigation of amino acids adjacent to the B/z insert did not give any additional information about involvement of some other important side-chains. Nevertheless, we could not completely fulfill our goals by mutational analysis, we gained on the other hand some new insights in the role of α-DG in activation of MuSK phosphorylation and consequently in the induction of the formation of the postsynapse. Furthermore, we also demonstrate that α-DG plays a crucial role in activation of MuSK. Here we present a new model, where α-DG as the main binding protein of agrin on the muscle surface, plays an auxiliary role in capturing agrin at the muscle surface and presenting the molecule to the receptor complex in order to efficiently activate MuSK. Moreover, our observations lead also to the hypothesis that AChR receptor clustering can be segregated in two different pathways, one that is driven through MuSK and the other through α-DG, as it was previously described for laminin (Sugiyama et al., 1997; Montanaro et al., 1998; Burkin et al., 2000; Marangi et al., 2002).

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Acknowledgments

First of all, I would like to express my gratitude to Prof. Dr. Markus Rüegg for giving me the opportunity to join his lab for doing my thesis. I am particularly thankful for his confidence, patience and support especially during the first project I was given, which was not as fruitful as initially expected.

I am also very thankful to Dr. Thomas Meier for his encouragement and support during my thesis, particularly during the time he spent at the Biozentrum. Furthermore, I really appreciate his agreement to join my thesis comity in this busy time. Also I would like to thank Dorothee Bleckmann from Myocontract, for her collaboration and for her moral and technical support. At this point I would also like to thank Dr. Florian Schärer from Myocontract for his big help and support in the YTH project.

I am indebted to our collaborators Dr. Joerg Stetefeld and Dr. Igor Stagljar for their precious scientific advices. Furthermore, I am very thankful to Prof. Dr. Tom Bickle to join my thesis comity.

I am particularly very grateful to all the members of the 'Rüegg-lab' for their help, fruitful scientific discussions, and especially for their friendship in and out of the lab, including Filippo Oliveri, Patrizia Barzaghi, Godela Bittcher, Sarina Meinen, Dr. Gabriela Bezakova, Iwona Ksianzek, Marcin Maj, Hinchi Kong, Milos Galic, Dr. Conny Burckhard, Dr. Shuo Lin, Dr. Joseph Mascarenhas, Mike Stebler and Eveline Engel. Especially, I would like to thank Patrizia Barzaghi for her friendship and cheering me always up and bringing some 'italian' temperament into the lab. Furthermore, I would also like to thank Filippo Oliveri for his technical assistance and for his moral support and friendship. Also I would like to thank Godela Bittcher for the nice atmosphere and support especially during all the weekends and nights working together in the lab. Then, I would like to thank also Marcin Maj for his humor, friendship and the fruitful scientific and non-scientific discussions.

Furthermore I would like to thank also Iwona Ksiazek, Hinchi Kong and Simon Hippenmeyer for the nice time we spent together at the neuroscience meeting in New Orleans, 2003.

Finally I would like to thank all people of the Biozentrum I had the pleasure to meet and to interact with at work and in private life.

Last but not least I would like to thank my parents and Mariafranca for their love, patience and support they gave me during the thesis.

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List of Publications

Scotton, P., D. Bleckmann, M. Stebler, T. Meier, J. Stetefeld, M.A. Rüegg. Site-directed mutagenesis of agrin splice inserts reveals amino acids involved in postsynaptic differentiation at neuromuscular junction. *In preparation.*

Posters/Presentations

Scotton, P., D. Bleckmann, M. Stebler, T. Meier, J. Stetefeld, M.A. Rüegg. The activity of neural agrin is dependent on single amino acids within the B/z splice site. Program No.36.2 2003 Abstract Viewer/Itinerary Plannar. Whashington, DC: Society for Neuroscience 2003.

Scotton, P., I. Stagljar and M.A. Rüegg: Screening for MuSK interactors using a YTH approach. Neurobiology retreat, Sils-Maria, Switzerland, September 2000 and September 2002.