

**Identification and Characterization of Agrin
in *Caenorhabditis elegans***

INAUGURALDISSERTATION

zur

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

von

Ana Hrus
aus Zagreb, Kroatien

Basel, December 2006

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von Prof. Ruth Chiquet-Ehrismann and Prof. Markus Rüegg.

Basel, den 19.12.2006

Prof. Dr. Hans-Peter Hauri
Dekan

Table of Contents

I. ABSTRACT	3
II. INTRODUCTION	4
II.1. AGRIN: PROTEIN WITH MANY FACES	4
II.1.1. HISTORICAL PERSPECTIVE	4
II.1.2. PROTEIN ARCHITECTURE OF VERTEBRATE AGRIN	4
II.1.3. CONSERVED ALTERNATIVE SPLICING AND ITS FUNCTIONAL IMPACT	6
II.1.4. AGRIN SIGNALING AT THE NEUROMUSCULAR JUNCTION	7
II.1.5. SIGNALING DOWNSTREAM OF MUSK AT THE NMJ	9
II.1.6. DYSTROGLYCAN AND THE CHANGES IN THE CYTOSKELETAL ORGANIZATION AT NMJ	10
II.1.7. FUNCTIONS OF AGRIN OUTSIDE THE NMJ	
II.1.7.1. AGRIN AND THE DYSTROPHIN-GLYCOPROTEIN COMPLEX (DGC) IN MUSCLE	12
II.1.7.2. AGRIN IN THE CENTRAL NERVOUS SYSTEM	13
II.1.7.3. FUNCTIONS OF AGRIN IN THE OTHER ORGANS	15
II.2. CAENORHABDITIS ELEGANS – MORE THAN JUST A WORM	16
II.2.1. <i>C. ELEGANS</i> AS A BIOLOGICAL MODEL ORGANISM	16
II.2.2. ANATOMY OF THE ADULT NEUROMUSCULAR-RELATED SYSTEMS	
II.2.2.1. NERVOUS SYSTEM	17
II.2.2.2. MUSCLE SYSTEM	19
II.2.2.3. NEUROMUSCULAR JUNCTIONS IN <i>C. ELEGANS</i> – DEVELOPMENT AND ANATOMY	20
II.2.3. PHARYNX	23
II.2.4. BASEMENT MEMBRANES	24
II.3 AIM OF THE WORK	27
III. RESULTS	28
III.1. SUBMITTED MANUSCRIPT: “<i>C. ELEGANS</i> AGRIN IS EXPRESSED IN THE PHARYNX, IL1 HEAD NEURONS AND THE DISTAL TIP CELLS OF THE GONAD AND IT DOES NOT GENETICALLY INTERACT WITH GENES IMPORTANT FOR SYNAPTOGENESIS OR MUSCLE FUNCTION”	28
III.2. UNPUBLISHED RESULTS	65
III.2.1. EXPRESSION AND PURIFICATION OF RECOMBINANT <i>C. ELEGANS</i> AGRIN FRAGMENTS	65
III.2.2. DETECTION OF THE ENDOGENOUS <i>C. ELEGANS</i> AGRIN BY IMMUNOFLUORESCENCE	66
III.2.3. ASSAYS <i>IN VITRO</i> WITH RECOMBINANT <i>C. ELEGANS</i> AGRIN FRAGMENT 2LAMG	
III.2.3.1. AChRS CLUSTERING ON MUSCLE MYOTUBES <i>IN VITRO</i>	69
III.2.3.2. AFFINITY CHROMATOGRAPHY EXPERIMENTS WITH <i>C. ELEGANS</i> AGRIN FRAGMENT AND WORM LYSATES	71
III.2.4. STUDIES OF AGRIN <i>IN VIVO</i> FUNCTION IN <i>C. ELEGANS</i>	
III.2.4.1. INVESTIGATING POSSIBLE AGRIN FUNCTION AT THE NMJ	
III.2.4.1.A. PHARMACOLOGICAL ASSAYS WITH LEVAMISOLE AND ALDICARB	73

III.2.4.1.B. INVESTIGATING POSSIBLE AGRIN GENETIC INTERACTIONS WITH ACETYCHOLINE ESTERASES (ACHE)	75
III.2.4.2. ANALYSIS OF PUTATIVE AGRIN FUNCTION IN <i>C. ELEGANS</i> MUSCLE	
III.2.4.2.A. GENETIC APPROACH TO TEST POSSIBLE AGRIN INTERACTIONS WITH COMPONENTS OF THE DGC	77
III.2.4.2.B. ADDRESSING POSSIBLE GENETIC INTERACTION BETWEEN AGRIN AND DYSTROGLYCAN IN <i>C. ELEGANS</i>	79
III.2.4.3. TESTING A POSSIBLE ROLE OF AGRIN IN PHARYNX-RELATED FUNCTIONS	
III.2.4.3.A. PUMPING PHENOTYPE	82
III.2.4.3.B. FEEDING PHENOTYPE – LARGE BACTERIA	83
III.2.4.4. SEARCH FOR PHENOTYPES RELATED TO IL1 HEAD NEURONS	84
III.2.4.5. GENETIC APPROACH TO TEST POSSIBLE AGRIN INTERACTIONS WITH DIFFERENT SIGNALING PATHWAYS INVOLVED IN GONAD DEVELOPMENT	86
III.2.4.6. AXON GUIDANCE EXPERIMENTS	88
IV. DISCUSSION	90
IV.1. OUR QUEST	90
IV.2. THE MOLECULAR STRUCTURE OF <i>C. ELEGANS</i> AGRIN IS SIGNIFICANTLY SIMILAR TO THE VERTEBRATE HOMOLOGUE	90
IV.3. THE <i>AGR-1</i> EXPRESSION PATTERN IN THE HEAD NEURONS AND PHARYNGEAL EPITHELIUM WAS UNEXPECTED	91
IV.4. AGRIN FUNCTION IS DISPENSABLE FOR <i>C. ELEGANS</i> IN THE LABORATORY CONDITIONS	92
IV.5. SIGNIFICANCE OF THE IDENTIFIED INTERACTING PARTNERS	94
IV.6. OPEN QUESTIONS AND POSSIBLE DIRECTIONS	95
V. APPENDIX	98
V.1. EXPERIMENTAL PROCEDURES (UNPUBLISHED DATA)	98
V.2. LIST OF ABBREVIATIONS	106
V.3. REFERENCES	108
CURRICULUM VITAE	120

I. Abstract

Agrin is a large basement membrane (BM) proteoglycan expressed in many tissues in vertebrates, with particularly important function at the neuromuscular junction (NMJ) where it clusters acetylcholine receptors (AChRs) and maintains structural stability of postsynaptic specializations. In order to cluster the receptors it has to activate muscle-specific kinase (MuSK) through an indirect interaction via an unidentified myotube-associated specificity component (MASC). Agrin has also been implicated in providing structural integrity to different tissues by connecting the extracellular matrix (ECM) to α -dystroglycan (α -DG) which is part of a large supramolecular dystrophyn-associated glycoprotein complex (DGC) spanning the cell membrane and binding the actin cytoskeleton.

Since an agrin orthologue was identified in the *C. elegans* genome, we decided to experimentally confirm its expression and characterize the protein. Based on the predicted sequences, we cloned the *agr-1* cDNA and assembled the ORF of 4422 bp from overlapping fragments. The putative protein domain architecture shared high similarity to the vertebrate agrin, except for missing one laminin G (lamG) domain, serine/threonine-rich regions and the SEA module. Since in vertebrates agrin exists in two main isoforms varying at the amino (N)-terminal side, it was surprising to identify only one isoform in *C. elegans*. Likewise, additional alternative splicing that occurs at conserved sites in the vertebrate agrin orthologues having strong impact on the AChRs clustering activity, was not identified in AGR-1. Reporter constructs revealed *agr-1* expression in the buccal epithelium of the pharynx, in four IL1 sensory neurons in the head, and the distal tip cell (DTC) of the gonad, but surprisingly no expression was found in the muscles or the motoneurons innervating them. The specific anti-AGR-1 antibodies detected the protein in the basement membrane of the pharynx.

We analyzed several *agr-1* mutant strains and performed many different assays with the goal to identify its function. No defects related to the NMJ could be found and some indications suggested that it might be implicated in the gonad migrations through genetic interaction with other factors. Based on the expression pattern in the head neurons and pharynx, we expected a sensory or feeding-related function but did not see clear defects. AGR-1 probably acts in parallel with several other proteins in a redundant fashion.

This is the first characterization of an invertebrate agrin orthologue which sets a substantial basis for further research.

II. Introduction

II.1. Agrin: protein with many faces

II.1.1. Historical perspective

Agrin is a large proteoglycan with a prominent function at the developing neuromuscular junction (NMJ) where it plays a pivotal role in the formation and maintenance of the acetylcholine receptor (AChR) clusters. Agrin was discovered more than two decades ago on the basis of its ability to induce clustering of AChRs on cultured myotubes. The observation that some trophic factors from the basal lamina extract of electric ray (*Torpedo californica*) were able to induce AChRs clustering on muscles *in vitro* [1] led to the identification of agrin. The protein was subsequently purified from the extract of the synapse rich *Torpedo* electric organ and, based on the observed aggregating activity, was named “agrín”, coming from Greek “ageirein” which means “to assemble” [2].

Further studies revealed that agrin is synthesized by motor neurons, transported down their axons, released into the synaptic cleft and stably integrated into the synaptic basal lamina (BL), a specialized thin layer of the extracellular matrix (ECM) [3-5]. Based on these findings McMahan proposed the ‘agrín hypothesis’, which states that agrin is a nerve-derived synaptic organizing molecule [6]. As an additional support to the hypothesis, it was demonstrated that purified agrin, when added to the cultured myotubes, induces the AChRs aggregation in a dose-dependent manner. Moreover, when mixed (chick and rat) nerve-muscle co-cultures *in vitro* were treated with the species-specific anti-agrin antibodies, it was confirmed that exclusively agrin released from motor neurons is responsible for the AChRs clustering [7].

II.1.2. Protein architecture of vertebrate agrin

So far agrin has been cloned from several vertebrate species including rat [8], chick [9,10], marine ray (*Torpedo californica*) [11], and man [12]. In all these species, the agrin gene encodes a large protein of more than 2000 amino acids with approximate size of 225 kDa. With additional glycosylation the molecular weight reaches up to 400-600 kDa (reviewed in [13]). Agrin exists in several isoforms with distinct expression patterns. The protein is localized particularly in the basement membrane, or stays attached to the membrane in tissues devoid of it.

Domain architecture of agrin is characterized by repetitive modules homologous to domains found in other basal lamina proteins, namely: nine Follistatin-like domains (FS), that bear homology to Kazal-type protease inhibitor domains; two laminin EGF-like domains (LE); two serine-threonine-rich regions (S/T)

flanking a domain common to sperm protein, enterokinase, and agrin (SEA); four EGF domains; and three laminin globular domains (lamG) (Introduction, Fig. 1) [8,9].

At least three O-linked carbohydrate attachment sites for glycosaminoglycan (GAG) side chains are found within the S/T-rich region [14]. It was experimentally confirmed that agrin can be glycosylated with both heparan and chondroitin sulphate glycosaminoglycan chains [15]. In addition, agrin harbors several consensus sites for N-linked sugar modifications, (for a review, see [13]). Glycosylation is important for agrin binding to a membrane-linked protein α -dystroglycan (α -DG) and for the clustering activity at the neuromuscular synapse development. Early *in vitro* experiments on glycosylation-deficient myotubes demonstrated the impairment of AChRs clustering, suggesting the importance of glycosylation for this process [16].

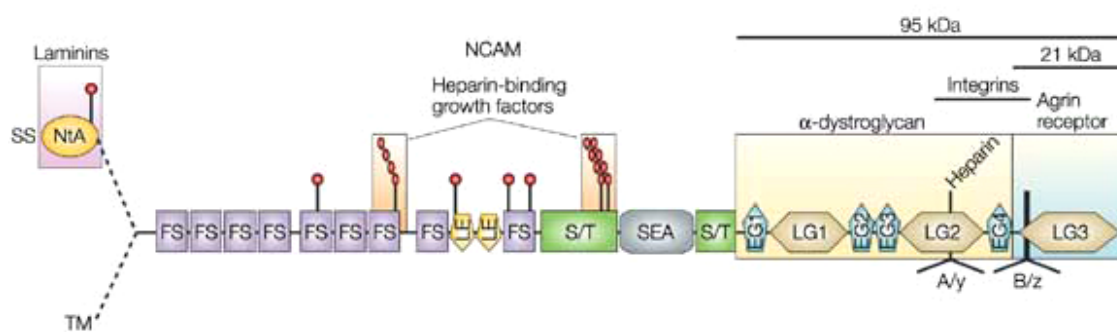


Fig. 1. Protein architecture of the vertebrate agrin isoforms. The SN and LN isoforms are presented; follistatin-like domains (purple, FS); laminin-type EGF domains (yellow, LE); serine-threonine rich region (green, S/T); sperm enterokinase and agrin domain (lilac, SEA); EGF domains (blue, EG1); laminin globular (beige, LG); (Source: [13])

Several alternative isoforms of agrin have been identified in vertebrates, with the variations at the amino (NH₂)-terminus and alternative splicing in the carboxy (COOH)-terminal part. A longer amino (N)-terminal isoform (LN) contains a cleaved secretion signal (SS) followed by an N-terminal agrin (NtA) domain [10]. The NtA domain confers binding to domain I/II of the laminin γ 1 chain in the basement membrane [17,18], thereby immobilizing agrin in the basal lamina. Originally identified in the chick, the NtA domain was found to be highly conserved in human, bovine, mouse [17,19] and predicted in the rat genome under the NCBI entry number AF250032.

The shorter N-terminal agrin isoform (SN), which was first identified in rat [8], lacks the secretion signal and the laminin-binding NtA domain, but instead has a non-cleaved internal signal sequence, which serves as a membrane anchor [19] and turns agrin into a type II transmembrane protein [20]. The amino terminal fragment of LN agrin is encoded by three exons, localized 8 kb upstream of the first SN exon. The presence

of such a big intron suggests that the two isoforms are transcribed from different promoters, rather than being a result of an alternatively spliced common mRNA precursor [21].

The expression of the two N-terminal agrin isoforms is highly tissue-specific. The LN agrin is expressed in both neural and muscle tissue, while the SN agrin is expressed in nervous system in a variety of different neuronal cell types. LN agrin is secreted and integrated into the basal lamina, whereas SN agrin stays attached to the plasma membrane [20]. Tissues devoid of the basement membrane, such as brain, predominantly express the SN trans-membrane agrin isoform.

II.1.3. Conserved alternative splicing and its functional impact

Additional alternative splicing occurs at two more conserved sites at the C-terminus, in both LN and SN agrin: termed A and B in chicken and y and z in rat (Intro, Fig. 1) [22,23]. At the A/y site an alternatively spliced exon encodes a four amino acid insert composed of lysine-serine-arginine-lysine (KSRK) which is important for heparin binding and modulating the binding to α -DG [24]. The splicing at the B/z site depends on the alternative usage of two short exons encoding 8, 11 or 19 (8+11) amino acid inserts. Splicing at both sites occurs in a synchronized tissue-specific manner. Agrin isoforms containing inserts at the B/z site (denoted as B/z⁺) are active in clustering of acetylcholine receptors at the NMJ and are selectively expressed by neuronal tissue [23,25]. Schwann cells are the only non-neuronal cells that express agrin isoforms with the B/z⁺ inserts.

Different combinations of alternatively spliced inserts at the A/y and B/z sites give rise to isoforms with significantly different clustering activity [26]. Isoforms A₄B₈ and A₄B₁₉ expressed by motoneurons are the most active in AChR clustering, while the A₀B₀ expressed in muscle are the least active. Recombinant C-terminal fragment, containing the three lam G domains with A₄B₈ splicing and total 95kD in size (C₉₅A₄B₈), has very similar activity compared to the full length A₄B₈ agrin. The fragment containing only the last lam G domain, of 21kDa in size, with B₈ splicing (C₂₁B₈), is sufficient for the clustering activity with several-fold lower potency (Intro, Fig. 1) [27]. Alpha-dystroglycan was originally proposed to be the agrin receptor involved in AChRs clustering, but the minimal active agrin fragment does not bind to it. Therefore, agrin probably binds to another protein, in a manner dependant on the splicing inserts at the B/z site [24].

On the other hand, binding to α -DG is much stronger in isoforms B/z⁻, lacking inserts at B/z site which suggests other physiological roles for this agrin isoform expressed in many non-neuronal tissues, e.g. muscle, heart, kidney [23,28]. Binding of agrin to α -DG at the NMJ might provide the structural support and stability of the already formed post-synaptic apparatus [24]. Outside the synapse, throughout the muscle

sarcolemma, this interaction might contribute to the connection between ECM and intercellular cytoskeleton therefore providing better tissue integrity.

II.1.4. Agrin signaling at the neuromuscular junction

At the developing neuromuscular junction (NMJ) the ingrowing nerve and the muscle membrane adopt numerous specializations in order to establish a functional contact (Intro, Fig. 2). Proper clustering of neurotransmitter receptors is one of the crucial post-synaptic specializations. In the rodent embryo, AChRs have a density of about $1000/\mu\text{m}^2$. In adults, by contrast, AChRs are concentrated at high density up to $20\,000/\mu\text{m}^2$ in the tiny fraction of synaptic muscle fiber membrane underneath the nerve terminal, whereas the remaining membrane (more than 99%) bears less than $10\text{ AChRs}/\mu\text{m}^2$ (for review see [29]).

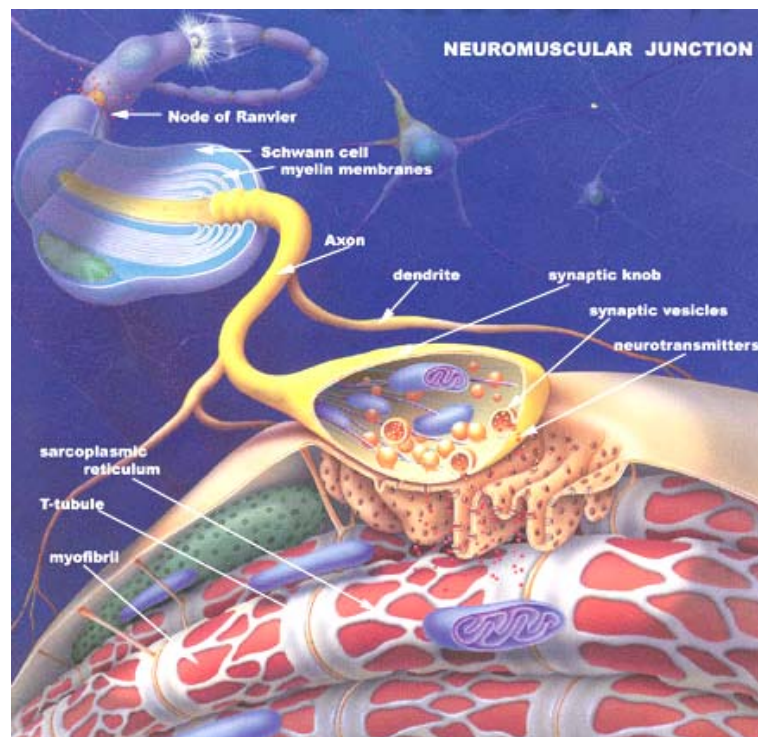


Fig. 2. Schematic presentation of a vertebrate neuromuscular junction. (Source: <http://fig.cox.miami.edu>)

Since the proposal of the ‘agrin hypothesis’, significant effort has been invested in identifying the receptor of agrin and its downstream signaling. A transmembrane receptor tyrosine kinase MuSK (Muscle-specific kinase) was considered a likely candidate [30]. Agrin induces rapid phosphorylation of MuSK on wild type muscle cells, but on $\text{MuSK}^{-/-}$ myotubes it fails to cluster AChRs [31]. Conversely, the AChRs-aggregating ability is restored upon MuSK expression [32]. Although agrin and MuSK are functionally correlated, there is no direct binding between them, suggesting that MuSK does not serve as a direct agrin receptor, but rather as a part of a common signaling complex. MuSK requires additional factors and/or modifications that bind and respond to agrin. Moreover, agrin can only activate MuSK in the context of a differentiated myotube,

and not in immature myoblasts or other cell types, suggesting a necessary role of other signaling factors present only in the differentiated muscle cells. Therefore, this putative missing link is termed myotube-associated specificity component (MASC) [31]. Even without a direct binding to MuSK, a conserved tripeptide “asparagine-leucine-isoleucine” within the B₈ insert of agrin is necessary and sufficient for the full MuSK phosphorylation activity [33].

Upon agrin binding to its receptor complex and the concomitant MuSK phosphorylation, downstream signaling cascades are activated leading to the activation of rapsyn, a 43 kDa peripheral membrane cytoplasmic linker [34-36]. The mobilization of the intracellular rapsyn depends on the extracellular part of MuSK, thus their contact is mediated through a putative linker protein which spans the membrane and binds the two proteins at opposite sides of the sarcolemma. This hypothetical link is termed rapsyn-associated transmembrane linker (RATL) [35].

Rapsyn binds to AChRs in equimolar ratio and contributes to their clustering, following phosphorylation of AChR β subunits [37]. *In vitro* experiments with cultured myotubes from rapsyn-deficient mice demonstrated that, although MuSK gets phosphorylated and activated, there is no phosphorylation of AChR β subunit and no concomitant AChRs clustering. These data suggested that rapsyn serves as a cytoplasmic linker between MuSK activation and the downstream signaling leading to the receptor clustering [35].

Genetic analysis of agrin and MuSK mutant mice supports the importance of the two proteins in forming functional NMJs *in vivo* [38,39]. Agrin mutant mice have significantly reduced number, size, and density of AChR clusters on the muscles and they die soon after birth due to breathing failure. The formation of both presynaptic and postsynaptic specialization is impaired. Surprisingly, despite the lack of agrin, postsynaptic differentiation is present to some degree: small uninervated AChR clusters distributed over the entire muscle fiber and some transcriptional specialization were present in the subsynaptic nuclei [38]. The importance of the B/z⁺ isoform was confirmed *in vivo*. Agrin knockout mice, with a specific deletion of the z exons only, gave the same phenotype as the null mutants [25].

MuSK deficient mice, as the agrin mutants, die at birth due to the inability to breathe. NMJ synapses are abnormal, nerve terminals lack arborization and no AChRs are clustered. Muscle structure, on the contrary, appears normal in comparison to the wild type control. No specializations are formed either on the postsynaptic membrane (AChRs, ErbB₄, utrophin, rapsyn) or in the basal lamina (AChE) and the synapse-specific transcription is abolished [39]. Moreover, muscle cells taken from MuSK-deficient mice fail to respond to the neural agrin by clustering AChRs [31].

Similarly like agrin and MuSK mutant mice, rapsyn knock-outs do not develop functional NMJs [40]. Their motor terminals grow more extensively than those present in wild type mice, but fail to establish synapses with clustered AChRs. In rapsyn knock-out mice MuSK is normally localized. This *in vivo* finding supported that agrin activation of MuSK presents the first step in the NMJ development, serving as a primary scaffold for docking rapsyn and aggregation of the receptors.

As a complementary approach to the knock-out studies, ectopic overexpression of agrin confirms its orchestrating function at the developing NMJ. Neuronal agrin isoform, when either injected or transfected into the extrasynaptic regions of innervated muscle fibers, can induce postsynaptic specializations and AChRs clustering [41,42].

II.1.5. Signaling downstream of MuSK at the NMJ

The agrin-induced signaling downstream of MuSK is still largely unknown. Upon stimulation and activation by agrin, MuSK binds the mammalian homologue of the *Drosophila melanogaster* protein Dishevelled (Dvl), a protein involved in the planar cell polarity signaling (Intro, Fig. 3) [43]. At the formed MuSK scaffold, protein PAK (p21-activated kinase) is recruited, and gets activated through Rac or Cdc42, thus mediating AChRs clustering through the planar cell polarity pathway (PCP). Either inhibition of Dvl or PAK interferes with AChR clustering at the neuromuscular synapse.

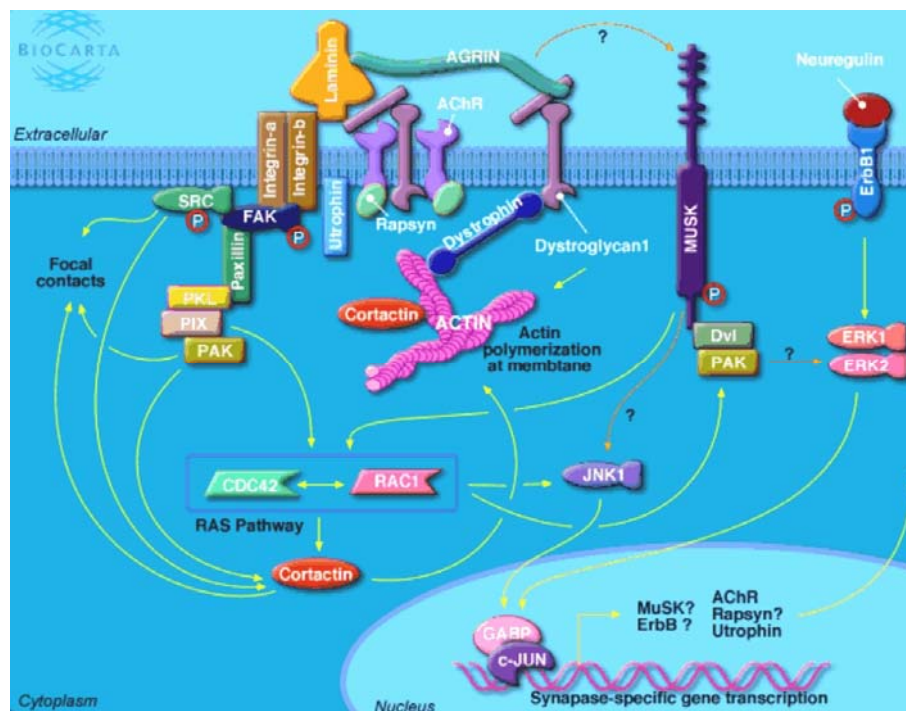


Fig 3. Signaling at the NMJ downstream of MuSK. Agrin indirectly activates MuSK, it gets phosphorylated and recruits Dvl and PAK, further activated by Cdc42/Rac pathway. (Source: www.biocarta.com)

Since recently, another cytoplasmic protein, Dok-7, has been implicated in neuromuscular synaptogenesis [44]. Dok-7 binds to MuSK through its phosphotyrosine-binding (PTB) domain, induces its autophosphorylation, therefore being essential for the activation of the MuSK signaling pathway leading to AChRs clustering in myotubes. Mutant mice lacking Dok-7 have the same defects as MuSK and agrin mutants, i.e. they are born immobile and die shortly after birth, probably due to the breathing failure.

Another protein, casein kinase 2 (CK2), binds MuSK *in vitro* and colocalizes with it at the sites of postsynaptic structures *in vivo* [45]. CK2 mediates phosphorylation of MuSK serine residues within the kinase insert domain (KI). In addition, the muscle-specific CK2 β knock-out mice develop a myasthenic phenotype due to defective NMJ morphology and its impaired function.

Several distinct proteins, functionally involved downstream of the agrin-MuSK pathway, have been identified so far, but the complete signaling pathway has yet to be elucidated.

II.1.6. Dystroglycan and the changes in cytoskeletal organization at the NMJ

As a part of a large transmembrane complex, dystroglycan is recruited to the NMJ where, it participates in the anchoring and recruitment of AChRs during the formation of synaptic as well as non-synaptic AChR clusters [46]. Alpha-DG binds agrin and plays a role in AChRs aggregation on muscle myotubes. The same blocking antibody, that inhibits receptor aggregation, is able to block agrin binding to α -DG, thereby suggesting the importance of their interaction at the NMJ. It is known that binding of agrin to α -DG depends on the glycosylation. Accordingly, myotubes with defective glycosylation do not form normal AChRs clusters and the binding of α -DG to agrin is abolished [47,48]. Dystroglycan chimeric mice develop progressive muscle degeneration and NMJs seem to be disrupted, although the basement membrane ultrastructure and laminin deposition look normal [49].

Based on the agrin- α -DG binding and the aberrant AChRs clustering due to the anti- α -DG blocking antibodies, it was expected that α -DG is the functional agrin receptor [47,48]. However, this is not the case because α -DG binds equally strongly both active and inactive agrin isoforms [24] and additionally, the smallest fragment sufficient to induce AChRs clustering does not bind to α -DG [24,50].

At the synapse, dystroglycan colocalises with agrin-induced AChRs clusters and modulates their density, but agrin signaling is not significantly changed in muscle cell lines with decreased α -DG level. MuSK phosphorylation at later time points does not differ significantly as compared to the normal control. Therefore, α -DG is a functional agrin receptor but it is not required for the signaling through MuSK. More likely α -DG acts downstream of the AChRs clustering induced by agrin-MuSK signaling to consolidate nascent clusters or their maintenance and growth [51]. Although not involved in the initial AChRs clustering, dystroglycan and integrins have been implicated in the stabilization of the preformed clusters [52,53].

Despite the prominent function of agrin at the NMJ, it is clear that AChRs clusters can spontaneously form even in its absence [38]. On the other hand, MuSK and rapsyn are required for these intrinsic postsynaptic patterning events [54,55]. Additionally, transcriptional specialization of synaptic nuclei can occur in the absence of innervation. According to more recent interpretation of these data, Agrin/MuSK signaling is presumed to stabilize the previously formed AChR clusters and induce their further growth. Following the innervation of the pre-formed clusters, the aneural AChR clusters are dispersed by a nerve-dependent mechanism [54]. More recent findings suggest that the neurotransmitter ACh acts as a signal for dispersal of clustered AChRs, while agrin counteracts it and stabilizes the clusters [56]. In mice lacking ACh, neuromuscular junctions can form independently of agrin, meaning that in double mutant mice, synapses do form relatively normally. According to this interpretation, agrin is considered to be an AChR clustering factor as well as an AChR anti-declustering factor (Intro, Fig. 4).

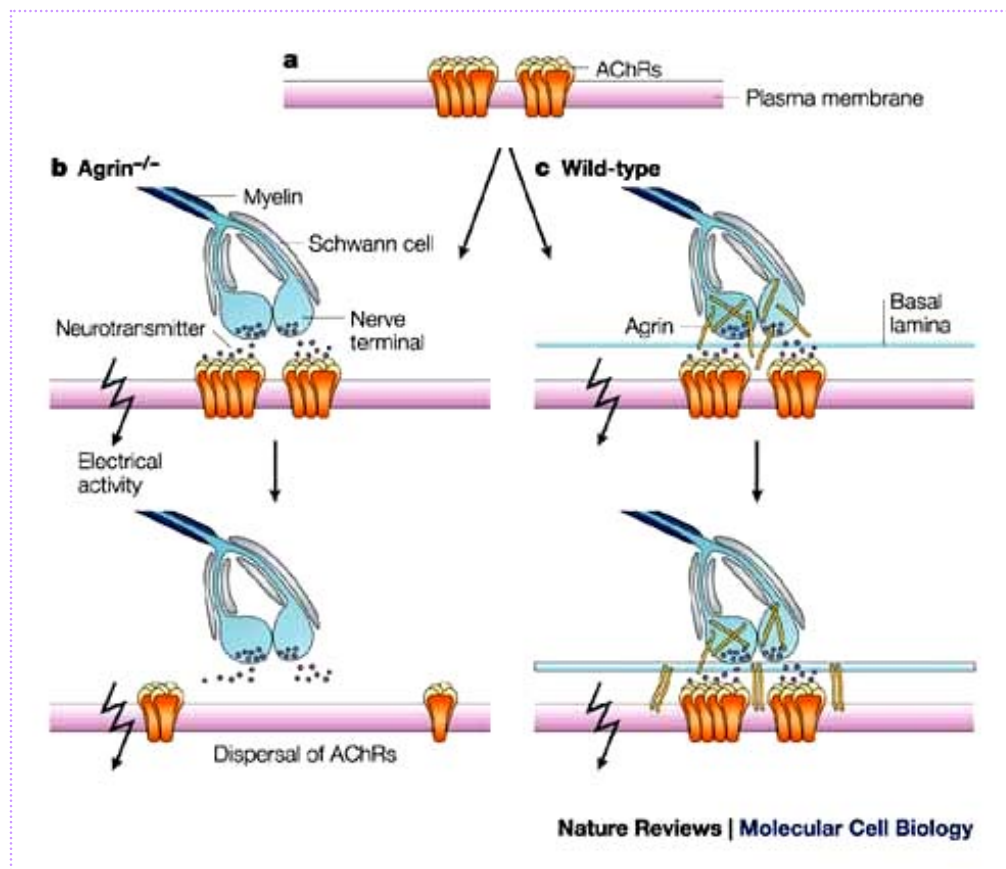


Fig. 4. Agrin stabilizes the AChR clusters at the NMJ. In Agrin-deficient mice (B) innervation induced dispersal of the spontaneously-formed AChRs clusters (A), while in the wild type animals the AChR clusters are stabilized (C). (Source: [13])

II.1.7. Functions of agrin outside the NMJ

II.1.7.1. Agrin and the dystrophin-glycoprotein complex (DGC) in muscle

The dystrophin-glycoprotein complex (DGC) is a large multi-protein complex connecting ECM to the intracellular cytoskeleton (Intro, Fig. 5) [57]. The central components of the DGC complex are α - and β -dystroglycan, post-translationally cleaved from a common precursor protein [58], where α -DG is an extracellular peripheral membrane protein that stays non-covalently associated to the transmembrane β -DG. Alpha-DG is targeted to the cell surface even in the absence of β -DG, suggesting that the two proteins may coordinate independent roles in skeletal muscle signaling [59]. On the extracellular side, α -DG interacts with several ECM proteins that contain laminin globular (lamG) domains, e.g. laminin, agrin, perlecan, while β -DG on the intracellular side binds to dystrophin which is in contact with the actin cytoskeleton. Moreover, β -DG associates with intracellular dystrobrevin and syntrophins at its C terminus, and within the membrane it is in direct contact with transmembrane sarcoglycans (α , β , γ , and δ) and sarcospan, assembling together a large DGC supramolecular complex (for a review, see [60,61]).

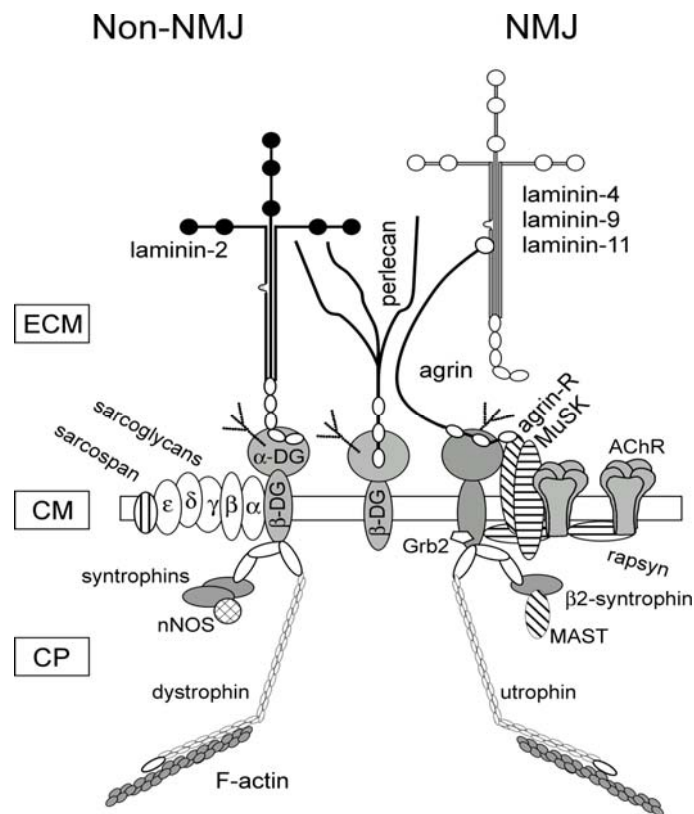


Fig. 5. Schematic representation of DGC complex at the muscle membrane outside the NMJ compared to the agrin complex at the NMJ. (ECM, extracellular matrix; CM, cell membrane; CP, cytoplasm; Source [61])

Genetic studies on animal models have shown that mutations in many components of the DGC lead independently to the outcome of muscular dystrophies. The underlying molecular mechanism is based on the interruption of the multi-protein complex between laminin, dystroglycan, and dystrophin which leads to the weakening of the connection between ECM and the muscle cytoskeleton (for a review, see [62]). More recent findings indicate that, beside the impaired structural integrity, the altered signaling pathways contribute to the outcome of muscular dystrophies, namely abnormal Ca^{2+} influx and defective MAPK pathways (for a review, see [63]).

Glycosylation is crucial for DG binding to its ligands: laminin, neurexin and agrin. DG is abnormally glycosylated in several muscular dystrophies what prevents it from establishing functional connections necessary for muscle structural integrity. The myodystrophy mouse model (*myd*) recapitulates the same defects in muscle as the patients suffering from muscular dystrophies. In addition, the *myd* mice display brain abnormalities, previously described in the human muscular dystrophy patients. Therefore, the DG-mediated link between cytoskeleton and ECM is pivotal in the pathology of muscular dystrophy with abnormal neuronal migration [64].

Denervation of muscles *in vivo* causes changes of muscle membranes and costameres similar to the features of muscular dystrophies. On the other hand, the over-expression of secreted B/z⁻ agrin isoform, by microinjecting it into an innervated muscle, preserves the organization of muscle structures even after denervation [65]. These data imply that agrin has a role in linking the ECM to the cytoskeleton, thus providing better structural integrity of the whole muscle membrane. In addition, the over-expression of a miniature form of agrin, composed of a laminin-binding NtA domain and lamG domains that bind to α -DG, can rescue the dystrophic phenotype of mice carrying mutation in the laminin-2 gene. Agrin is believed to make up for the defective link by binding to the other laminins with its N-terminal part and α -DG with its C-terminus [66]. Therefore, although it is not the only extracellular ligand of dystroglycan, agrin plays a role in the DGC complex and the concomitant cytoskeletal dynamics.

II.1.7.2. Agrin in the central nervous system

In the central nervous system (CNS) agrin is expressed in many cell types, but its function and signaling mechanisms are not yet completely clear. The transmembrane (TM) agrin isoform, which lacks the laminin-binding domain, is the dominant agrin isoform in the brain [19]. Agrin is expressed both by neurons and non-neuronal cells in the brain, early in development, and high levels are present in developing brain and spinal cord. The isoform lacking the inserts at the z site which has the highest affinity for α -DG is present in the blood-brain barrier at the side of the microvasculature. By binding to α -DG, agrin might serve as a link

between the ECM and the astrocytic cytoskeleton. Increased permeability of the blood-brain barrier is a feature of Alzheimer's disease, where agrin may be correlated to the disease etiology since it was detected as a major component of amyloid plaques and accelerates the fibril formation (reviewed in [67]).

Agrin was shown to inhibit neurite growth in a variety of neuronal cell types *in vitro*: in ciliary ganglia (CG) neurons, dorsal root ganglia, retina and hippocampus [68-72]. Its expression was detected in, and near, multiple axon pathways during development [70], suggesting physiological relevance of agrin function as a neurite growth inhibitor.

In vitro experiments demonstrated that agrin can induce *c-fos* expression on cultured cortical neurons, as it would be expected for a signal transduction pathway activated by a cell surface receptor [73]. Interestingly, the alternative splicing does not have any effect on *c-fos* activation, as compared to the NMJ where it influences the clustering activity. Therefore, the *in vitro* experiments provide evidence for a functional agrin receptor in CNS where agrin might have a role in the neuron-neuron synaptogenesis. As expected, the agrin-deficient neurons are less sensitive to *c-fos* induction by *in vitro* experiments. Agrin mutant mice show lower mortality and seizure activity in response to kainate, an excitatory drug and a potent agonist of glutamate receptors, in comparison to the wild type mice. Additionally, the *in vivo* induction of *c-fos* by kainate, is much lower in agrin heterozygotes than in wild type mice, suggesting an *in vivo* role of agrin in neuronal signaling by excitatory neurotransmitters [74].

The attempts to identify the agrin receptor in CNS indicated that agrin binds to several molecules on the neuronal surfaces: to $\alpha V\beta 1$ integrin, through its second laminin G domain, to $\beta 1$ integrin with its fourth EGF repeat, and with the last laminin G domain to a, yet unidentified, molecule on neuron surface [20].

Colocalization studies on cultured neurons and biochemical binding experiments demonstrated that the CNS agrin binds to the Na^+/K^+ -ATPase (NKA) family of membrane ion pumps [75]. Agrin binding evokes fast and reversible depolarization of neuronal membrane. The minimum active fragment is the agrin C-terminus, of 20 kDa in size, independently of alternative splicing. Interestingly, the sequences flanking the conserved alternative splicing site seem to be important, because their deletion, which leaves a fragment of 15kDa, acts as an agrin antagonist [76]. Agrin induces similar effects as the cardiac glycoside ouabain, a naturally-occurring NKA inhibitor, which is used in clinics for the treatment of heart failure and for the *in vitro* NKA inhibition studies. It was demonstrated that the inhibition of NKA activity is responsible for a form of long-term plasticity in hippocampal interneurons [77], therefore agrin is speculated to act as an endogenous NKA inhibitor involved in synaptic plasticity [75].

II.1.7.3. Functions of agrin in the other organs (i.e. kidney, lung, and immunological system)

The widespread expression of agrin in many adult tissues, e.g. kidney [78,79], lung [12,80], heart, and skeletal muscle [23,28], suggests multiple functions outside the developing NMJ. Interestingly, agrin in these tissues does not contain the inserts at the conserved B/z site. The agrin isoform A₀B₀ from several tissues (i.e. kidney and lung) binds to α -DG with high affinity. The two proteins were also found colocalised in the developing organs [80]. This widespread distribution of agrin isoforms which lack the AChRs clustering activity, but have the α -DG affinity instead, indicates agrin's function as a linker between basal lamina and the cytoskeleton.

In kidney agrin was found to be expressed primarily in the glomerular basement membrane [79], where it contributes to linking the podocyte cytoskeleton to the basement membrane, supporting the membrane integrity [81]. In several forms of nephropathy agrin glycosylation is defective, possibly due to increased expression of the deglycosylating enzyme heparanase [82].

In lung, agrin is expressed in the alveolar basement membranes where it is speculated to inhibit the tissue proteases, thus participating in membrane stabilization which has to be tightly balanced with proteolysis [12].

Agrin plays a role in the formation of the immunological synapse, the functional contact between T lymphocytes and antigen presenting cells (APCs), by setting the threshold for T-cell activation and further signaling. Agrin is believed to activate T-cells by inducing lipid raft aggregation. Interestingly, T-cells express only agrin isoform without alternatively spliced inserts at the z site (z₀). In resting T-cells agrin is glycosylated, but due to activation it gets extensively deglycosylated, contrary to the activity in the NMJ. When incubated with muscle myotubes *in vitro*, only agrin purified from activated (agrin_{act}) not resting (agrin_{rest}) T-cells, was able to induce AChRs clustering despite its lack of inserts at the z site [83]. Agrin is expressed on lymphocytes and is upregulated in their activation. Alpha-DG is also expressed on lymphocytes and colocalises with agrin. Down-regulation of α -DG expression inhibits lymphocyte activation even in the presence of agrin, suggesting that both factors together mediate lymphocyte activation [84].

II.2. *Caenorhabditis elegans* – more than just a worm

II.2.1. *C. elegans* as a biological model organism

C. elegans is a free-living nematode with the natural habitat in the soil. Its body is approximately 1mm long and 0.1mm wide. It is built of various types of cells, with an invariable number of 959 cells in hermaphrodites and 1031 cells in males [85]. The relatively simple anatomy, fast generation time (3 days at 25°C), large number of progeny (300 – 350 from a single parent), and easy maintenance in the laboratory make it a very attractive model organism. Sydney Brenner was the one who recognized the potential of *C. elegans* for powerful genetics and in 1965 pioneered studies of it in the laboratory [86]. The worms' transparent body made it possible to dissect developmental lineages for each cell in the developing worm [87].

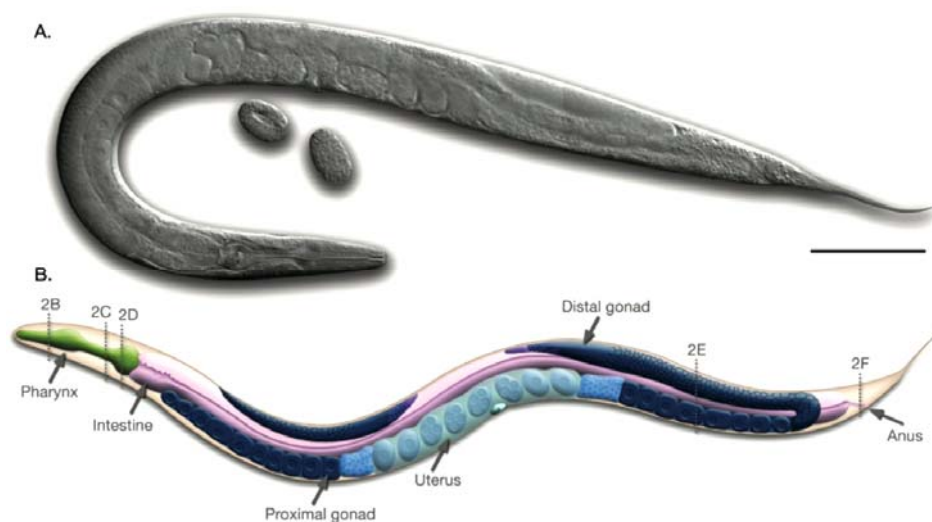


Fig 6. Anatomy of an adult hermaphrodite (Source: www.wormatlas.com). **A**, DIC image of an adult hermaphrodite, as seen from left lateral side. Scale bar 0.1 mm. **B**, Schematic drawing of anatomical structures, left lateral side. Dotted lines and numbers mark the level of transverse sections from the figure source (not shown here).

Although quite simple, the organs in *C. elegans* are highly sophisticated and therefore of interest to be studied (Intro, Fig. 6). They include a gastrointestinal tract, a reproductive system, epithelial, nervous, muscle, excretory tissues, and immune system. In addition, most of the underlying molecular mechanisms for the major physiological processes are highly conserved when compared to vertebrates [88]. Therefore,

the experimental data obtained from the worm proved to be highly informative and applicable in elucidating the analogous mechanisms in mammals.

Different genetic approaches have been taken to investigate the functions of the vertebrate gene homologues identified in *C. elegans* genome (reverse genetics) or to identify the previously unknown genes which, if mutated in the worm, result in interesting phenotypes (forward genetics). For forward genetics, worms are commonly mutated with chemical mutagens (such as ethyl methane sulphonate, EMS) often in combination with UV irradiation [86,89], followed by large-scale screens to identify certain phenotypes, such as resistance to a particular drug (e.g. levamisole, aldicarb, ivermectin). On the other hand, in reverse genetics approaches. The goal is to learn more about a particular, already known, gene and address its mechanisms of action in *C. elegans* [90]. Both approaches are commonly used to identify novel candidate drug targets for treatment of human diseases (reviewed in [91]).

As mentioned above, there are two *C. elegans* sexes, a self-fertilizing hermaphrodite (XX) and a male (XO). In a normal population males arise infrequently (0.1%), but through mating they occur at higher frequency (up to 50%). Hermaphrodites can be induced to generate male progeny spontaneously at a higher rate by treatment at 30°C. Hermaphrodite self-fertilization allows the generation of genetically identical progeny from homozygous mutants, while mating facilitates the isolation and maintenance of mutant strains as well as moving mutations between strains.

II.2.2. Anatomy of the adult neuromuscular-related systems

II.2.2.1. Nervous system

In adult *C. elegans* hermaphrodites there are 302 neurons and 56 neuron-related glial cells, together making up for 37% of the total somatic cell number in the adult hermaphrodite (reviewed in [92]). Since many of the neurons are not essential for the animals' survival in laboratory conditions, even mutations causing severe impairments can still be maintained and experimentally studied, a fact that makes *C. elegans* a wonderful model for neurobiology.

The neurons in *C. elegans* can be classified by at least four different criteria: general function (sensory, interneurons, and motoneurons); “neurotransmitter phenotype” (GABAergic, glutamatergic, cholinergic); according to the functional units (e.g. sensory sensilla); based on the specific morphological features (cell body position, axodendritic morphology, synaptic connectivity) (Intro, Fig. 7). Unlike in most species, in *C. elegans* a well defined neuronal precursor does not exist, therefore even the cells within the same class (according to the classifications above) do not originate from the same lineage.

Remarkable work on the reconstructions of serial electron microscopic sections from the whole *C. elegans* body provided significant insight into the anatomy of its nervous system and helped define 118 main neuron classes according to morphological criteria [93]. Sensory neurons and interneurons occur mostly in bilaterally symmetric pairs. The entire nervous system has in total 5000 chemical synapses, 600 gap junctions and 2000 neuromuscular junctions [94].

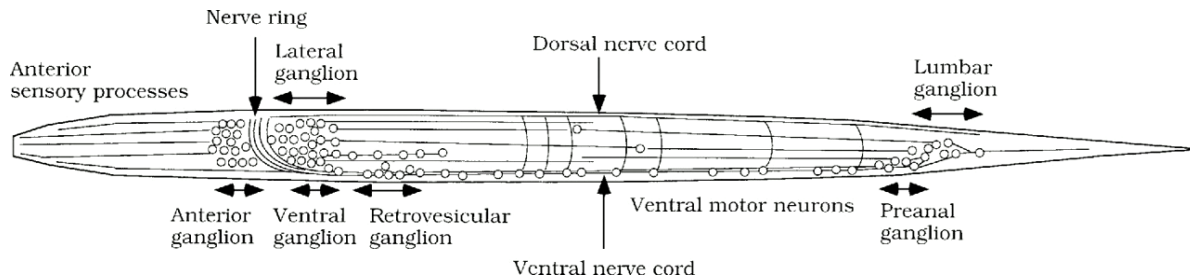


Fig 7. The structure of the *C. elegans* nervous system with depicted positions of the main ganglia and process bundles (only the left side of the bilaterally symmetrical neurons presented). Neuronal processes run along the body mainly in the ventral and dorsal cord (connected by commissural processes) and along lateral axonal tracts. (source: [95])

Sensory neurons have openings at the cuticle with specialized sensory endings (e.g. ASH amphid neurons). The chemosensory neurons can be classified as follows: two bilaterally symmetrical amphids each containing endings of 12 sensory neurons; two symmetric phasmids in the tail, each consisting of two neurons; and six inner labial (IL) sensory organs in the head [96].

There are several classes of mechanosensory neurons based on the stimuli they are specialized for and the responses they induce. The ASH, FLP and OLQ neurons are responsible for the light nose touch, each class accounting for a fraction of the normal response. In addition, ASH neurons have a unique polymodal role in sensing high osmolarity and volatile repellents [97]. Glutamatergic neurons IL1 and OLQ synapse to the RMD neuron and are responsible for spontaneous foraging (i.e. head movements) and head withdrawal reflex [98]. Touch cells ALM, AVM, PLM are responsible for sensing light body touch, experimentally scored by an eyelash touch along the body. Those cells respond also to gentle tapping sensation [99]. The worms can distinguish between light and harsh body touch because different neurons are stimulated. The harsh touch stimulates PVD neurons which synapse to the AVA and PVC interneurons, experimentally scored by a thin platinum wire.

In order to evoke movement, a sensory input is transmitted to the motoneurons, either directly or through the interneurons. Most of the synapses are localized at the nerve ring, a neuropile around the pharynx. The motoneurons and interneurons in *C. elegans* extend their axons mainly along the dorsal and ventral midline of the body, in the fasciculated tracts of the dorsal and ventral nerve cord. Their cell bodies are localized in one of the ganglia (Intro, Fig. 7) or along the ventral nerve cord, from where they can extend circumferential processes, commissures, towards the dorsal cord. Many different molecules are implicated in the axon guidance along the ventral midline (e.g. netrin/UNC-6 via the attractant DCC/UNC-40 receptor, EphR/VAB-1, collagen XVIII, nidogen) and the commissural guidance towards the dorsal cord (e.g. UNC-6/netrin via a repulsive UNC-5 receptor). The mutations of two heparan sulphate-modifying enzymes were identified in genetic screens as responsible for defects in axon branching, therefore indicating the importance of glycosylation in axon guidance (reviewed in [100]). A recent report provided evidence that the heparan sulphate syndecan (SDN-1) regulates cell migration and axon guidance in *C. elegans* [101].

II.2.2.2. Muscle system

In *C. elegans* there are several groups of muscles with different structure and function, namely two major groups: striated body-wall muscles, supporting locomotion, and non-striated muscles such as pharyngeal, intestinal, vulval, uterine and anal muscles [102]. Sarcomeres are the main structural components of all muscles: striated muscles contain many highly-organized sarcomeres, while non-striated ones have only one.

In the body-wall muscles of *C. elegans*, sarcomere assembly is a highly orchestrated process, giving rise to the sarcolemma attachments with remarkable similarity to vertebrates [103]. The body wall muscles are positioned longitudinally, assembled from 95 cells organized in four quadrants (two dorsally, two ventrally) each consisting of two rows of muscle cells in staggered pairs (Intro, Fig. 8A,B). Each spindle-shaped muscle cell makes attachment complexes to the neighboring muscle cells as muscle-muscle plaques, as well as attachments to the hypodermis. Additional strong attachments are established between muscles and hypodermis, through the basal lamina, particularly through the contact between integrin (PAT-2/PAT-3) and perlecan (UNC-52). Within the muscle cells, precisely aligned with the hypodermal contacts, dense bodies and M-lines are positioned and attached to the actin thin filaments and myosin thick fibers (Intro, Fig. 8C). On the side towards hypodermis, hemidesmosome-like structures make connections the cuticular exoskeleton [104]. In the regions of muscle contacts, the hypodermis contains tightly compressed structures rich in organized intermediate filaments (IFs) called fibrous organelles [105]. MUA-3 is one of the proteins

implicated in these attachments localized on the apical side of the hypodermis [106]. Their development is tightly coordinated with the muscle development, and regulated by several factors, such as myotactin [107].

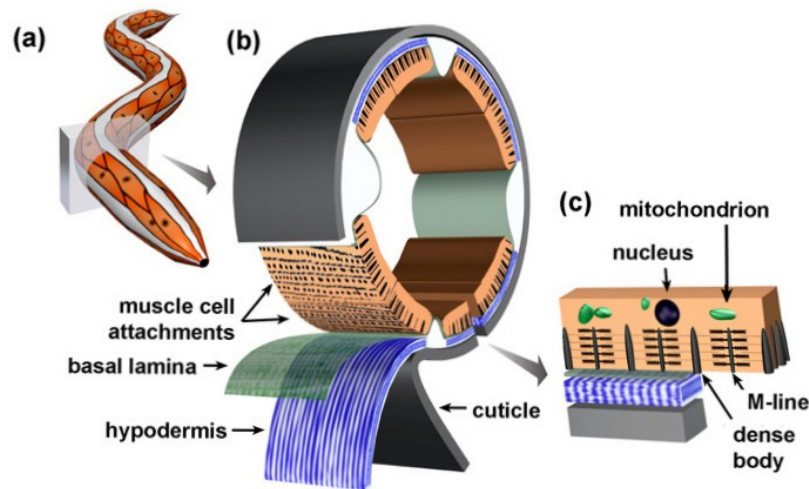


Fig 8. Schematic diagram of the *C. elegans* body-wall muscle structure. **A**, Worm seen from the front, muscle bundles are shown in orange. **B**, Cross-section through the body wall. **C**, Enlarged segment of the body-wall and the muscle attachments to the hypodermis. (Source: [103])

The nonstriated muscles, such as the pharyngeal muscles, contain a single sarcomere, with the attachment points localized at the end of a cell, connecting myofilaments to the epithelium or basal lamina.

II.2.2.3. Neuromuscular junctions in *C. elegans* – development & anatomy

Neuromuscular junctions are specialized chemical synapses between presynaptic motoneurons and postsynaptic muscles. There is a significant insight into the development and structure of *C. elegans* NMJs, mainly due to their physical accessibility and excellent possibilities for genetic analysis.

Muscles in *C. elegans* have a unique property to make specialized cell projections, called muscle arms, which extend from the muscle bundles and reach the proximal nerve cord (Intro, Fig. 9) [92,100]. Since in *C. elegans* the motoneurons and the body-wall muscles are separated by a basement membrane, the muscles have to pass through it and make direct contact to the motoneuron axon [93]. On the sites of contact, the muscle arms make *en passant* synapses to the motor axons that run along the anterioposterior axis. Depending on the type of the neurotransmitter, the NMJ synapses can be excitatory (cholinergic with ACh) or inhibitory (GABAergic).

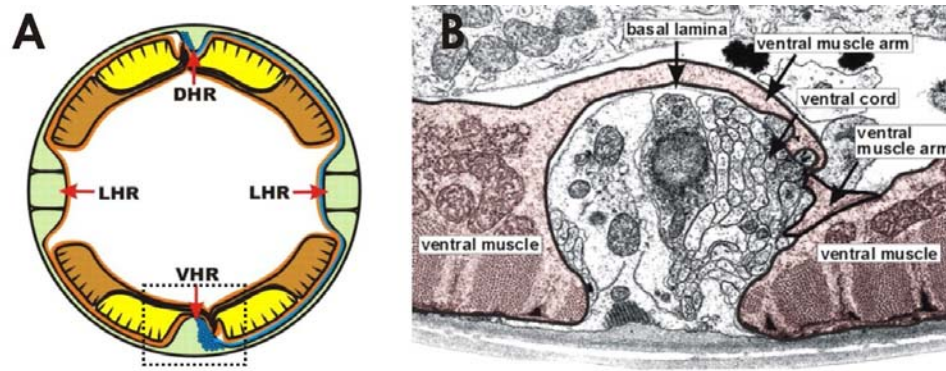


Fig 9. Transverse cut through the mid-body of *C. elegans*. **A**, Schematic view of the ventral and dorsal muscle bundles (yellow and brown), touching the nerve cords through the muscle arm extensions. Rectangle labels the regions enlarged in **B**. Ventral (VHR), dorsal (DHR) and lateral (LHR) hypodermal ridges are pointed with red arrows. The right axon bundle of the ventral nerve cord and the left bundle of the dorsal cord are shown in blue. **B**, A false-colored transmission electron micrograph of the transverse section of the ventral midline. The muscles are shaded in pink and separated from the nerves by basal lamina, except in the point of NMJ contacts. (Sources: [108,109])

Genetic screens for synaptogenesis mutants have identified key players in NMJ formation and structure. Animals carrying mutations at the synaptic components often exhibit uncoordinated movements (*unc*), egg-laying defects (*egl*), defecation defects or paralysis. Pharmacological assays with nematocidal drugs, such as cholinergic agonist levamisole or the acetylcholine esterase inhibitor aldicarb, have been extensively used in screening for mutants (Intro, Fig. 10) [86]. The drugs which are toxic for the wild type worms cause resistance in the synaptic mutants. The genes for several postsynaptic AChR subunits were identified on the basis of their resistance to levamisole, e.g. *unc-29*, *unc-38*, *unc-63*, *lev-1* [86,110]. Altogether, at least 16 nAChR subunit genes are thought to be expressed in *C. elegans* [111].

Neuromuscular junctions in *C. elegans* are highly dynamic structures. Several proteins have been identified as crucial factors for the normal NMJ development. One of them is a transmembrane protein LEV-10. The mutant was identified as weakly resistant to levamisole due to significantly reduced postsynaptic density of AChRs [112]. Interestingly, the LEV-10 extracellular protein domain alone is sufficient to rescue the *lev-10* mutant phenotype, suggesting a novel AChRs clustering mechanism.

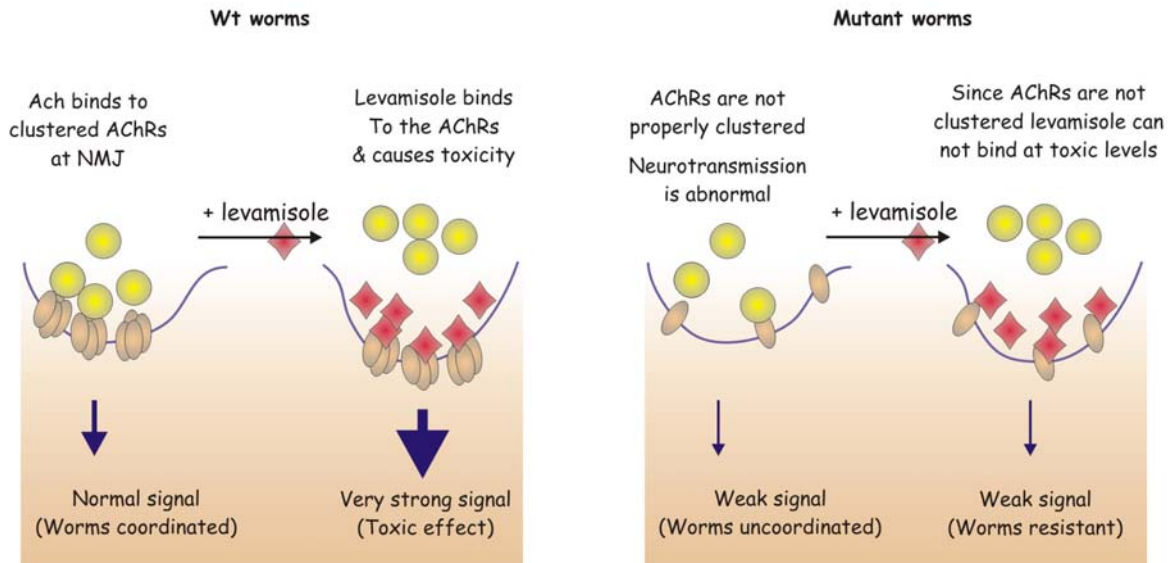


Fig 10. Schematic diagram of the levamisole mechanism at the nematode NMJ. In the wild type worms, where AChRs are clustered normally, the drug binds irreversibly and causes toxicity. In the mutant worms, with defective AChR clusters, the agonist effect is sub-toxic and the animals appear resistant.

In vertebrates one of the key factors involved in AChR clustering is the receptor tyrosine kinase MuSK. The gene with the highest similarity to MuSK in *C. elegans* is an orphan receptor KIN-8 (CAM-1) [113,114]. In addition to the impairment in cell polarity and neuron migration, the *kin-8/cam-1* mutants are uncoordinated and have mislocated AChR subunit ACR-16 [115]. Therefore, *kin-8/cam-1* in *C. elegans* might be a protein with a similar role as MuSK in vertebrates.

Synaptic ECM proteins have been implicated in NMJ formation in the worm, namely collagen XVIII (CLE-1) and nidogen (NID-1) [116]. Single mutants in each of the genes generally cause reduced number of the enlarged and diffuse GABA receptor clusters.

Aggrin, which is the main postsynaptic organizer at the vertebrate NMJ, has been identified in the *C. elegans* genome [117], but has not been further investigated.

II.2.3. Pharynx

The pharynx is an alimentary muscle organ with its own nervous system, gland cells, and structural cells surrounded by a basal lamina. Its precise structure was identified from serial electron micrographs that revealed its main components: 20 syncytial muscle cells, 9 marginal, 9 epithelial, 5 gland cells and 20 neurons [118]. The pharynx starts at the anterior part of the worm's body with the buccal cavity (mouth), followed by the procorpus, the anterior bulb (metacorpus), a narrower part called isthmus that ends with the terminal bulb which contains the grinder for crushing bacterial food (Intro, Fig. 11) (reviewed in [119]).

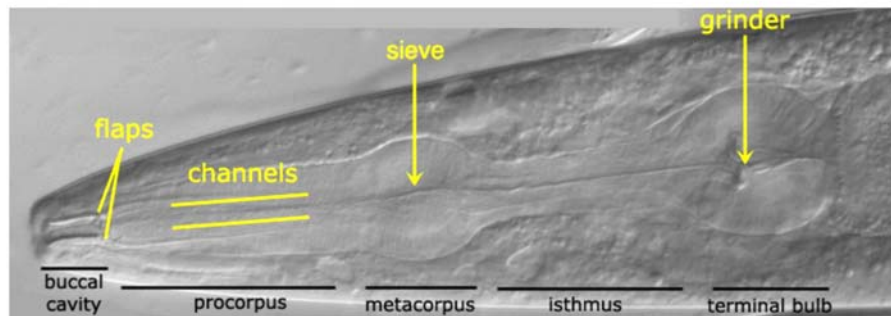


Fig 11. DIC image of the *C. elegans* pharynx. All the major segments are labeled in black and the interior cuticular structures in yellow. (source: www.wormatlas.org)

Pharyngeal muscles are organized in eight segments (pm1-pm8) forming consecutive rings that radially encircle its lumen. Most of the pharyngeal muscle rings are composed of three muscle cells giving rise to a three-fold symmetry (see Fig 15, Unpublished results). The majority of the muscle cells are separated by marginal cells, epithelial cells rich in the intermediate filaments that provide structural integrity to the entire pharynx.

Of the 20 neurons innervating the pharynx, five are motoneurons (M1-M5), six are interneurons (I1-I6), and the others are neurosecretory motoneurons (NSMs), marginal cell neurons (MCs) and a motor-interneuron (MI) [118]. Most of their cell bodies localize to one of the pharyngeal bulbs, sending processes to the anterior and /or posterior part. The motoneurons make NMJs directly to the pharyngeal muscles, while the others synapse to each other, either within a small local plexus or “en passant”. Since the pharynx is surrounded by its own basement membrane, the only link with the somatic nervous system is through a pair of RIP neurons that receive input from extrapharyngeal IL1, IL2, URA, and RME (reviewed in [119]).

The pharynx is specialized to pump and concentrate food (bacteria suspended in liquid) before grinding it within the grinder of the posterior bulb and pushing it further into the gut [120]. Its complex movements

involve highly coordinated isthmus peristalsis and the contractions (pumping) of the posterior bulb. The pharynx of a wild type worm pumps at the rate of about 4 pumps/second in the presence of food and 1 pump/second in its absence [121]. Many mutations, that have been discovered so far, cause reduced pharyngeal pumping rate or defects in the coordination of pharyngeal movements [122]. Based on the phenotype, the mutants are called *eat*, and their underlying mutations affect different proteins involved in pharyngeal function, e.g. *eat-2* and *eat-18* code for the nicotinic AChRs subunits [123], *eat-4* for a sodium-dependent inorganic phosphate transporter [124], and *eat-5* is related to the connexin family involved in the cell-cell interactions [125], etc. The size of the food particles (bacteria) influences edibility and regulates subsequently the worms' growth rate [120]. Larger bacterial strains, such as *Bacillus megaterium*, are generally less well tolerated and wild type worms grow more slowly when fed on them.

II.2.4. Basement membranes

Basement membranes (BMs), specialized thin layers of extracellular matrix (ECM) in *C. elegans* have been extensively studied giving rise to substantial insight in the major components and functions (for a review, see [126]). In addition to supporting structural integrity, BMs provide the scaffold for signaling molecules. In *C. elegans*, BMs cover the surfaces of all internal tissues and surround the pharynx, body wall muscles, intestine, gonad, and hypodermis on the pseudocoelomic side.

Most of the key proteins essential for the assembly of the BMs are conserved between the nematode and vertebrates [117]: laminin, perlecan, collagen IV, osteonectin/SPARC, and collagen XVIII and nidogen, involved in the organization of the nervous system. Almost all of those proteins play a certain role in gonad development. As expected, the gene families for the ECM genes are generally smaller in *C. elegans*, making it easier for the experimental set-up. Different BM proteins are expressed in a different spatio-temporal manner and often regulated on the level of alternative splicing.

Laminins are heterotrimeric glycosylated proteins comprised of an α , β , and γ chain, which in different combinations result in more than 15 isoforms in vertebrates. In *C. elegans* there are two α (α A LAM-3 and α B EPI-1) only one β (LAM-1) and γ (LAM-2) chain, giving rise to two laminin isoforms. They have distinct localization patterns with some overlap [127]. Putative null mutation of the α B chain, *epi-1(rh199)* causes embryonic and larval arrest in more than two thirds of the population and sterility in almost one third [128]. In addition, the *epi-1* mutation has been implicated in neuron migration [129]. Mutant animals in the α A chain, *lam-3(n2561)* arrest as embryos or L1 larvae [127]. Since LAM-3 is dominantly expressed in the pharynx, the pharyngeal BMs display obvious defects.

The perlecan ortholog in *C. elegans* is encoded by the *unc-52* gene and its protein product can be detected exclusively in the BMs surrounding muscle cells [130]. Complex alternative splicing of a common transcript gives rise to several isoforms [131]. The most severe phenotypes caused by *unc-52* mutations result in Pat phenotype (paralysis at the two-fold stage) due to defects in muscle development. Mutations in some of the components necessary for RNA splicing cause a synthetic lethal phenotype together with *unc-52*, confirming the importance of this tightly-regulated process. Viable *unc-52* alleles display defects in the migration of the distal tip cell of the gonad in a cross-talk with the netrin/UNC-6 system [132].

In vertebrates two nidogen/entactin genes encode ubiquitous glycoproteins consisting of two globular domains spaced from the third by a rod domain mainly composed of EGF repeats. In *C. elegans* there is only one gene *nid-1*, with three alternatively spliced variants [133]. NID-1 is primarily concentrated in the nerve ring and the BM of the gonad, but it is not essential for the BM assembly, since all mutants are viable and fertile. NID-1 is closely associated with NMJ synapses and was shown to affect synaptic organization together with collagen XVIII [116].

Collagen XVIII exists in *C. elegans* as a single orthologue *cle-1*, which is highly conserved at its C-terminus to the vertebrate protein. The usage of different promoters and alternative splicing give rise to at least three major isoforms. CLE-1 localizes primarily to the nervous system where it has been implicated in synaptic organization and function [116].

Collagen IV is the most abundant BM protein, highly conserved among species, from sponges to humans. Two genes that encode collagen IV have been identified and characterized in *C. elegans*, *emb-9* and *let-2* [134]. Multiple mutant alleles for both genes cause embryonic lethality, demonstrating that normal collagen IV is necessary for embryogenesis.

Integrins are transmembrane α/β heterodimers that bind to many components of ECM and serve as the ECM receptors, involved in cell migration, matrix assembly and remodeling. In vertebrates there are at least 20 distinct integrins, but in *C. elegans* only two are predicted, assembled from the proteins encoded by two α (*ina-1* and *pat-2*) and one β (*pat-3*) genes [135,136]. INA-1 is the most similar to the laminin-binding integrins and PAT-2 to the RGD-binding integrins of vertebrates. Both genes are broadly expressed, but *ina-1* becomes restricted to some tissues at the L1 larval stage. Strong alleles for *ina-1* cause lethality while weaker ones have mispositioned neurons and defasciculated axonal tracts suggesting defects in adhesion between axons [135]. The *pat-2* and *pat-3* mutants got their name after the Pat phenotype (paralysis at the

two-fold stage) because they fail to form normal muscle structures [136], similarly like *unc-52* perlecan mutants [130]. Therefore, this integrin was suggested to bind perlecan as its transmembrane receptor.

Several other BM / ECM components have been identified and characterized in *C. elegans*, such as: SPARC/osteonectin (*ost-1*, [137]) an essential protein restricted to body wall and sex muscles; fibulin (*fbl-1* [138] that binds to laminin and nidogen and its loss can suppress the mutations in ADAM-like genes; and hemicentin (*him-4*, [139]) which, when mutated, causes defects in adhesion.

II.3. Aim of the work

The work presented in this thesis was done with the primary goal to confirm experimentally the existence of an agrin orthologue in *C. elegans*, to characterize its expression pattern and to elucidate its function. Since in vertebrates the mechanistic link between agrin and MuSK is still unclear together with the downstream signaling at the NMJ, we intended to lay the basis for the identification of the missing links by taking various genetic and biochemical approaches. We investigated putative agrin interacting factors in search for conserved and/or novel molecules that could help us add another small building block to the understanding of the extracellular matrix organization.

III. Results

III.1. Submitted manuscript

***C. elegans* agrin is expressed in the pharynx, IL1 head neurons and the distal tip cells of the gonad and it does not genetically interact with genes important for synaptogenesis or muscle function.**

Ana Hrus^a, Stefano Canevascini^a, Gordon Lau^a, Harald Hutter^b, Susanne Schenk^a, Jacqueline Ferralli^a, Marianne Brown-Luedi^a and Ruth Chiquet-Ehrismann^{a,*}

^a Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, CH-4058 Basel, Switzerland

^b Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC V5A 1S6, Canada

* Corresponding author: Ruth Chiquet-Ehrismann
Friedrich Miescher Institute for Biomedical Research
Maulbeerstrasse 66
CH-4058 Basel
Switzerland

Tel.: 41-61-697 24 94
Fax: 41-61-697 39 76
e-mail: Ruth.Chiquet@fmi.ch

Abstract

Agrin is a basement membrane protein crucial for development and maintenance of the neuromuscular junction in vertebrates. The *C. elegans* genome harbors a putative agrin gene *agr-1*. We have cloned the corresponding cDNA, to determine the primary structure of the protein, and expressed its recombinant fragments to raise specific antibodies. The domain organization of AGR-1 is very similar to the vertebrate orthologues. It contains a signal sequence for secretion, seven follistatin domains, three EGF-like repeats and two laminin G domains. AGR-1 null mutants did not exhibit any overt phenotypes and did not acquire resistance to the acetylcholine receptor agonist levamisole. Furthermore, crossing them with various mutants for components of the dystrophin-glycoprotein complex, that have impaired muscle function, did not lead to an aggravation of the phenotypes. Promoter-GFP translational fusions, as well as immunostaining of worms, revealed expression of agrin in buccal epithelium and the protein deposition in the basal lamina of the pharynx. Furthermore, dorsal and ventral IL1 head neurons and the distal tip cells of the gonad arms are sources of agrin production, but no expression was detectable in body muscles or the motoneurons innervating them. Recombinant worm AGR-1 fragment is able to cluster vertebrate dystroglycan in cultured cells, implying a conservation of this interaction, but since neither of these proteins is expressed in muscle of *C. elegans*, this interaction may be required in different tissues. The connections between muscle cells and the basement membrane, as well as neuromuscular junctions, are structurally distinct between vertebrates and nematodes.

Key words: Agrin, basement membrane, pharynx, neuromuscular junction, *Caenorhabditis elegans*

Introduction

Agrin is a large proteoglycan with a prominent function at the developing neuromuscular junction (NMJ) where it plays a pivotal role in the formation and maintenance of the acetylcholine receptor (AChR) clusters. Agrin was discovered more than two decades ago through the observation that trophic factors from the basal lamina extract of electric ray (*Torpedo californica*) were able to induce AChRs clustering on muscles *in vitro* [1]. The protein was subsequently purified from the extract of the synapse rich *Torpedo* electric organ and, based on the observed aggregating activity, was named “agrin”, coming from Greek “ageirein” which means “to assemble” [2]. Further studies revealed that agrin is synthesized by motor neurons that release it into the synaptic cleft where it stably integrates into the synaptic basal lamina (BL), a specialized thin layer of the extracellular matrix (ECM) [3-5]. Based on these findings, McMahan proposed the ‘agrin hypothesis’, which states that agrin is a nerve-derived synaptic organizing molecule [6], (reviewed in [13,140]).

Agrin has been cloned from several vertebrate species including rat [8], chick [9,10], marine ray (*Torpedo californica*) [11], and man [12]. All described agrin gene orthologues encode a large protein of more than 2000 amino acids with an approximate molecular weight of 225 kD. Additional O-linked glycosylation by heparan and chondroitin sulphate glycosaminoglycan chains together with N-linked carbohydrates raise the molecular weight up to 400-600 kDa [14,15], reviewed in [13]. The domain architecture of agrin is characterized by several repeated structural motifs which share homology with follistatin (Kazal-type protease inhibitors), laminin epidermal growth factor (EGF) and laminin globular (lamG) domains. In addition the protein contains a SEA module common between sea urchin sperm protein, enterokinase and agrin flanked by serine/threonine (S/T)-rich regions [8,9]. Differential transcription of the first exon results in a longer form which is secreted and binds to the basal lamina via its laminin-binding N-terminal agrin (NtA) domain [10,17,18] and a shorter isoform which lacks the NtA domain and remains in the membrane as a type II transmembrane protein [19,20]. Additional alternative splicing, in a tissue-specific manner at two conserved sites, termed A and B in chicken or y and z in the rat, respectively, gives rise to isoforms with significantly different activities in clustering AChRs [23,24,26]. Isoforms expressed by motoneurons, which contain inserts at the B/z splice site, are active in AChR clustering, whereas agrin expressed by muscle, lacks the inserts and does not cluster AChRs.

Despite of numerous studies available, the mechanism of agrin action has not been completely solved yet. Muscle specific kinase (MuSK) is a transmembrane receptor tyrosine kinase necessary for agrin-induced AChR clustering without direct interaction with agrin. The missing link in this signaling pathway is a hypothetical protein termed MASC (myotube-associated specificity component) able to mediate the interaction between agrin and MuSK [31,38]. Genetic analysis of agrin and MuSK deficient mice support

the common function in AChRs clustering [38,39]. Both mutants die at birth due to breathing failure. Agrin null mutants and mice lacking only B/z⁺ agrin exons have a significantly reduced number, size, and density of AChRs clusters on the muscle, although, some postsynaptic differentiation is present [25,38]. In MuSK mutant mice, NMJ synapses and the related specializations can neither be found on the postsynaptic membrane nor the basal lamina [39]. Signaling downstream of MuSK is still largely unknown. Several reports demonstrated MuSK-related activation of different proteins, leading to AChRs clustering. Among them were dishevelled (Dvl), a protein involved in planar cell polarity signaling [43], a cytoplasmic protein Dok-7 [44], and protein casein kinase 2 (CK2) [45], essential for NMJ synaptogenesis *in vitro* and *in vivo*.

In addition to the NMJ, many non-neuronal tissues, such as muscle, heart and kidney, express an agrin isoform without inserts at the B/z site [23,28]. *Alpha*-dystroglycan (α -DG) binds to this alternative splice variant in different tissues with strong affinity, by a carbohydrate-dependent mechanism [47,80]. In vertebrates, muscle dystroglycan is a central component of a large dystrophin-glycoprotein complex (DGC) connecting the ECM with the intracellular cytoskeleton (reviewed in [60,141]). Genetic studies on animal models have shown that mutations in many components of the DGC independently lead to the outcome of muscular dystrophies (reviewed in [62-64]). Agrin binding to α -DG might contribute to the connection between the ECM and the cytoskeleton thus improving tissue integrity [65]. The interaction between agrin and α -DG is functionally conserved in the formation of the immunological synapse between antigen presenting cells (APC) and T-cells [83,84].

The nematode *C. elegans* is a useful model organism with many experimental advantages, e.g. short generation time, easy maintenance, transparent body, simple but specialized organs which make it a powerful tool for genetic analysis [86,88]. *C. elegans* harbors a gastrointestinal tract, a reproductive system, epithelial, nervous, muscle, excretory cells, and even innate immunity pathways [142]. In addition, most of the molecular mechanisms underlying major physiological processes are highly conserved when compared to vertebrates [88]. Therefore, the experimental data obtained from the worm proved to be highly informative and applicable in elucidating many analogous mechanisms in mammals, for a review, see [143].

NMJ in *C. elegans* have some distinct morphological features when compared to the vertebrate counterparts. Instead of motoneurons growing axons towards the muscles they innervate, muscles in *C. elegans* make specialized cell projections, called muscle arms, which extend from the muscle bundles to reach the proximal nerve cord [92,100]. At the sites of contact, the muscle arms make *en passant* synapses to the motor axons that run along the anterioposterior axis. Depending on the type of the neurotransmitter, the NMJ synapses can be excitatory (cholinergic) or inhibitory (GABAergic). Genetic screens for synaptogenesis mutants have identified key players in NMJ formation and structure. Animals carrying

mutations in synaptic components often exhibit uncoordinated (*unc*), egg-laying defects (*egl*), defecation defects or paralysis. Pharmacological assays with nematocidal drugs, such as the cholinergic agonist levamisole or the acetylcholine esterase inhibitor aldicarb, have been extensively used in screening for mutants that are resistant to these drugs [86]. The genes for several postsynaptic AChR subunits were identified on the basis of the resistance to levamisole, e.g. *unc-29*, *unc-38*, *unc-63*, *lev-1* [86,110]. Neuromuscular junctions in *C. elegans* are highly dynamic structures. Several proteins have been identified as crucial factors for normal NMJ development. One of them is a transmembrane protein LEV-10. The mutant was identified as weakly resistant to levamisole due to significantly reduced postsynaptic density of AChRs [112]. Interestingly, the LEV-10 extracellular protein domain alone is sufficient to rescue the *lev-10* mutant phenotype, suggesting a novel AChRs clustering mechanism.

In vertebrates one of the key factors involved in AChR clustering is the receptor tyrosine kinase MuSK. The gene with the highest similarity to MuSK in *C. elegans* is an orphan receptor KIN-8 (CAM-1) [113,114]. In addition to the impairment in cell polarity and neuron migration, the *kin-8/cam-1* mutants are uncoordinated and have mislocated AChR subunit ACR-16 [115]. Therefore, KIN-8/CAM-1 in *C. elegans* might be a protein with similar role as MuSK in vertebrates. Several other synaptic ECM proteins have been implicated in the NMJ formation in the worm, namely collagen XVIII (CLE-1) and nidogen (NID-1) [116]. Single mutants in each of the genes causes reduced numbers of the enlarged and diffuse postsynaptic receptor clusters.

Different genetic approaches have been taken to investigate the functions of the vertebrate gene homologues identified in the *C. elegans* genome (reverse genetics) or to identify the previously unknown genes which, if mutated in the worm, result in interesting phenotypes (forward genetics). In reverse genetic approaches, the goal is to learn more about a particular gene of interest and address its mechanisms of action in *C. elegans* [90]. Since in *C. elegans* and *C. briggsae*, two closely related nematode worm species, putative agrin orthologues have been identified on the basis of genomic sequence analysis [117], we decided to take a reverse genetics approach to clone the *C. elegans* agrin cDNA, characterize the protein, and describe its expression pattern. We found expression of agrin in four head neurons, the distal tip cell of the gonad, and in epithelial cells of the pharynx. We could not detect any agrin in muscle or at NMJs and genetic analysis of agrin mutants did not provide any evidence for a major function of agrin in AChR clustering or muscle function in the worm. However, the known binding of agrin to α -DG in vertebrates, seems to be conserved by *C. elegans* agrin, pointing to an ancestral role of this interaction.

Results

C. elegans expresses an agrin-like gene *agr-1*

A nematode agrin gene, with sequence homology to vertebrate agrin, was identified in the *C. elegans* genome. The analysis was based on queries by BLAST searches of Wormpep followed by reciprocal BLAST searches of insect or mammalian orthologs in GenBank [117]. In WormBase, the online database of the *C. elegans* genome, the agrin gene was mapped to the cosmid F41G3, originally as two separate open reading frames (ORFs) named F41G3.12 and F41G3.15, one corresponding to the 5' and the other to the 3' part of vertebrate agrin, respectively. Based on the predicted gene sequences, the agrin-specific primers (Table 1; Fig.1) were used to amplify overlapping fragments of each of the predicted ORFs and of a putative common transcript from cDNA reverse transcribed from RNA isolated from mixed stages of worms. As a result, three overlapping fragments gave rise to one unique agrin sequence instead of the two ORFs predicted by WormBase. The incorrect prediction was probably due to three sequence mistakes present in WormBase which resulted in false stop codons. The additional bases identified in our cDNAs are highlighted with red rectangles in Fig.1 and their positions in the genome are indicated by black arrows in Fig.2. The identified *agr-1* coding region is 4422 bp long with 5' and 3' untranslated regions of 212 and 160 bp, respectively (Fig. 1). The agrin ORF is encoded by 29 exons which span a chromosomal region of almost 14.5 kb (Fig. 2). The gene harbors two very big introns, one following exon 4 and the other following exon 24, but no alternative splicing was found. We attempted to identify putative alternative exons corresponding to the B⁺/z⁺ splicing described in vertebrates [23,26] by PCR with primers on neighboring exons, but could not detect any differential splice variants.

AGR-1 protein domain architecture is similar but not identical to its vertebrate homologues

The predicted agrin protein AGR-1 sequence consists of 1474 amino acids (Fig. 1). Domains were predicted by computational analysis of this protein sequence using the SMART bioinformatics tool package (Fig. 3) [144]. A putative 22 amino acids-long signal sequence (purple) is followed by seven Follistatin domains (F, blue), two epidermal growth factor domains of the laminin-type (LE, gray), another follistatin domain (F, blue), an epidermal growth factor (EGF)-like domain (EG, orange) and two laminin G domains (LamG, yellow).

When compared to the known vertebrate agrin orthologues, the *C. elegans* protein shares a high similarity in terms of modular architecture, but is missing certain domains (Fig. 3). Vertebrate agrin molecules exist in

two different forms, one with a signal sequence followed by a laminin-binding NtA domain (Fig. 3, light purple) and another one with a non-cleaved signal sequence (TM, empty rectangle) serving as a transmembrane anchor [10,19]. Using a 5' RACE approach in *C. elegans* only one isoform was found, containing a signal sequence, but no laminin-binding NtA domain. The signal sequence, together with a corresponding cleavage site between the amino acids 22 and 23, was predicted with 0.74 probability by Signal P3.0 Server [145]. However, we could not find any potential exon encoding a domain similar to the NtA domain in the genomic sequence. The N-terminal part of AGR-1 has seven repetitive follistatin domains, while vertebrate agrin contains eight of them. Further differences are present in the C-terminal part of the AGR-1 protein where the serine/threonine-rich regions (S/T, light yellow) are missing as well as the SEA (sea urchin sperm protein, enterokinase, agrin) domain. Vertebrate agrin is a heavily glycosylated protein, carrying large O-linked heparan sulphate and chondroitin sulphate chains at several positions in the protein [14,15] shown as branched structures in Fig. 3. This is probably not the case for AGR-1 since the S/T-rich region is missing. Finally, vertebrate agrins have three laminin G domains (lam G), while AGR-1 has only two and no EGF-like domains separating them. The overall similarities of the different agrin segments to the corresponding regions of chicken agrin are indicated in Fig. 3.

When the LamG domains from the *C. elegans* protein were used as a query against Swissprot, Trembl and Refseq databases the best hits besides the *C. briggsae* predicted agrin homologue (Q61GM7_CAEBR) included vertebrate agrins as well as laminins themselves and perlecan, shown in the alignments of Fig. 4A and 4B. In addition, a reciprocal analysis was done to see if some vertebrate agrin lamG domains produce best similarity hits with the nematode one. It turned out that the LamG domains of *C. elegans* agrin are quite distinct from those in vertebrates, and that the most similar lamG domains in vertebrates do not produce clear reciprocal best hits. This is not surprising since the LamG domains of the different proteins revealed equal similarities to *C. elegans* agrin LamG domains (Fig. 4A,B). However, blast searches with each AGR-1 LamG separately resulted in a better match between the first *C. elegans* lamG and the second LamG from several vertebrate homologues, as well as between the second *C. elegans* LamG and the third LamG domain of vertebrate agrins (Fig. 4A and B). This implies that the two LamG domains of *C. elegans* agrin rather correspond to the last two LamG domains of vertebrate agrin. This is further supported by the alignment of the region preceding the second LamG domain of AGR-1, which aligned best to the chicken B₀ agrin isoform, i.e. it does not contain any inserts at the conserved B/z site (Fig. 4C). Based on the overall domain architecture of *C. elegans* agrin we conclude that AGR-1 is the nematode agrin orthologue and not an orthologue of any other lamG domain-containing protein.

***Agr-1* is expressed in *C. elegans* buccal epithelium, dorsal and ventral IL1 head neurons, and distal tip cells of developing gonad, but not in body wall muscles**

To determine the expression pattern of the *agr-1* gene, we made different *agr-1::reporter* fusion constructs, containing up to 5421 bp upstream of the first exon and up to 3275 bp of sequence downstream of the translational start codon including the large intron after exon 4 (Fig. 5A). Another construct containing the *gfp* gene flanked by *agrin* genomic sequences was co-injected with the cosmid F09G5. Upon recombination the *gfp* gene was integrated in the cosmid sequence and fluorescence could be observed in the transgenic lines (Fig. 5A). All five constructs gave the same expression pattern. Fluorescence started to be visible in cell clusters of young embryos at the 100-cell stage (Fig. 5B and C). Starting from the comma stage, expression became restricted to the head region of the embryo (Fig. 5D and E). The L1 and L2 larvae (Fig. 5H and I, respectively) and young adults (Fig. 5F and G) expressed the marker in the nine buccal epithelial cells (dotted-line arrows), four head neurons (arrows), some unidentified cells in the head, and in the posterior-most cells of the gut (asterisk). The expression in the four neurons and buccal epithelium remained constant throughout the four larval stages and in the adult animal. Starting from the L2 stage, when gonad development and migration begins, fluorescence became also visible in the distal tip cells of the gonad (Fig. 5I).

To identify the four head-neurons expressing *agr-1*, we stained amphid neurons with DiI in the *hdEx25* transgenic lines, with or without 50mM CaAcetate. We did not find any co-staining between the red and yellow fluorescing dyes in these experiments, suggesting that the four neurons were neither amphids, nor IL2 neurons (Fig. 6A-F). We then analyzed *agr-1::dsRED* expression in the *kdEx71; otIs107(ser-2::gfp)* [146] and in the *kdEx71; adIs1240(eat-4::gfp)* [124] transgenic lines. No co-staining was observed in *kdEx71; otIs107* (results not shown), suggesting that the neurons expressing *agr-1* are not the OLQ neurons. In the *kdEx71; adIs1240* animals the dorsal and the ventral, but not the lateral IL1 neurons co-stained for the red and green markers, allowing us to identify the *agr-1* expressing neurons as the dorsal and ventral IL1 neurons (Fig. 6G-I).

In vertebrates *agrin* plays an important role in synaptogenesis at the neuromuscular junctions and in muscle stability (reviewed in [13]), therefore we carefully looked whether *agr-1* is expressed in muscles and neurons in the nematode. We did not observe any marker expression in muscles and motoneurons in the worm, implying that the gene is not expressed in these tissues or is expressed at undetectable levels.

***Agr-1* does not interact genetically with genes important for synaptogenesis and muscle stability in the worm.**

To further investigate the possible involvement of *agr-1* in synaptogenesis and muscle stability, we tested possible genetic interactions between *agr-1* and factors involved in these processes in the worm. The *agr-1(eg1770)* single mutant did not show any obvious phenotype and its movements and co-ordination seemed normal. We tested the sensitivity to aldicarb and levamisole of the *agr-1(eg1770)* single mutant and in the *lev-1(e211)* [111], and *dys-1(cx18); dyb-1(cx36)* [147,148] backgrounds. The *eg1770* mutation did not influence cholinergic activity in these backgrounds suggesting that the *agr-1* gene does not play a role in the biogenesis and activity of cholinergic synapses in the worm.

In order to investigate possible genetic interactions of agrin and the components of the DGC complex in *C. elegans*, we generated double and triple mutants with *agr-1(eg1770)* and the components of the dystrophin-glycoprotein-complex. The crosses were done with mutants for dystrophin *dys-1(cx18)*, dystrobrevin *dyb-1(cx36)*, *dys-1(cx18); dyb-1(cx36)*; transcription factor MyoD1 *hlh-1(cc561)* that was shown to enhance the phenotypes of hypomorphic muscle mutants [149]; components of the extracellular matrix perlecan *unc-52(gk3)*, *unc-52(e444)*, collagen *cle-1(cg120)*, and nidogen *nid-1(cg119)* [150]; and with the cell adhesion factor *dig-1(n1321)*. Locomotion in normal conditions or under stress in thrashing assays, response to touch, viability, and muscle integrity by staining the muscle fibers either with rhodamine-phalloidin [151] or in *stEx30(myo-3::gfp)* [152] were investigated in the single, double and triple mutant strains. The *agr-1(eg1770)* mutation did not aggravate any of the phenotypes of the different mutant backgrounds, suggesting that *agr-1* function is dispensable in the muscles of the worm.

Specific monoclonal and polyclonal antibodies detect agrin in the basal lamina of the pharynx

To confirm the agrin expression pattern at the protein level and to obtain more information on its possible function in the nematode, we raised specific antibodies against the lamG domains (Fig. 7A). The first lamG domain was expressed in *E. coli*, purified and used as antigen to raise monoclonal antibodies. A fragment containing both lamG domains was fused to a short sequence of chicken tenascin-C (Tn-C), expressed in HEK293_{EBNA} cells, purified from the conditioned media and used to raise polyclonal antibodies. The specificity of both polyclonal and monoclonal antibodies was tested by western blotting and immunostaining of COS cells transiently transfected with the TN-C-agrin fusion construct (Fig. 7). In the conditioned medium, a band of around 80 kDa, which corresponds to the size of the recombinant protein,

was detected by all antibodies. The polyclonal serum recognized additional smaller fragments which probably correspond to protein degradation products. Transfected and non-transfected cells were stained with the anti-agrin monoclonal antibody pool (Fig. 7C-F) as well as the monoclonal antibody against the TN-C epitope tag (Fig. 7G-J). In the permeabilised cells (Fig. 7D and H), the overexpressed fragment could be detected mainly in the Golgi apparatus while in non-permeabilised cells (Fig. 7C and G) the agrin protein could be detected in patches bound to the plasma membrane. This represented a first indication that the COS cells may express a surface receptor that binds agrin. Non-transfected COS cells did not give any immunofluorescence signal (Fig. 7 E-J).

In order to detect the endogenous worm agrin protein, we analyzed worm extracts by western blotting with purified anti-agrin polyclonal antibodies. We compared extracts from wild type worms with the three *agrin* mutants available, *agrin-1(eg1770)*, *agrin-1(eg153)* and *agrin-1(tm2051)* described in Experimental procedures. Extracts were prepared from about 50µl of synchronized young larval stages (L1 and L2). The fractions soluble in RIPA buffer, including 6M urea, are presented in Fig. 8A. Two bands, one of about 160 kDa and the other of about 75 kDa, were detected by anti-agrin antibody in the lysate of wild type worms, but not in any of the three *agrin* mutant strains. The 160 kDa band corresponds to the calculated weight of the full length agrin protein, while the smaller band could represent a degradation product. Two additional bands (asterisks) which were present in all analyzed strains, seem to be due to a non-specific reactivity.

To localize the agrin protein in worms, they were co-stained with the monoclonal antibody pool against *C. elegans* agrin and anti-rim, a pre-synaptic marker prominent in the nerve ring [153]. The major site of agrin expression was around the pharynx and the staining was particularly enriched in the anterior part (Fig. 8B). The posterior bulb staining was weaker probably due to poor antibody penetration. Polyclonal antiserum staining resulted in the same staining pattern in wild type worms of different developmental stages (Fig. 8C and D). Young larvae (L1) generally showed stronger agrin staining compared to young adults (C compared to D, respectively). Pharyngeal staining was absent in all three *agrin* mutant strains, which is an additional confirmation for their lack of agrin expression (Fig. 8F-H). In addition to the pharynx staining in the wild type worms, the polyclonal antiserum stained the gut lumen (arrowhead) both in the wild type worms as well as in the *agrin* mutants, but not when preimmune serum was used instead. The staining of the lumen of the gut represents an unrelated cross-reactivity of our antiserum, possibly related to the background bands detected on the western blots.

The strong pharyngeal localization led us to suspect that agrin has a pharynx-related function, namely in feeding behavior or structural stability. Therefore, we investigated the pharyngeal pumping rate of normal

versus mutant animals, but could not detect any differences. Despite the absence of agrin in the pharynx of the mutants, pharyngeal morphology was normal in young as well as in the older animals (data not shown). To challenge the pharynx function, we fed agrin mutants with different strains of bacteria of various sizes [120]. However, even ingestion of the largest bacteria, strain *Bacillus Megaterium*, did not result in a different growth rate as compared to the wild type animals.

Recombinant fragment of *C. elegans* agrin binds to purified chicken α -dystroglycan

Based on the well described binding of agrin to α -dystroglycan in vertebrates [47,80], we addressed this possible interaction in the case of *C. elegans* agrin. The interaction between agrin recombinant fragment containing two lamG domains and dystroglycan was tested biochemically by a protein overlay assay. Purified chicken α -dystroglycan was run on an SDS PAGE and blotted to a nitrocellulose membrane. Separate strips of this membrane were incubated either with conditioned medium containing the recombinant *C. elegans* agrin fragment, or with purified recombinant chicken agrin fragments representing the muscle agrin isoform lacking inserts in the A/y and B/z sites or with the neuronal isoform with 4 and 8 amino acid inserts at the respective sites. Following several wash steps, agrin bound to α -DG was detected with anti-Tn60 antibodies that recognize the short TnC part fused to agrin (Fig. 9). The chicken muscle agrin fragment, which served as positive control bound efficiently to dystroglycan, appearing as a dark smear caused by the migration behaviour of the highly glycosylated dystroglycan (Fig. 9, lane 1), while the neuronal isoform gave a very weak signal at the dystroglycan protein core size of approximately 200 kD (lane 2). The *C. elegans* agrin fragment also bound to DG and was detectable as a smear on the membrane strip similar to the chicken muscle agrin (Fig. 9, lane 3). Conditioned medium from non-transfected cells was used as negative control and did not result in a signal (lane 4). This result indicates that the *C. elegans* agrin retains the binding to α -dystroglycan.

***C. elegans* agrin induces endogenous dystroglycan clustering in COS cells**

The interaction between *C. elegans* agrin and DG was further investigated in cell cultures of COS cells, transfected with the recombinant fragment of *C. elegans* agrin (fragment 2 in Fig. 3). Transfected cells secreted the agrin fragment into the medium where it bound to the cell surface in a patchy pattern (Fig. 10 A and D). Remarkably, the dystroglycan staining followed the same pattern (Fig. 10 B and E) and overlapped with the agrin staining in patches (Fig. C and F), while the dystroglycan staining of untransfected cells showed a diffuse membrane staining (Fig. 10 H and K). The anti- β -DG antibody recognizes the intracellular part of the protein, therefore only permeabilized cells showed strong staining (Fig. 10E). Immunostaining experiments with the pool of the monoclonal anti-agrin antibodies resulted in the same pattern as with polyclonal antibodies (data not shown), but co-staining with anti- β -DG was not possible due to the same host species in which the antibodies were raised. Our results suggest that the recombinant agrin fragment containing the two lamG domains bound to the cell membrane, probably through direct interaction with dystroglycan. Interestingly, the endogenous dystroglycan had a diffuse pattern in cells without agrin overexpression, but it appeared clustered by agrin secreted from the transfected cells, suggesting that agrin bound to the cells and induced clustering of DG.

Discussion

We have identified and characterized the first invertebrate agrin. In terms of domain structure, a high degree of similarity was found between *C. elegans* and vertebrate agrins, although some domains were missing. In the worm, only one N-terminal variant was detectable containing a secretion signal, but no NtA domain [10]. Several other domains were missing, namely the Ser/Thr-rich region, the SEA module, and one of the three lamG domains at the C-terminal end of the molecule. We analyzed the two lamG domains present in the worm and compared them to the vertebrate lamG domains to determine whether they corresponded to a particular domain pair of vertebrate agrin. Blast searches revealed that the AGR-1 LamG domains aligned better to the last two LamG domains of vertebrate agrin. However, we could not detect any small inserts known to be important for the clustering function of the vertebrate neuronal agrins [23,24]. There still remains the possibility of an alternatively spliced agrin isoform present in the worm, much less abundant or expressed in a particular developmental stage, which was not possible to be amplified and detected in the presence of the major isoform. Nevertheless, when we searched the intron sequences between the last few exons encoding the LamG domains in all three frames, we could not detect any potential alternative exons coding for conserved amino acid resembling inserts present in the A/y and B/z sites of vertebrates. Therefore, we concluded that in *C. elegans* there is only one major agrin isoform expressed.

With the goal to detect the endogenous agrin by western blot, we raised specific polyclonal antibodies against the fragment comprising two lamG domains. Purified antibodies detected protein bands of about 160 kDa and 75 kDa, present only in the lysates of the wild type worms and not in any of the agrin mutants. The bigger band corresponded to the protein core size and the smaller possibly to a degradation product or a shorter isoform. In the null mutants *agr-1(eg1770)* and *agr-1(eg153)* we expected a complete lack of the protein, but *agr-1(tm2051)* carried an in-frame deletion and was expected to express a shorter protein. However, in *agr-1(tm2051)* mutant the deleted exons 26 and 27 encode the majority of the region against which the polyclonal antibodies were raised. Therefore, such a truncated protein may not be recognized and no band detected although the protein may be expressed. Another possibility is that the protein with the deletion does not fold properly and gets degraded. In any case, it is clear that the agrin mutants do not express the normal agrin protein as the wild type worms do.

The most surprising result of our study was the fact that we could not detect any agrin expression neither in body wall muscles nor in motoneurons innervating them. The lack of agrin expression at the NMJs suggested that agrin may not have a conserved function in AChR clustering in the worm. Nevertheless, we investigated the possible phenotypes in *C. elegans agr-1* mutants related to muscle and/or NMJ function. In

the case of defective muscle or NMJ formation, worms should have an uncoordinated (*unc*) movement, a defective thrashing pattern, and/or egg laying deficiency. However, we did not observe any significant impairment neither in *agr-1* single mutants, nor in combination with other related mutations. Pharmacological assays with levamisole, a potent nematode-specific cholinergic agonist [110] and aldicarb, an ACh esterase inhibitor [154], did not reveal any resistance in *agr-1* mutants. Therefore, in *C. elegans* we could not find any evidence for a role of agrin in muscle or synaptic functions.

Therefore, we concentrated more on the sites of agrin expression visualized by reporter genes and antibody staining. Prominent expression was found in four head neurons and some pharyngeal cells. This relatively restricted expression pattern was quite unexpected but was confirmed by several reporter constructs with varying portions of the gene as well as by antibody staining.

We identified the four agrin-expressing neurons by crossing *agr-1::dsRED* worms with transgenic lines expressing GFP in specific neuron subtypes [124]. This approach suggested that the agrin-expressing neurons corresponded to IL1, inner labial sensilla polymodal neurons. These are mechanosensory, motoneurons and interneurons at the same time [98]. There are six IL1 neurons in total (three pairs) in the head of the worm [93], but agrin was found to be expressed only in the dorsal and ventral pairs. Such a sub-specialization might be significant to distinguish very fine sensory inputs from the environment. It is known that IL1 neurons together with OLQ neurons are responsible for the sensing of a light nose touch and the regulation of spontaneous foraging movements [98]. Therefore, we tried to test agrin mutants for light nose-touch-avoidance by an eyelash test [97], but agrin mutants seemed to be as sensitive to touch as the wild type worms. We cannot exclude, however, that our technique was too crude to find a difference.

The pharyngeal cells expressing agrin belong to the buccal epithelium which surrounds the anterior-most part of the pharyngeal lumen ([118], Wormatlas, <http://www.wormatlas.org/>). In early larvae agrin is in addition expressed in other, unidentified cells of the pharynx, most likely marginal cells. The epithelial tissue forms a rigid narrow cylinder restricting the food entry into the pharynx [118]. Agrin expressed in this tissue might have a function in the structural support of the pharynx. The immunostaining with anti-agrin specific monoclonal and polyclonal antibodies detected the protein around the pharynx, resembling perlecan immunostaining in the pharynx basal lamina [131]. Thus, agrin is probably secreted from the pharyngeal cells and integrated into the basal lamina. This pharyngeal staining was missing in all three agrin mutant strains. Therefore, we analyzed pharynx function in the mutants. The pharynx is a muscle pump important for food intake, exposed to strong contractions with the frequency of about 4 pumps per second in the presence of bacterial food [155]. Therefore, strong mechanical support of the pharynx is necessary to endure the shear forces to which it is exposed. Pharynx function was tested by measuring the pumping rate, which

did not differ between the agrin mutants and the wild type worms. To challenge pharynx function more drastically, we tested whether bacterial size had any influence on the feeding and ingestion capability of the worms. For that purpose we grew the worms on *Bacillus megatherium*, a large bacterial strain and compared it to the regular *E. coli* OP50 strain used in regular laboratory maintenance [120]. Pumping defective mutant strains *eat-4(ky5)* and *eat-5(ad464)*, which grow much more slowly on large bacteria, were used as positive controls. On both food sources our agrin mutant *agr-1 (eg1770)* grew equally fast as wild type worms. Also pharynx morphology did not seem defective in the *agr-1 (eg1770)*, suggesting that if agrin has a function in pharynx-related functions, it is subtle or redundant.

Some expression of agrin was present in the distal tip cells of developing gonads in young larvae. The distal tip cell is critical for germ line proliferation and guides gonad migration by sensing environmental cues [132,156]. We have investigated a possible effect of agrin deficiency in gonad migration or brood size, but the mutants could not be distinguished from the wild type worms.

Interaction between agrin and α -dystroglycan in vertebrates is important in different tissues and processes (reviewed in [13,157]). At the NMJ, DG stabilizes mature synapses by connecting the basal lamina to cortical F-actin. In the muscle sarcolemma DG is a central component of a large dystrophin-glycoprotein complex (DGC) where it serves again as a linker between the ECM and intracellular cytoskeletal proteins (reviewed in [60,141]). At the NMJ and the muscle membrane agrin interacts with α -DG with its C-terminal lamG domains and with its N-terminal part to laminin therefore providing additional support. Interaction between agrin and DG is found to be conserved also in the immune system, where this binding mediates lymphocyte activation via a lipid raft-dependent mechanism [84]. Based on the established knowledge on the vertebrate agrin and DG, we decided to investigate whether this interaction is conserved in *C. elegans*. We could demonstrate direct binding of a recombinant fragment of *C. elegans* agrin to purified vertebrate α -DG by a membrane overlay assay. In COS cells, transfected with the same recombinant fragment, endogenous DG was clustered and colocalized with the agrin bound to the cell membrane.

Taken together, our experiments suggest that the interaction between agrin and dystroglycan may be conserved in *C. elegans* but this interaction does not play a role at the NMJ or in muscle in general. This is supported by the recent observation that also *C. elegans* dystroglycan, *dgn-1*, is not expressed in muscle, but rather in epithelia and neurons, it localizes to basement membrane surfaces and is not involved in muscle function, either [158]. Therefore, the structural organization at the molecular level of the NMJ, as well as the DGC complex, is clearly distinct in *C. elegans* as compared to vertebrates. Our studies demonstrate that the existence of protein orthologs in evolutionarily distant organisms [117] does not necessarily imply identical functions, at least not in every aspect. In *C. elegans* it appears that the muscle basal lamina

depends rather on the function of the perlecan orthologue *unc-52* [104,130,131,150] and on the integrin chain orthologues *pat-2* and *pat-3* [126,136,159,160] and not on dystroglycan and its ligands [158]. On the other hand dystroglycan and agrin appear to function in epithelia and certain neurons but not in muscle.

Experimental procedures

C. elegans culture conditions and preparation for RNA and protein extraction

The worm strains were grown at 20°C on NGM agar plates seeded with *E. coli* OP50 [86]. For growth in large amount and further protein extraction, worms were grown at 20°C on 10 cm culture dishes with NGM medium and the addition of egg yolk [161]. In order to prepare synchronized cultures of young larvae, gravid worms were washed with M9 buffer and sucrose flotation [162], then bleached [163] followed by extensive washing in M9. The larvae hatched and, when they reached the L2 larval stage, were rinsed off the plates with M9 buffer, washed in M9, deionized water, and quick-frozen in liquid nitrogen.

Sequence identification and cDNA cloning

Total RNA was isolated from a mixed-stage worm population (Trizol reagent, Gibco) and reverse-transcribed into cDNA using oligo dT primers and MMLV reverse transcriptase from AdvantageTM RT-for-PCR Kit (Clontech) according to the supplier's protocol. Agrin cDNA was amplified by using sets of primers designed according to the predicted gene sequences in the Wormbase (ACeDB: F41G3.15 and F41G3.12, as of January 1, 2003; presently, only one common ORF exists in the database under the name F41G3.12) in order to obtain overlapping PCR products using the following primers: agr3 and agr6; agr1 and agr2; agr9 and agr12; agr25 and oligo dT primers listed in Table 1. In parallel, a commercial *C. elegans* cDNA library (OriGene Technologies) and the EST clone Yk1264e03 (vector pME18S-FL13, kindly provided by Dr. Yuji Kohara) were used as templates. The 5'UTR was determined with 5' RACE approach (Roche) following the supplier's protocol. Agrin cDNA sequence was assembled from overlapping fragments resulting in an open reading frame (ORF) of 4422 bp.

Protein architecture analysis and alignments with vertebrate orthologues

Protein architecture was analysed with SMART (EMBL, [144]), and with Blast at ExPASy [164]. Several overlapping domains were predicted, but in this report only a representative structure is presented based on the similarity to the vertebrate orthologues. Laminin G domains of *C. elegans* agrin were used as a query in myHits [165] against Swissprot, Trembl and Refseq. After the analysis of a more extensive alignment, the most informative Swissprot hits were selected. Visualization was done in Jalview, using Zappo colors without conservation threshold, to analyze subgroups [166]. Alignments were submitted to the Boxshade server at Pasteur (<http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html>) for producing the greyscale shading. Pairwise comparison of sequences (% identity / % similarity) was performed using Smith Waterman alignments (as implemented in water, a tool in the EMBOSS package; [167]). The homology searches between fragments of *C. elegans* agrin sequence and chicken agrin were done at myHits (SIB)

using iterative PSI-BLAST searches [165]. The *C. elegans* fragment composed of 2 lamG, yielded different Blast scores for alternatively spliced chicken agrin isoforms (Swissprot), which was due to the presence, or absence, of the spliced exons. The alignments of the conserved alternative splicing site were produced by Blast at ExPASy.

Agr-1::reporter expression constructs for expression pattern analysis

Agrin fragments were cloned following standard procedures [168]. The constructs used to create transgenic animals were the following:

Pagr-1::dsRED (p251). A 1926 bp genomic DNA fragment immediately upstream of the *agr-1* ATG start codon was amplified using the primers E149 and E150 (the sequences of the primers are listed in Table 1). The *Sall* site near the start codon was removed by introducing a point mutation in the sequence of primer E150 (bold and underlined in Table 1). The PCR fragment was then cloned into the *SphI-Sall* sites of the vector pVH14.05 that contains the *dsRED* gene (pVH).

Pagr-1::yfp (pVH11.07). A 1527 bp genomic DNA upstream of the *agr-1* ATG start codon was amplified with the primers XY1 and XY2 and cloned into the *PstI-XbaI* sites of pVH20.01 (promoterless *yfp* vector)

Pagr-1::gfp (5.5Kb) (p130). A 5431 bp genomic DNA fragment including 5421 bp of promoter sequence and the first 12bp of the *agr-1* ORF was amplified with the primers E32 and E33. The fragment was then ligated into the *SphI-XbaI* sites of the pPD95.75 vector (Fire Lab vector kit). The *NotI* and *ClaI* sites introduced with the primer E33 are needed for further cloning (see below).

Pagr-1::gfp (8.6Kb) (p143). A 3198 bp genomic DNA fragment from the 5' region of the *agr-1* gene was amplified with the primers E34 and E35 and cloned into the *ClaI-NotI* sites of the *pagr-1::gfp* (5.5Kb) plasmid. The *pagr-1::gfp* (8.6Kb) plasmid contains 5421 bp of promoter region and the *agr-1* genomic region covering the first seven exons and introns (the primer E35 primes on the 5'end of exon 8). Furthermore, primer E34 was chosen downstream of the putative signal sequence on exon1; the signal sequence is therefore not present in the construct.

Pagr-1::gfp::agr-1 (p233). The *gfp* ORF was amplified from pPD95.75 with the primers E36 and E37 and ligated into the *ClaI* site of *pagr-1::gfp* (8.6Kb). A TAG stop codon terminates translation at the end of the *gfp* ORF. Orientation and sequence of the insert were checked. The *gfp* gene flanked by *agr-1* sequences was then excised from the plasmid with *AflIII* and *SgrAI*, gel purified, and co-injected (20ng/μl) with the cosmid F09G5 (100ng/μl).

Transgenic strains

The *agr-1::reporter* constructs were injected at a final concentration of 50 ng/μl in wild type N2 animals or in *dpy-20(e1282)*. As markers, a plasmid containing a *dpy-20+* genomic fragment (primers E42 and E43, plasmid p133) or pRF4 (*rol-6(su1006)*) were co-injected at a final concentration of 10ng/μl [169]. Integration of the extrachromosomal arrays was induced by UV294 irradiation and the integrated strains were then backcrossed 10 times in N2 to remove unwanted mutations.

Agrin mutant strains

Three mutations in the agrin gene were isolated. The *agr-1(eg1770)* and *agr-1(eg153)* mutants were created by *Mos*-driven mutagenesis and were kindly provided by Dr. Jean-Louis Bessereau [170]. In the *agr-1(eg1770)* mutant strain the *Mos1* transposon was inserted into the seventh exon (after the base pair at the position 4948, according to the numbering in the cosmid T13C2) and brings the transcript out of frame, therefore causing a putative null mutation. The *agr-1(eg153)* agrin mutation was created by transposon mobilization which led to an imprecise excision and left an insertion of five base pairs (“TGATA” at the position after 4948 of cosmid T13C2) from the agrin ORF and gave rise to another putative out-of-frame null mutation. The third mutant, *agr-1(tm2051)*, was kindly provided to us by the National BioResource Project, the Japanese *C. elegans* knock-out consortium. The mutant carries an in-frame deletion of 423 bp including exons 26 and 27 (nucleotides 22165-22587 of cosmid F41G3), which encode the last laminin G domain and therefore disrupt a potentially functionally important site of the gene [27].

Analysis of agrin mutants

Agrin mutants were analyzed for potential defects at the neuromuscular junction, by pharmacological treatments with aldicarb and levamisole, following previously described procedures [110,154].

Based on the expression of agrin in the worms' pharynxes, different related phenotypes were investigated as described previously: pharyngeal pumping rate, pharyngeal morphology [171], and worms' feeding on large bacteria [120].

Staining of amphid neurons

Amphid neurons were stained with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen D-282) [172]. Young adult hermaphrodites were incubated in M9, 5μg/ml DiI (with or without 50mM CaAcetate) for two hours at room temperature, washed three times in M9 and transferred to a NGM plate with *E. coli* OP50. After two hours the worms were mounted on agarose pads with 30mM Na Azide [154] and analyzed with a Zeiss LSM510 confocal microscope.

Cloning, expression and purification of agrin fragments

Cloning of the first lamG domain, bacterial expression, and purification

Agrin cDNA coding for the first laminin G domain was amplified with primers containing restriction sites: lam3 (*SphI*) and lam6 (*HindIII*) listed in Table 1. The 513 bp PCR product was digested by *SphI* and *HindIII* restriction enzymes and cloned into the pQE30 vector (Qiagen) containing a 6xHistidine (6xHis) tag just upstream of the multiple cloning site. Expression and purification were done following the QIAexpressionist protocol (Qiagen) under denaturing conditions. The purified agrin fragment of 25kD was analyzed by SDS-polyacrylamide gel electrophoresis (SDS PAGE) and stained with Coomassie blue (GelCode, Pierce). Fractions of the elution peak were pooled and dialysed against PBS⁻ for further experiments.

Cloning of both lamG domains and expression in eukaryotic cells

The agrin fragment encoding both laminin G domains and the C terminus of the protein, was amplified by specific primers, named overlap5' and lam8euk4 (Table 1). The amplified agrin fragment of 1203 bp was fused to an N-terminal fragment of chicken tenascin-C containing a signal sequence and the epitope for the monoclonal anti-tenascin-C (TNC) antibody anti-Tn60 [173]. The TNC fragment was amplified from pCTN 230 [174] with T3 as the upstream primer and an antisense primer of which the 3' half was homologous to the template sequence and the 5' part reverse complementary to the beginning of the agrin fragment (primer agr/TN antisense, Table 1). The two fragments were fused by PCR using the T3 and lam8euk4 primers. The resulting fusion product of 2100 bp was digested with restriction enzymes *Cla I* and *Not I* and cloned into pKSII. Insertion was verified by sequencing and the fragment cloned into the *Kpn I* and *Not I* restriction sites of the expression vector pCEP for expression in HEK293EBNA cells. The cells were transfected with the FugeneTM6 reagent (Roche) following the supplier's protocol. Since the recombinant agrin fragment contained the signal peptide of vertebrate TNC, the protein product was secreted into the conditioned medium which was then tested by western blotting with anti-Tn60.

For large scale protein expression the cells were grown in 15cm tissue culture dishes in DMEM/10%FCS until they reached 75% confluency. Then the cells were washed with DMEM without FCS and 24 to 48 hours after the medium change two liters of serum-free conditioned media were collected. The agrin in the conditioned medium was precipitated with 50% ammonium sulphate at 4°C. The pellet was resuspended in PBS⁻/0.1% Tween and dialysed overnight against the same buffer. Agrin was purified over a anti-Tn60 affinity column as described previously for recombinant TNCs [174]. Elution fractions were analysed by SDS-PAGE and western blotting. The fractions containing peak amounts of agrin fragment were pooled and dialysed against PBS⁻. Alternatively, precipitated conditioned media were dialysed against PBS⁻ overnight and DMEM for one hour to be used for *in vitro* binding assays with dystroglycan (see below).

Monoclonal and polyclonal antibodies against C. elegans agrin

The recombinant agrin fragment expressed in *E. coli* was used as antigen for the immunization of mice to raise monoclonal antibodies. Conditioned media from hybridoma clones were tested for antibody production and specificity. The activity was determined by ELISA tests and western blots using recombinant agrin protein encompassing both lamG domains expressed in eukaryotic cells. Four hybridoma clones were positive, their cultures were expanded, and the antibodies from conditioned media were concentrated over protein G columns. In further experiments a pool of the purified monoclonal antibodies was used.

Polyclonal antibodies were raised in two rabbits (AG1 and AG2) against the recombinant agrin fragment containing both lamG domains fused to a short epitope of TNC. Antisera were tested by western blot and immunofluorescence for the specificity to the agrin fragment and preimmune sera, taken from the rabbits just before the immunization, served as negative control. To purify monospecific antibodies from these antisera the recombinant agrin fragment, which was used as antigen was bound to CNBr-activated Sepharose™ 4B resin (Amersham Pharmacia) and packed into a column. Polyclonal antiserum from rabbit AG1 was loaded on the column, the column was washed, bound antibodies eluted, and their activity tested by western blots of the recombinant protein. These highly purified antibodies were used for the detection of endogenous agrin in *C. elegans*.

Western blots

The purified agrin fragment of two lamG domains was run on 7.5% SDS PAGE [175] and transferred onto Immobilon membrane (Millipore). The membrane was blocked for one hour in 5% non-fat dry milk (Fluka) in TBS/Tween-20 (0.05%) (Fluka) with gentle agitation at room temperature. Incubation with primary antibodies was carried out overnight at 4°C slowly rocking. The anti-agrin purified monoclonal antibody pool was diluted 1:1000 and whole polyclonal antisera 1:8000 in blocking solution (5% non-fat dry milk in TBS/Tween-20). The following day membranes were washed over one hour with several changes of TBS/Tween-20, followed by incubation with goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRP) - conjugated secondary antibodies (Cappel, MP Biomedicals), diluted 1:2000 in 5% milk, during one hour at room temperature. After extensive washing in TBS/Tween over more than one hour, protein on the membrane was visualized by ECL reagent (Amersham Biosciences) and exposed on Kodak Biomax MR film.

To detect endogenous worm agrin by western blotting young larval stages were homogenized in ice-cold RIPA buffer (NaCl 150mM, TrisHCl pH8 50mM, NP-40 1%, deoxycholic acid 0.5%, SDS 0.1%, NaF 50mM) containing a cocktail of protease inhibitors (Complete Mini EDTA-free, Roche). The suspension was centrifuged for 30 minutes at 14000 rpm in a tabletop centrifuge at 4°C. The supernatant was kept and the pellet resuspended in RIPA buffer including 6M urea and centrifuged for additional 30 min at RT. The

supernatant was separated and the pellet resuspended in reducing sample buffer (SB⁺) with 6M urea. All fractions were heated for 5 minutes at 95°C and run on a 6% SDS PAGE, followed by western blotting, as described above. Purified polyclonal antibodies (AG1) were diluted 1:500 in the blocking solution and anti-rabbit secondary antibody 1:10000. Immunoreactive proteins were visualized by the HRP substrate Super Signal (Pierce) exposed to double-coated ML film (Kodak) for 1 minute.

Immunofluorescence

COS cells were grown in 35mm tissue culture dishes and transfected with pCEP-Agrin using Fugene 6 reagent (Roche). 24 hours after transfection the cells were rinsed with PBS⁺ and fixed with 4%PFA in PBS⁻ for 15 minutes at room temperature. Some dishes were permeabilized with 0,1% TritonX-100 (Fluka) in PBS⁻ during 5 minutes at room temperature, while some were left non-permeabilized for control. After rinsing with PBS⁻, the cells were blocked with 3% goat serum in PBS⁻ during 15 minutes at room temperature. Primary antibodies were diluted 1:100 in blocking solution and incubated 2 hours at room temperature. After washing in PBS⁻, the secondary antibody was diluted 1:1000 in blocking solution and incubated on cells for one hour at room temperature in the dark. Goat anti-mouse and goat anti-rabbit FITC antibodies (Alexa 488, Molecular Probes) were used on separate samples of cells. Together with the secondary antibodies, Hoechst dye (Fluka) was added at dilution 1:1000 for visualization of cell nuclei. Cells were washed with PBS⁻, rinsed with deionized water to remove traces of salt, and mounted with ProLong Gold antifade reagent (Invitrogen). The pictures were obtained on a Zeiss Z1 automatized upright fluorescence microscope for multi-dimensional acquisition. The same conditions were used for all the samples, 100x magnification, exposure of 500 ms for FITC and 80 ms for Hoechst.

To simultaneously stain for agrin and endogenous β -dystroglycan in COS cells, cells were treated as described above. The anti- agrin polyclonal antiserum was used at 1:100 dilution and the monoclonal anti- β -dystroglycan antibody 43DAG1/8D5 (Novocastra, kindly provided to us by Prof. Markus Rüegg) at 1:100 dilution in blocking solution. The β -dystroglycan antibody was visualized by a goat anti-rabbit secondary antibody coupled with a red dye (Alexa Fluor[®] 594, Molecular Probes) and anti-agrin by green-labelled goat anti-mouse (Alexa Fluor[®] 488, Molecular Probes).

Immunofluorescence staining of endogenous worm agrin

Worms were immunostained following a modified Finney & Ruvkun protocol [176]. Mixed stages of worms, grown on NGM plates, were extensively washed in M9 buffer. The last wash was done with deionised water and the worms were quick-frozen on dry ice/ethanol in Ruvkun fixation buffer mix diluted from a 2x stock (160mM KCl, 40mM NaCl, 20 mM Na₂EGTA, 10mM spermidine-HCl, 30mM Pipes, pH 7.4, and 50% methanol) with the addition of 1.5% formaldehyde. Following permeabilization by freeze-thaw in three cycles, worms were fixed for 1 hour on ice. The cuticle reduction and final permeabilisation was done by TTB and 1% β -mercaptoethanol overnight, slowly rotating at 37°C. Permeabilization was completed the following day by 10mM dithiothreitol (DTT) in 1x BO₃ buffer (diluted from 20x stock: 1M H₃BO₃, 0.5M NaOH), including 0.01% Triton and 0.3% H₂O₂ in the same buffer for additional 15 minutes. The worms were blocked in AbB buffer (1x PBS, 0.1% BSA, 0.5% TritonX-100, 0.05% Na-azide, and 1mM EDTA) and immunostained with the antibody solution in the AbA buffer (1% BSA) overnight at 4°C with gentle rocking. The mouse monoclonal anti-agrin antibody pool used at 1:200 dilution, the rabbit polyclonal anti-agrin antibody at 1:100, and a rabbit polyclonal anti-rim antibody 1:6000 [153]. The anti-rim was kindly provided by Prof. Michael Nonet. Worms were washed with AbB during several hours and incubated with the secondary antibody at 1:1000 dilution in AbA buffer at room temperature gently rocking in the dark during two hours. For agrin monoclonal antibodies anti-mouse secondary antibody conjugated with a green dye (Alexa Fluor[®] 488, Molecular Probes), for polyclonal anti-agrin secondary anti-rabbit labeled green (Alexa Fluor[®] 488, Molecular Probes) and for rim polyclonal antibody red-labeled anti-rabbit (Alexa Fluor[®] 546, Molecular Probes) was used. Following extensive washing in AbB during two hours, the worms were mounted on glass slides with Mowiol (Dabco) mounting medium. Images of agrin / rim co-staining were obtained by a confocal LSM510 META Axioplan2 microscope and for single agrin stainings by the polyclonal antibody a Zeiss Axioscope Bio microscope was used.

In vitro assay for agrin - β -dystroglycan binding

Purified chicken α -dystroglycan [33]; kind gift from Prof. Markus Rüegg) was run on 7.5% SDS PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for two hours in blocking buffer (PBS, 0.05% Tween-20, 1mM CaCl₂, 1mM MgCl₂, 5% milk). The membrane was cut in strips which were incubated with different samples of agrin. Chicken recombinant agrin proteins were kindly provided by Prof. M. Rüegg [27]. The chicken agrin fragments were 120 kDa in size, containing 25 kDa of N-terminal laminin-binding domain (NtA) fused to all three C-terminal lam G domains of 95 kDa, differing only in the alternative splicing essential for dystroglycan binding [24]. The fragment of a splice variant of chicken agrin enriched in muscles was used as positive control for DG binding, the chicken agrin neural isoform as negative control, and *C. elegans* recombinant fragment containing two lamG domains was tested. Final

concentration of both agrin control samples was 4µg/ml. The incubation was carried out overnight at 4°C with gentle agitation. The following day, membrane strips were washed in blocking buffer (5% milk in TBS/Tween-20). Detection of bound agrin fragments was performed as described for western blotting with minor modifications. The polyclonal anti-chick agrin antibodies were prepared by Dr. Shuo Lin and kindly provided to us by Prof. Markus Rüegg. Incubation with the primary antibodies was done during 3 hours at room temperature. Polyclonal anti-chicken agrin antibody was diluted 1:2000 in 5% milk blocking solution. The *C. elegans* agrin fragment was detected by anti-Tn60 [173] diluted 1:1000 in 3% BSA blocking solution. Following washing in 5% milk blocking solution, membrane strips were incubated with HRP-conjugated goat anti-mouse antibody diluted 1:2000, during one hour at room temperature. After extensive washing in TBS/Tween proteins were visualized by ECL reagent (Amersham Pharmacia) and the membranes exposed to Kodak BioMax MR film.

References*

* The references are listed in the Appendix together with all the references from the Thesis.

Fig. 1. *C. elegans* agrin DNA and protein sequence with predicted domain architecture. The *C. elegans* agrin coding sequence was assembled from overlapping cDNA fragments, amplified by RT-PCR. The positions of the primers are shown by black arrows, where the corresponding pairs are depicted with the same line pattern (full line, dotted line, “dash-dot-dash” line). The three nucleotides missing in the genomic sequence of the database entry are framed with red rectangles. Based on the nucleotide numbering in cosmid F41G3, their positions are: C after 30028, A after 29776 and C after position 28351. The coding region of the gene is 4422 bp long with 5' and 3' untranslated regions of 212 and 160 bp, respectively (dark gray boxes). The predicted protein sequence is 1474 amino acids long and the domain architecture is shown in different colors. A putative signal sequence (purple box) is followed by seven follistatin domains (blue), two epidermal growth factor domains of the laminin-type (light gray), a follistatin domain (blue), an EGF-like domain (orange) and two laminin G domains (yellow).

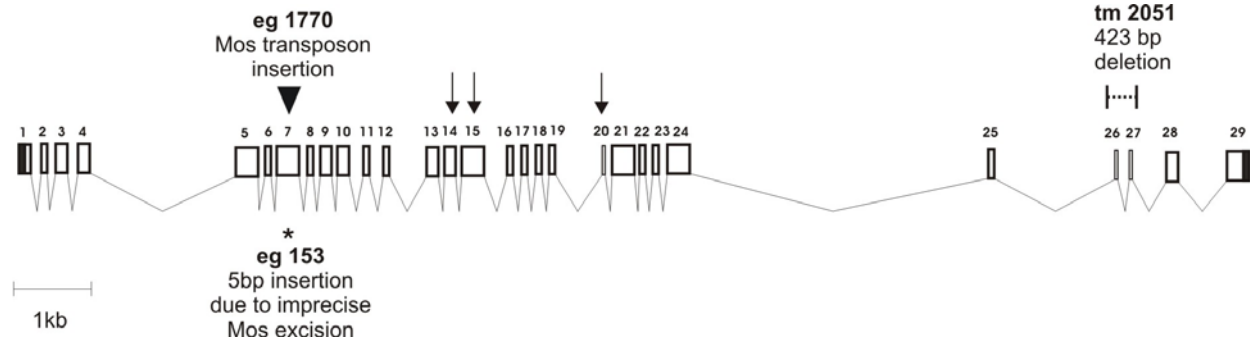


Fig. 2. Genomic organization of the *agrin* gene and mutant alleles. The assembled transcript consists of 29 exons which span over the chromosomal region of almost 14.5 kb on chromosome 2. Black arrows indicate the three locations where a nucleotide is missing in the database genomic sequence (exons 14, 15 and 20; cf. Fig.1). Three mutations in the *agrin* gene were isolated. In the *eg1770* mutant strain (black arrowhead) *Mos1* transposon was inserted into the seventh exon what brings the transcript out of frame, therefore causing a putative null mutation. The *eg153* strain (asterisk) was created by imprecise excision of the *Mos1* transposon leaving 5 bp at the insertion site and resulting in a +2 frameshift mutation. Mutant *tm2051* (dotted line) carries a deletion of 423 bp including exons 26 and 27 resulting in an in-frame loss of 42 amino acids.

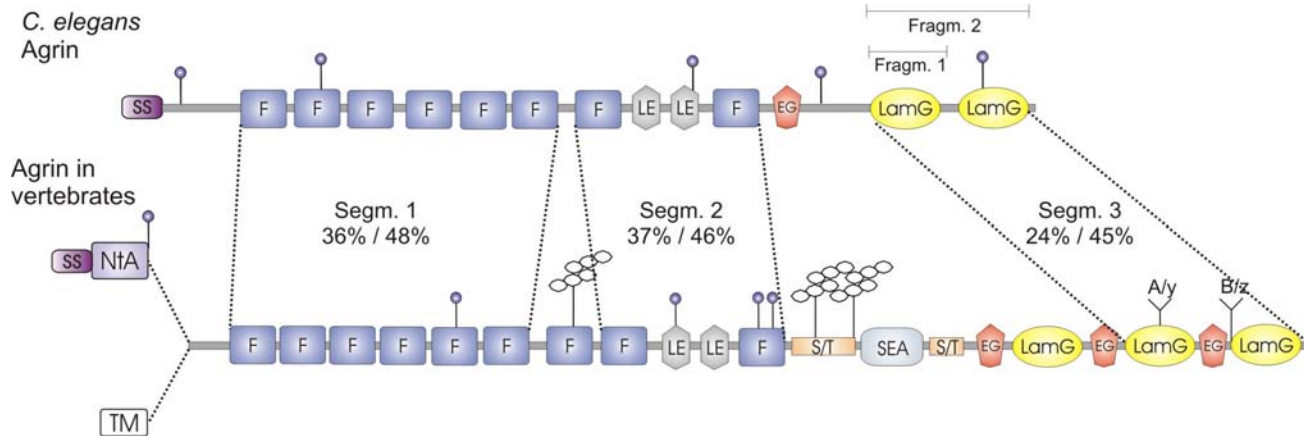


Fig. 3. Domain architecture of the *C. elegans* agrin protein in comparison to the vertebrate orthologues. *C. elegans* agrin starts with a signal sequence (SS; purple), followed by seven follistatin-like domains (F; blue), two epidermal growth factor (EGF) domains of the laminin-type (LE; gray), one follistatin-like domain (F; blue), an EGF-like domain (EG; orange), and two laminin G domains at the C terminus (LamG; yellow). The color scheme follows the same pattern as presented in the Fig. 1. Predicted N-glycosylation sites are shown with blue circles. Vertebrate agrins have two alternative N-termini: a secreted form, with a signal sequence (SS; dark purple) and a laminin-binding N-terminal agrin domain (NtA; light purple). These are followed by one follistatin domain more than *C. elegans* (F; blue), a sea urchin sperm protein, enterokinase and agrin domain (SEA; light blue), two serine/threonine rich regions (S/T; light orange), and three laminin G domains (LamG; yellow). O-linked heparan sulphate and chondroitin sulphate chains are schematically shown as branches and several N-linked glycosylation sites as blue circles. Alternative splicing at the last two LamG domains of vertebrate agrin (A/y and B/z) gives rise to several agrin isoforms with different functions, but no alternative splicing was found in *C. elegans* agrin. Three separate segments of *C. elegans* agrin marked by dashed lines were used in a Blast search. The resulting degree of identity/similarity to the corresponding parts of chicken agrin (Swissprot entry P31696-2) are indicated. Recombinant fragments 1 and 2 of *C. elegans* agrin indicated above the LamG domains were used as antigens for raising monoclonal and polyclonal antibodies, respectively.

A

```

Agrin_LamG1_C.elegans  FKP YRTNG I L F Y - - - - - W S V P S D P - - - - - H T D F I A F A M I D A K P H F V Y E L G S G L S Y I R - G - E
Agrin_LamG1_C.briggsae  - - P Y R T N G L L F Y - - - - - W S V P S D P - - - - - H T D F I A F A M I D A K P H F V Y E L G S G L S Y I R - G - E
Agrin_LamG2_Human      - - - - - S G L L L Y - - - - - N G Q K T D G - - - - - K G D F V S L A L R D R R L E F R Y D L G K G A A V I R - S R E
Agrin_LamG2_ElectricRay - - - - - N G M I F Y - - - - - N G Q K T D G - - - - - R G D F V S L N L R D G Y L E F R Y D L G K G A A V I R - S - K
Agrin_LamG2_Chicken    - - - - - S G M I F Y - - - - - N G Q K T D G - - - - - K G D F V S L A L H D G Y L E F R Y D L G K G A A V I R - S K E
Perlecan_LamG2_Human   F K P L A P D G V L L E - - - - - S G G K S G P - - - - - V E D F V S L A M V G G H L E F R Y E L G S G L A V I R S A - E
Perlecan_LamG1_Human   F R P D S A D G M L L Y N G Q K R V P G S P T N L A N R Q P D F I S F G L V G G R P E F R E D A G S G M A T I R - H P T
Perlecan_LamG2_Mouse   F R P D S A D G M L L Y - - - - - N G Q K R S P T N L A N R Q P D F I S F G L V G G R P E F R E D A G S G M A T I R - H P T
LAML2_LamG4_C.elegans  - R P T S D N G I I F - - - - - I A T N K - - - - - R T D H I A V M L E H G R V V F T Y D T G S G Q V I I K - S - D

Agrin_LamG1_C.elegans  - P I P L N S - W H T V R I E R L A K D V S M F V N - - E T L V K K H T S Q S K N A - H L D I S K K D A L Y V G F V P -
Agrin_LamG1_C.briggsae - P I P L N S - W H T V R I E R F A K D V S M Y V N - - G T L A K K Y T S Q S K N A - H L D I L K N D V L E V G F V P -
Agrin_LamG2_Human      - P V T L G A - W T R V S L E R N G R K G A L R V G - - D G - - P R V L G E S P V P - H T V L N L K E P L Y V G G A P -
Agrin_LamG2_ElectricRay - A P I P L N V - W N V V T V E R N G R K G L M K I N K - D E L V S G E S P K S R K A P H T A L N L K E A F Y V G G A P -
Agrin_LamG2_Chicken    - P V P L N T - W I S V L L E R S G R K G V M R I N N G E R V M G E S P K S R K V P - H A F L N L K E P F Y V G G A P -
Perlecan_LamG2_Human   - P L A L G R - W H R V S A E R L N K D G S L R V N - - G G R P V L R S S P G K S Q - G L N L H T - - L Y L G G V E -
Perlecan_LamG1_Human   - P L A L G H - F H T V T L L R S L T Q G S L I V G - - D L A P V N G T S Q G K F Q - G L D L N E E - - L Y L G G Y P -
Perlecan_LamG2_Mouse   - P L A L G Q - F H T V T L L R S L T Q G S L I V G - - N L A P V N G T S Q G K F Q - G L D L N E E - - L Y L G G Y P -
LAML2_LamG4_C.elegans  - K S I I D G R W H T I K V S R R G K S A H L I V D - - D N S Y E S E G A A N Q N E - D L - I E T Q P P F Y V G G V P A

Agrin_LamG1_C.elegans  - - E - G I S H K V R K L N V P F E G E L Q E L R I N E L — Reference
Agrin_LamG1_C.briggsae - - N - G E I S H K V R K L N V P F E G E L Q E L R I N E L — 91% / 94%
Agrin_LamG2_Human      - - D - F S K L A R A A A V S S I G F D G A I Q - - - - - — 29% / 52 %
Agrin_LamG2_ElectricRay - - D - F N K F A R A A G I I S G F T G A I Q N L S L K S I — 33% / 52%
Agrin_LamG2_Chicken    - - D - F S K L A R A A A I S T S F Y G A V Q R I S I - - - - - — 29% / 52%
Perlecan_LamG2_Human   - - P - S V P L S P A T N M S A H F R G C V G E V S V N - - — 35% / 51%
Perlecan_LamG1_Human   - - D Y G A I P K A - - G L S S G F I G C V R E L R I - - - - - — 33% / 51%
Perlecan_LamG2_Mouse   - - D Y G A I P K A - - G L S S G F V G C V R E L R I - - - - - — 33% / 50 %
LAML2_LamG4_C.elegans  D L A - G F A R N L V V G V R S Q F S G C I K D F K L N - - — 26% / 49%

```

B

```

Agrin_LamG2_C.elegans  V I W Q E G A W S Q A E D G G D F L L V F I E E G K L Y V G V N L G A D V H L K - P I - - S T N V T V A D N H W H S V S
Agrin_LamG2_C.briggsae V L W L E G A W S Q A E D G G D F I L V F I D E G K L Y V G V N L G A D V H L K - P I - - S T N V T V A D N H W H S V A
LamininA4_LamG4_Human M I F - - - - Y V S D Q E E N D F M T L F L A H G R L V Y M E N V G - - - H K K L K I - - R S Q E K Y N D G L W H D V I
Perlecan_LamG3_Human  L L W Q G V E V G E A G Q G R D F I S L G L Q D G H L V F R Y Q L G S G - E A R - L V - - S E D - P I N D G E W H R V T
Agrin_LamG3_Human     V L W - - - - S G K A T E R A D Y V A L A I V D G H L Q L S Y N L G S Q - P V V - L - - R S I V P V N T N R W L R V V
Agrin_LamG3_Rat       V L W - - - - I G K A A E R A D Y M A L A I V D G H L Q L S Y D L G S Q - P V V - L - - R S I V P V N T N R W L R I R
Agrin_LamG3_Chicken   I L W - - - - S G K G L E R S D Y I A L A I V D G F V Q M M Y D L G S - - - - K - P V V L R S I V P I N T N H W T H I K

Agrin_LamG2_C.elegans  F R R K E R K C E L W V D S K K I L H V V A S P G D V N L D S N G L V Y L G G A N P K K H K L L K S L N L S N K F V G C
Agrin_LamG2_C.briggsae F R R K E R K C E L W V D S K K I L H V V A S P G D T N L D S N G L V Y L G G V N P K K H K L L K S L D L T N R F V G C
LamininA4_LamG4_Human F I R E R S S G R L V I D G L R V L E E S L P P T E A T W K I R G P I Y L G G V A P G K A V K N Q V I N S I Y S F S G C
Perlecan_LamG3_Human  A L R E G R R G S I Q V D G E E L V S G R S P G P N V A V N A K G S V Y I G G A P - - D V A T L T G G R F S S G I T G C
Agrin_LamG3_Human     A H R E R E G S L Q V G N E A P V T G S S P L G A T Q L D T D G A L W L G G L P E L P V G P A L P K A Y G T G F V G C
Agrin_LamG3_Rat       A H R E R E G S L Q V G N E A P V T G S S P L G A T Q L D T D G A L W L G G L Q K L P V G Q A L P K A Y G T G F V G C
Agrin_LamG3_Chicken   A Y R V Q R E G S L Q V G N E A P I T G S S P L G A T Q L D T D G A L W L G G M E R L S V A H K L P K A Y S T G F I G C

Agrin_LamG2_C.elegans  V K N L K I F G — Reference
Agrin_LamG2_C.briggsae V K N L R I F G — 89% / 98%
LamininA4_LamG4_Human L S N L Q L N G — 30% / 44 %
Perlecan_LamG3_Human  V K N L V L H S — 31% / 51%
Agrin_LamG3_Human     L R D V V V - - — 28% / 49%
Agrin_LamG3_Rat       L R D V V V - - — 25% / 47%
Agrin_LamG3_Chicken   I R D V I V - - — 28% / 52%

```

C

```

Agrin_LamG2_C.elegans  L V F P N N V T F S - - - - - V G S S K S S N F S F D F R L K Q F G M I - W Q
Agrin_LamG3/B0_Chicken ME Y H N A V T K S - - - - - E K A L Q S N H F E L S I K T E A T Q G L I L W S
Agrin_LamG3/B11_Chicken ME Y H N A V T K S - - - - - P D A L D Y P A E P S E K A L Q S N H F E L S I K T E A T Q G L I L W S
Agrin_LamG3/z0_Rat    I E Y L N A V I E S - - - - - E K A L Q S N H F E L S I R T E A T Q G L I L W I
Agrin_LamG3/z19_Rat   I E Y L N A V I E S E L T N E I P A P E T L D S R A L F S E K A L Q S N H F E L S I R T E A T Q G L I L W I

```

8aa 11aa

Fig. 4. Alignment of the *C.elegans* lamG domains to the corresponding domains of other proteins.

A. The first LamG domain of the *C.elegans* protein (Agrin_LamG1_ *C.elegans*) was used as a query against Swissprot, Trembl and Refseq databases. After the analysis of a more extensive alignment, the best hits were selected for this representation and include: the predicted agrin orthologue of *C. briggsae* (Agrin_LamG1_ *C.briggsae*), the agrin LamG2 domains of the human, electric ray and chicken proteins, the LamG2 and LamG1 of human perlecan and the LamG4 of a laminin-like protein 2 (LAML2) identified in *C. elegans*. The similarities between each of the sequences compared to the *C. elegans* lamG1 are expressed as % identity / % similarity.

B. The second LamG domain of the *C.elegans* protein (Agrin_LamG2_ *C.elegans*) was used as a query against Swissprot, Trembl and Refseq databases. After the analysis of a more extensive alignment, the best hits were selected for this representation and include: the predicted agrin orthologue of *C. briggsae* (Agrin_LamG2_ *C.briggsae*), the LamG4 of human lamininA4, the LamG3 of human perlecan and the agrin LamG3 domains of the human, electric ray and chicken proteins. The similarities between each of the sequences compared to the *C. elegans* lamG1 are expressed as % identity / % similarity.

C. The *C.elegans* agrin sequence aligns best with the B₀/z₀ isoforms of chick and rat agrin, respectively. The conserved alternatively spliced agrin exons, encoding 8 aa, 11 aa or 19 aa inserts, at this site do not exist in *C. elegans*.

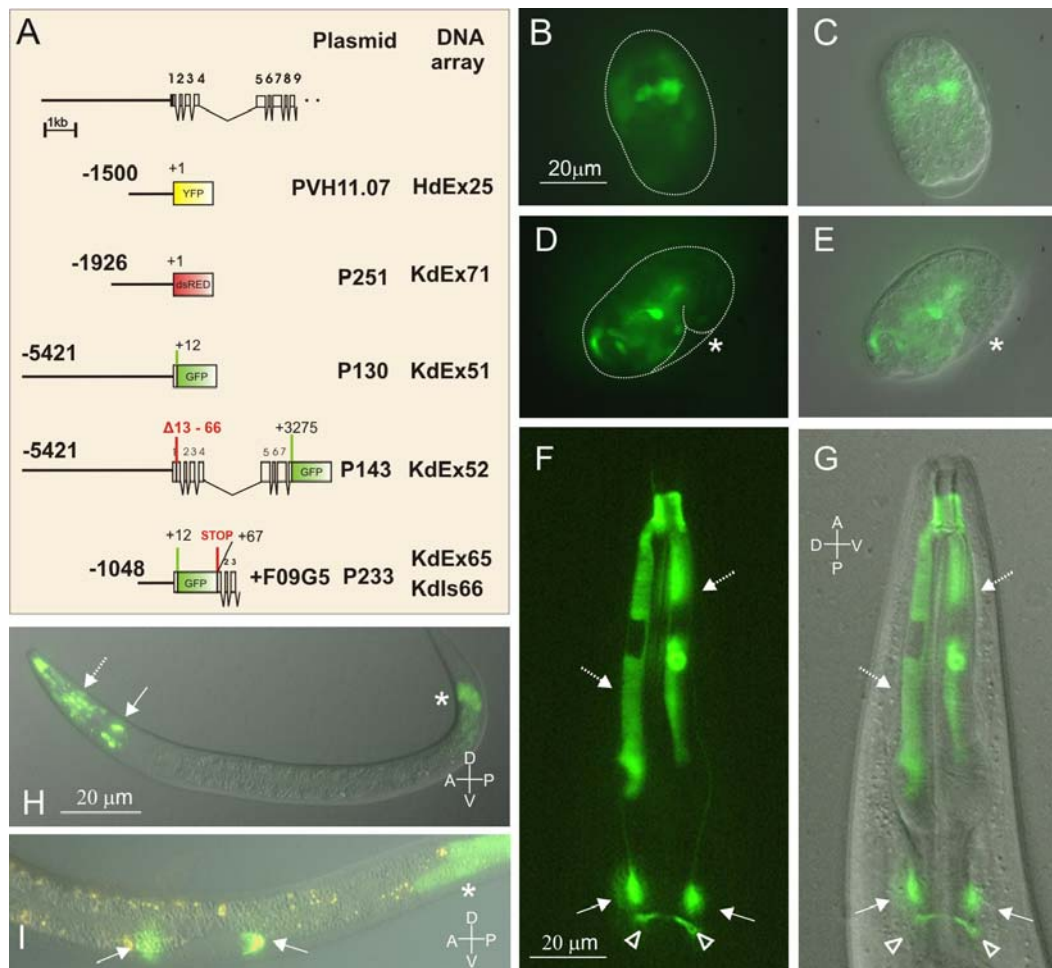


Fig. 5. *Agr-I::reporter* expression in transgenic animals. A, Reporter genes were fused to different portions of agrin non-coding and coding sequence as shown below the schematic representation of the genomic region containing the *agr-I* promoter and *agr-I* 5'-end. The lengths of the promoter or gene sequences and the names of the the *pagr-I::reporter* plasmids and DNA arrays are indicated. Since all of these constructs resulted in the same expression patterns, representative micrographs of the *kdIs66* transgenic worms are shown in B-I. **B and C**, small groups of cells express GFP under the *agr-I* promoter in early gastrula (≈ 100 min). **D and E**, expression in the anterior body of a late gastrula (≈ 240 min); no expression is visible in the tail (asterisk) of the animal. **F and G**, head of a young adult worm; expression in the buccal epithelial cells (dashed arrows) and in the IL1v and IL1d neurons (arrows); open arrowheads point at the IL1 processes in the nerve ring. **H**, L1 larval stage; expression in the buccal epithelial cells and other non identified cells near the pharynx (dashed arrow), IL1v and IL1d neurons (arrow) and posterior gut cells (asterisk). **I**, L2 larva; expression in the migrating distal tip cells (arrows) and posterior gut (asterisk).

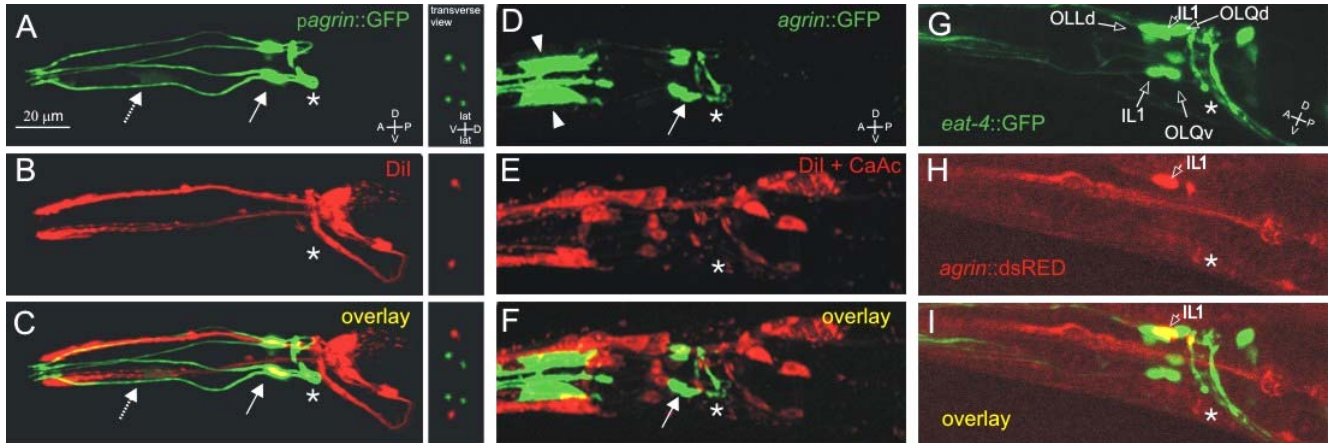


Fig. 6. *Agr-1* expression in IL1d and IL1v neurons. A-C, DiI staining in *hdEx25* transgenic worms; no costaining is observed between *agr-1::YFP* (A) and DiI (B). D-F, no costaining is observed between *agr-1::GFP* (D) and DiI + CaAcetate (E) in *kdIs66* transgenic animals. G-I, costaining is observed in *eat-4::GFP* (G) and *agr-1::dsRED* (H) in *adIs1240; kdEx71* transgenic worms. (C, F, I) merged channels. In all panels dashed arrows point out dendrites; arrows neuronal cell bodies; arrowheads buccal epithelial cells and asterisks the nerve ring.

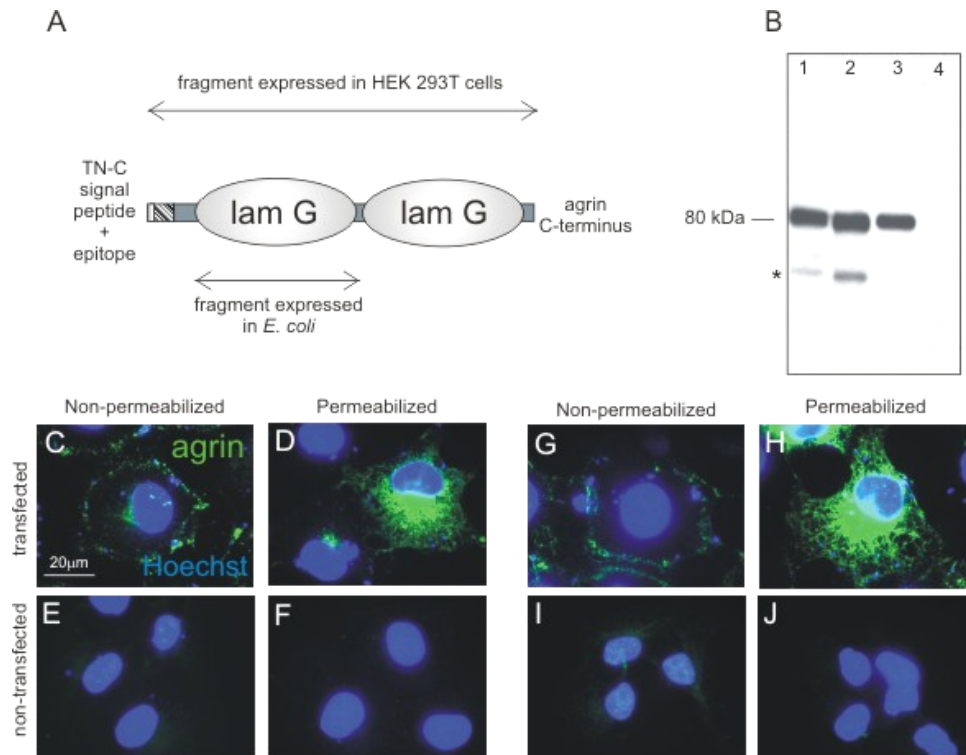


Fig. 7. Antibodies against *C. elegans* agrin. **A.** Schematic representation of the recombinant fragments used as antigen to raise monoclonal and polyclonal antibodies. For eukaryotic expression the C-terminal lamG domains were fused to a short fragment of chicken tenascin C (Tn-C), including a secretion signal and the epitope of anti-Tn60. The specificity of both polyclonal and monoclonal antibodies was tested by western blotting on conditioned medium of COS cells transfected with the construct encoding the two LamG domains with the Tn-C-tag (**B**). Lanes 1 and 2 were incubated with polyclonal antisera from two different rabbits, respectively; lane 3 with the monoclonal antibody pool raised against the bacterially expressed fragment, and lane 4 with pre-immune serum. All antibodies detected a band of about 80 kDa, which corresponds to the size of the recombinant protein. Additional smaller bands (asterisk) most likely correspond to degradation products which are not recognized by the monoclonal antibodies. **C-J**, Immunofluorescence staining of transfected COS cells was performed with the anti-agrin monoclonal antibody pool (**C-F**) and compared to the anti-Tn60 control (**G-J**). In transfected cells (**C and D**; **G and H**) the secreted agrin fragment was detected on cell surfaces of non-permeabilized cells (**C and G**) or in the endoplasmatic reticulum / Golgi apparatus of permeabilized cells (**D and H**). Non-transfected cells were used as a negative control (**E and F**; **I and J**).

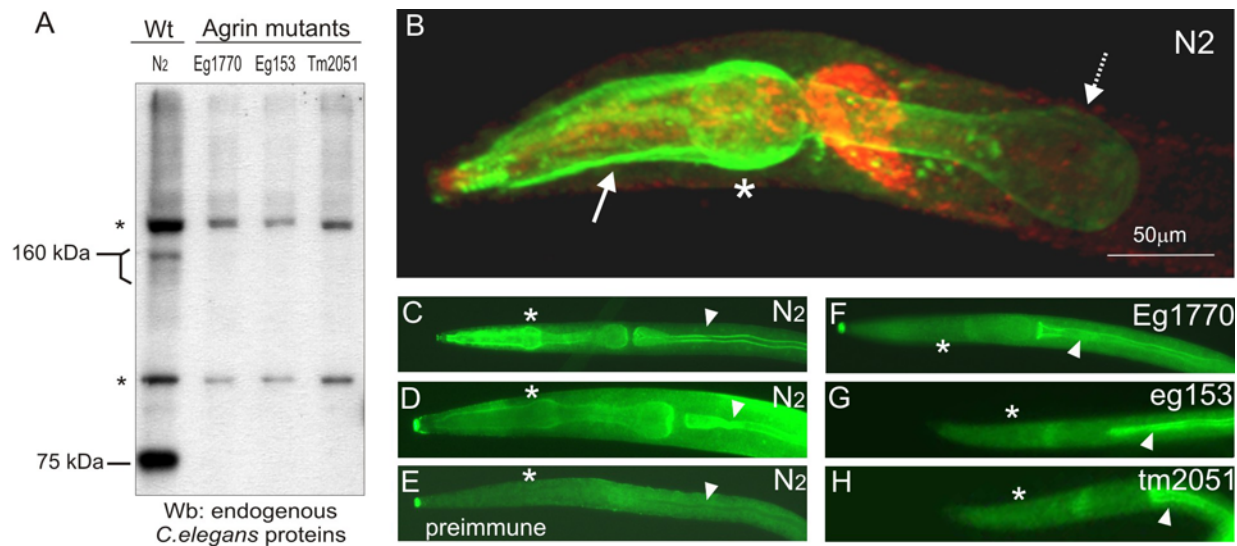


Fig. 8. Detection of endogenous *C. elegans* agrin by western blot and immunofluorescence. Lysates of wild type (N_2) and agrin mutant worms (*eg1770*, *eg153*, *tm2051*) were analysed on western blots **A**. Two prominent bands of about 160 kDa and 75 kDa were present exclusively in the wild type (wt) worms and not the mutants. The larger band corresponds to the calculated size of the full length AGR-1 protein and the smaller band may represent an agrin degradation product. Asterisks denote two additional background bands present in all the strains. **(B)** Worms were immunostained with the monoclonal antibody pool against *C. elegans* agrin (green) and Rim, a synaptic marker prominent in nerve ring (red). Agrin was detected in the basal lamina around the pharynx procorpus (arrow) and anterior bulb (asterisk). Posterior bulb staining was weaker possibly due to poor antibody penetration (dashed arrow). **(C-H)** Polyclonal antiserum staining resulted in the same pattern in the pharynx of wild type worms (**C and D**, asterisk for anterior bulb) where it was clearly absent in agrin mutants (**F-H**). Prominent background staining of the gut was present in all strains (**C-H**, arrowhead). Preimmune serum of the same rabbit was used as negative control on wild type worms (**E**) where both pharyngeal and gut staining was clearly missing.

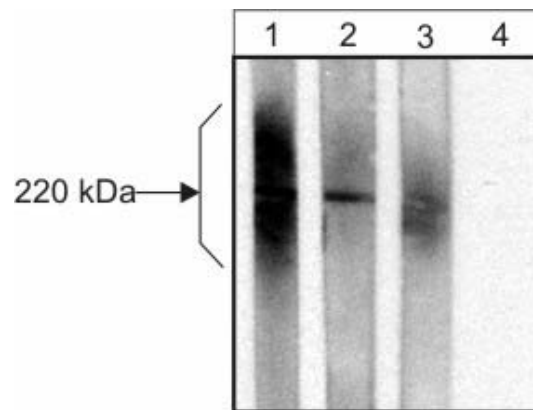


Fig. 9. *In vitro* interaction between *C. elegans* agrin and vertebrate α -dystroglycan. Purified chicken α -DG was transferred to nitrocellulose membrane after separation by SDS-PAGE and membrane strips were incubated with different samples of agrin: lane 1, chicken muscle agrin isoform; lane 2, chicken neuronal isoform; lane 3, *C. elegans* agrin in conditioned medium of transfected COS cells; lane 4, conditioned medium of non-transfected COS cells. Binding of the respective agrins was detected by anti-chick agrin antibody (lanes 1 and 2) or with the anti-Tn60 antibody raised against the short tenascin C fragment which was fused to the *C.elegans* agrin fragment (lanes 3 and 4). Binding of *C.elegans* agrin to α -DG was detected in lane 3, but not in the negative control (lane 4).

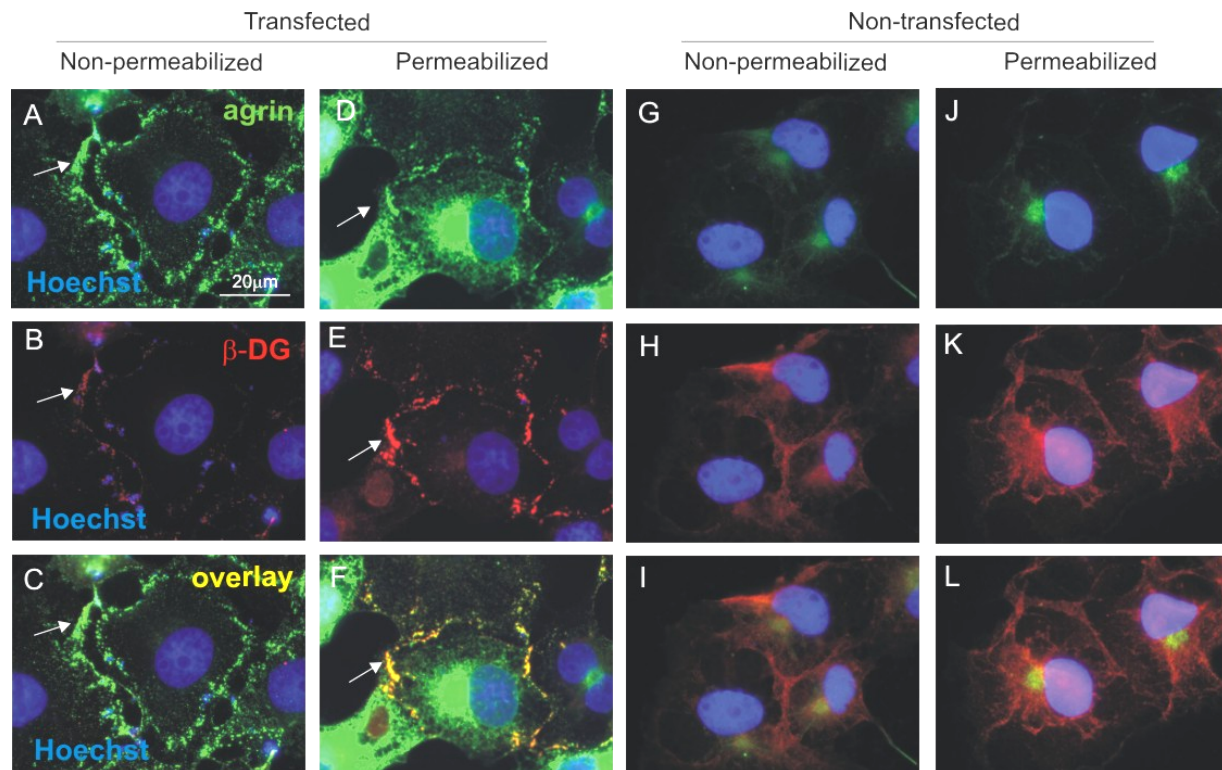


Fig. 10. Recombinant *C. elegans* agrin clusters endogenous dystroglycan in COS cells.

COS cells were transfected with the recombinant fragment of *C. elegans* agrin and immunostained for agrin and endogenous β -DG. (A-F) In transfected cells, agrin secreted into the medium bound to the cell surface in a patchy pattern (A and D, arrows). The cells were co-immunostained for endogenous β -DG (B and E) which, in transfected permeabilized cells, colocalized with agrin (F, arrow). Weak β -DG staining in non-permeabilized cells (A-C) is probably due to minor antibody diffusion into cells. In non-transfected cells (G-L), no agrin staining was present (G and J) and β -DG showed diffuse, homogeneous membrane staining (H and K).

Table 1. Primer sequences

Primer name	Sequence (5' → 3' direction)
agr 1	TGATGAAGCTGGAAGTCCCTGTGAG
agr 2	AATTCCCAGATGACTTTTCCAGCC
agr 3	ACGCGCATTGGCACCTTTCTC
agr 6	TGGGCATACACATTTGGGTTTTCCG
agr 9	GCCTCCATCTCATTGTCATTCATC
agr 12	TCGGATTGGCTCCTCCAAGATATAC
agr 25	GCAAGAAGGAGCATGGAGTCAG
agr/TN (antisense)	GAAATTTTCAGGCGCCATGGAGCAGTTGGGGCCTTTC
E32	AAGCATGCGGAGTTGAGTGGAGACGC
E33	AACTCTAGAGCGCCGCAAGATCGATGTGAAGTCTCATGTCGAC
E34	CACATCGATCCTCGTCCAACCTCGTTCC
E35	TAGAGCGGCCGCTTCATTATCAAAGTCTCTCC
E36	AAATCGATATGAGTAAAGGAGAAGAAC
E37	TTATCGATCTAGGCGCCTTTGTATAGTTCATCCATGCC
E149	TTGGTACCGCATGCCCGAGAGGAGTACGCGTCC
E150	TTGAGCTCAGTCGACTGCAGCATGCGGACATGTGAAAGTGAAAATG
lam 3	GAAGCATGCATGGCGCCTGAAATTTCAAGAAC
lam 6	GTGAAGCTTAGATATCAAATTGATTGGAAGT
lam 8	GTGAAGCTTTTAGTTATAGCAGTACTTGGGT
lam 8 euk 4	CAGCGGCCGCCATCTAGATTAGTTATAGCAGTACTTGGG
oligo dT (for 3'UTR)	GGCATGGTT(TTT) ₆
overlap5' agr (sense)	ATGGCGCCTGAAATTTCAAG
T3 primer	TTAATTGGGAGTGATTTCCC
XY1	GCCTGCAGTGTTATGAATTTTTCTTGAG
XY2	CCTCTAGATGAAAGTGAAAATGTTTCGTTTC

III.2. Unpublished Results

III.2.1. Expression and purification of recombinant *C.elegans* agrin fragments

With the goal to raise anti-agrin monoclonal antibodies and for the *in vitro* assays, we cloned the first laminin G domain of *C. elegans* agrin (see section III.1.)

Following expression in *E. coli*, the protein lysates were solubilized in denaturing buffer (prepared following the QiaExpressionist protocol, Qiagen) and the agrin fragment was purified over Ni-NTA agarose beads packed in a column. The interaction between the agrin fragment and Ni⁺ ions on the column material was due to the six-histidine tag fused to agrin. Following extensive washing, the 25 kDa agrin fragment was eluted from the column by decreasing pH (Results, Fig. 11). Only odd-numbered fractions were loaded on the SDS PAGE and detected by Coomassie blue staining. The fractions richest in agrin fragment (C8 to D3) were pooled and dialysed against PBS⁻ before they were injected into mice to raise monoclonal antibodies.

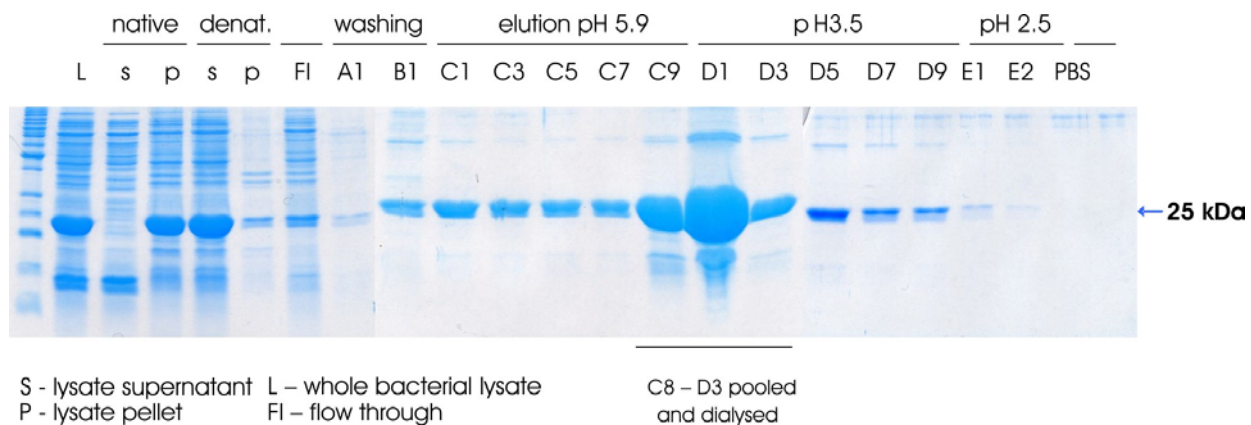


Fig 11. SDS PAGE of the protein lysates and of the fractions eluted from the Ni-NTA agarose column, and purification of the recombinant agrin fragment comprising the first lam G domain

A second recombinant worm agrin fragment composed of both lam G domains (2LamG) was fused to a short fragment of chicken tenascin C coding for a secretion signal and an oligomerization domain (Results, Fig. 12). The cloning and expression procedure is explained in detail in the chapter containing Submitted manuscript.

HEK293_{EBNA} cells were transfected with the 2lamG construct and Puromycin resistance served to select the transfected cells. The expression of the agrin fragment 2lamG was detected in the conditioned medium by western blot with an anti-TN-C epitope antibodies (Results, Fig. 12A). In order to additionally confirm the correct cloning, we analyzed the conditioned media containing the recombinant fragment, by reducing and

non-reducing conditions. The presence of the TN-C oligomerization domains caused the formation of protein hexamers which were intact under non-reducing conditions (SB⁻) and disrupted in reducing conditions (SB⁺) (Results, Fig. 12B). Conditioned media from the cell line, stably expressing the agrin fragment, were concentrated and the fragment was purified over an affinity column containing anti-TN-C antibody. The purified fragment was further used for raising polyclonal antibodies and for *in vitro* assays.

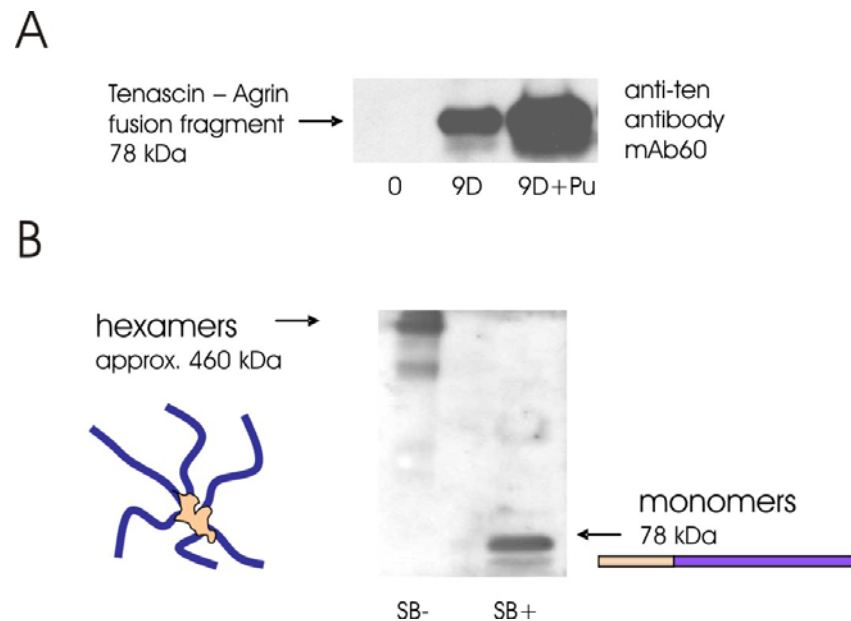


Fig 12. **A**, western blot of the conditioned media from HEK293_{EBNA} cells, either non-transfected (negative control, 0), transiently transfected with the clone called 9D or a stably transfected cell line selected by resistance to the antibiotic puromycin (Pu). **B**, western blot of conditioned media prepared for the gel in non-reducing (SB⁻) and reducing (SB⁺) conditions. Oligomerization happens due to the short fragment of TN-C fused to the agrin 2LamG fragment.

III.2.2. Detection of the endogenous *C. elegans* agrin by immunofluorescence

[This part was done in collaboration with **Gordon Lau**, an IAESTE student in our laboratory.]

As one of the first steps towards understanding agrin function in *C. elegans*, we extensively analysed its localization by immunofluorescence. In order to set up the staining protocol, we optimized the staining conditions for previously described markers (Results, Fig. 13). Antibodies against the presynaptic vesicle marker Rim [153], kindly provided to us by Prof. Michael Nonet, stained the nerve ring and most of the neurons in the worms' bodies (Fig. 13A). Furthermore, we used antibodies against prominent muscle and basement membrane proteins, the anti-myosin monoclonal antibody 5-6 [177] (Fig. 13B) and the anti-

perlecan monoclonal antibody MH2 [104] (Fig. 13C), both obtained from the Developmental Studies Hybridoma Bank, University of Iowa in Iowa City. Those proteins served as markers to investigate the morphology and structural integrity of the corresponding tissues in agrin mutants. No difference was observed between the staining of wild type and agrin mutants *agr-1(eg1770)* worms suggesting that nerve, muscle and basement membrane structure was not impaired due to agrin loss (results not shown).

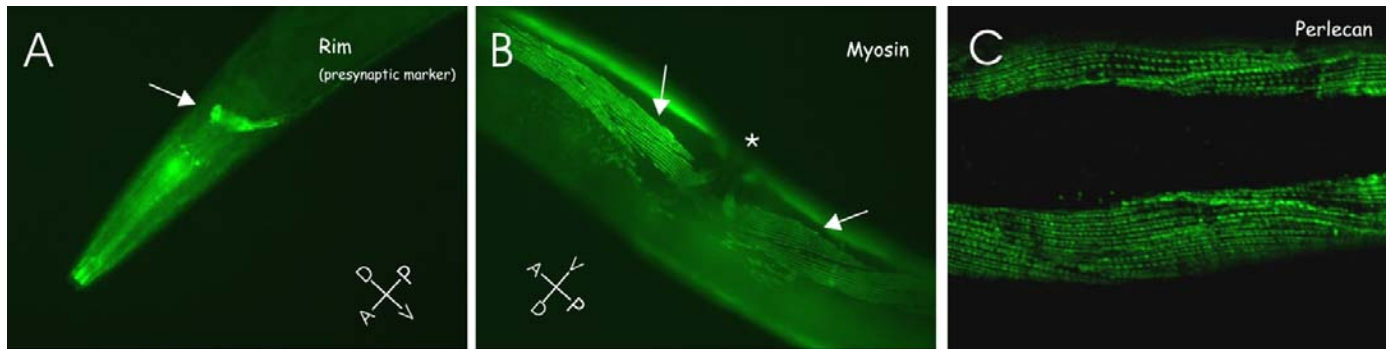


Fig 13. Immunostaining of wild type worms for the pan-neuronal marker Rim (A, arrow points to the nerve ring), the muscle marker myosin (B, arrows point to body wall muscles and the asterisk shows the position of the vulva), and perlecan for basement membrane (C).

The immunostaining procedure is based on the “Finney & Ruvkun protocol” (see section III.1.) [176]. For high-resolution visualization of the staining, we analyzed the samples by confocal microscopy. Most prominent immunostaining was observed in the pharynx of the worms (Results, Fig. 14). Agrin was detected primarily in the basement membrane of the pharynx (Fig. 14) and the marginal cells (Results, Fig. 15). This protein localization led us to further investigate pharynx function and morphology in the agrin mutants, as it is explained in a later paragraph.

Beside pharynx, some immunostaining was detected along the dorsal and ventral nerve cords, in the area of hypodermal ridges, a thickenings of the hypodermis (Fig. 16) known to be involved in axon guidance and neuronal patterning along the midline [100]. It is important to mention that, to some extent, agrin immunostaining was also present in the hypodermal ridges in the agrin mutant *agr-1(eg1770)*, suggesting that the staining might not be specific.

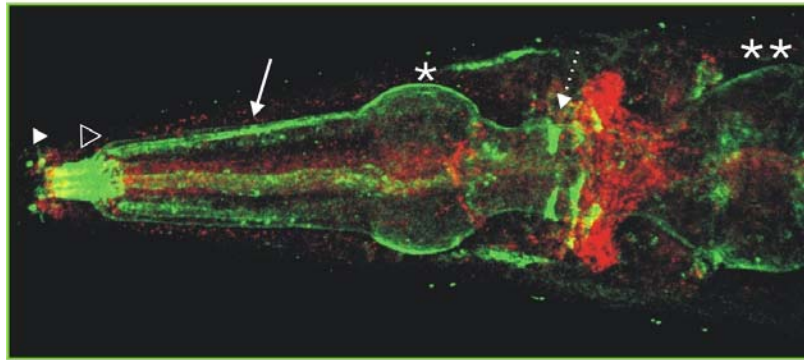


Fig 14. Immunostaining of the wild type worm with the pool of anti-agrin monoclonal antibodies (green) and anti-Rim antibodies, (red, pre-synaptic neuronal marker). Arrow points to the pharynx procorpus, asterisks label the first and second pharyngeal bulb, dashed arrow points to a subset of head neurons, full arrowhead shows the mouth of the worm (including the labial sensilla of IL1 neurons), and empty arrowhead points at buccal hypodermis. Red staining of Rim protein localizes mainly to the nerve ring.

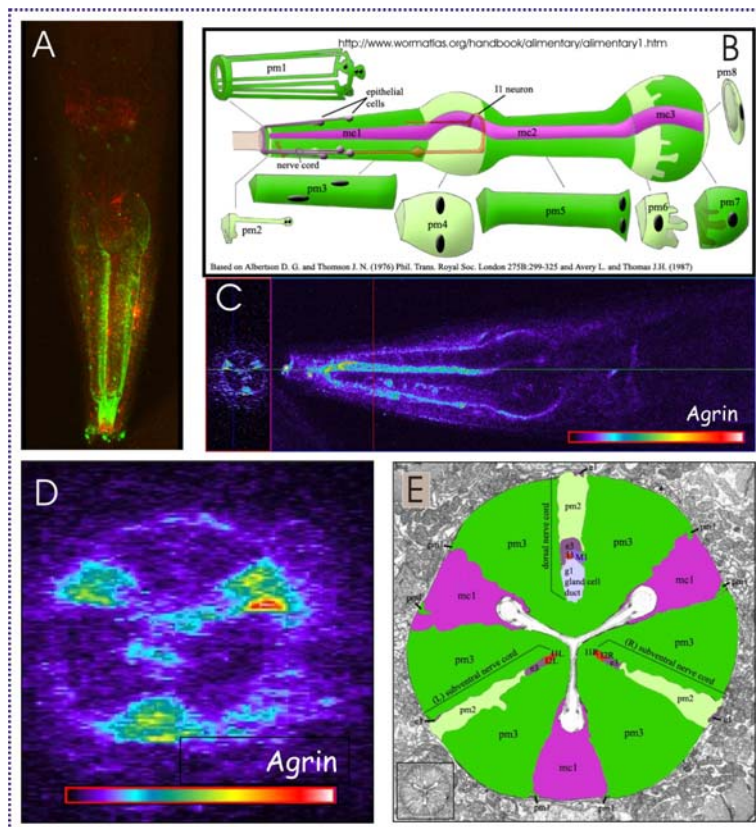


Fig 15. Immunostaining of marginal cells in the pharynx. A, Marginal cells are stained in green, mostly in the anterior part of the pharynx, probably due to better antibody penetration. B, Schematic representation of the main components of the pharynx with marginal cells shown in purple. C, Lateral view of the anti-agrin immunostaining presented in false colors for better visualization. D, Transverse view of the false-coloured agrin immunostaining. E, Schematic transverse view of the pharynx. (B and E were adapted from www.wormatlas.org)

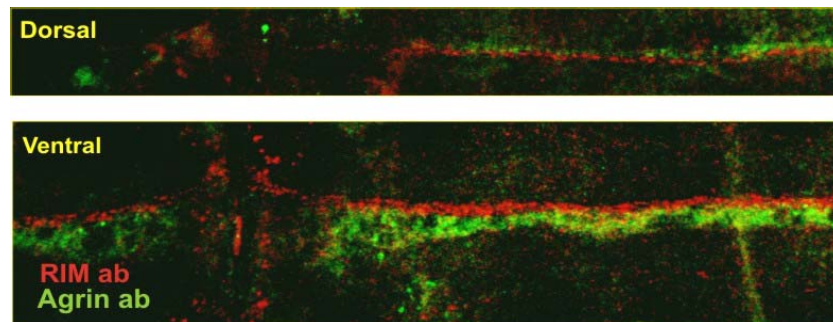


Fig 16. Agrin immunostaining (green) in the area of hypodermal ridges follows dorsal and ventral nerve cord (stained red for presynaptic protein Rim).

III.2.3. Assays *in vitro* with recombinant *C. elegans* agrin fragment 2LamG

[Materials & protocols for these experiments were kindly provided by **Prof. Markus A. Rüegg**, University of Basel]

III.2.3.1. AChR clustering on muscle myotubes *in vitro*

In vertebrates agrin has a prominent function at the neuromuscular junction where it contributes to the clustering of AChRs and their maintenance (reviewed in [13]). The clustering activity was demonstrated by *in vitro* assays on cultured muscle myotubes [178] [2]. Based on described properties of vertebrate agrin, we investigated whether the *C. elegans* 2LamG (see above) agrin fragment, comprised of the two C-terminal lam G domains, retains the clustering activity on muscle myotubes.

We performed the experiment following the standard protocol from the Rüegg lab. The procedure is explained in the Appendix. Tissue culture dishes were coated with gelatin and mouse muscle precursor cells (myoblasts, C₂C₁₂ line) were grown until about 80% confluency (Fig. 17). Differentiation of the cells was induced by the exchange of fetal calf serum for horse serum at a lower concentration. Under these conditions, the cells were cultured during 5 days and by the end of that time the muscle precursor cells had fused into syncytial myotubes (Fig. 17).

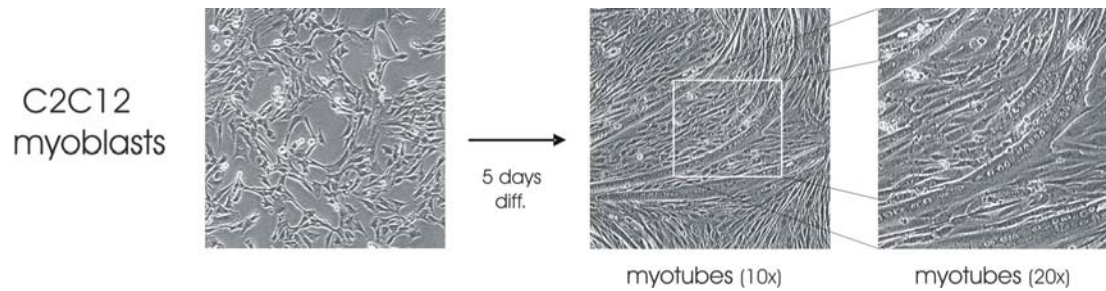


Fig 17. Differentiation of muscle C₂C₁₂ myoblasts into muscle myotubes. Syncytial organization is clearly visible in differentiated muscle cells.

The cultures were incubated with recombinant 2LamG fragment of *C. elegans* agrin. Chick agrin fragments, composed of all three lam G domains, served as controls. A neuronal isoform, which contains inserts at the B⁺/z⁺ site was used as positive control, while the muscle isoform, lacking the inserts (B⁻/z⁻), served as negative control. After an overnight incubation, cells were rinsed, fixed and stained for the AChRs subunits by fluorescently labeled bungarotoxin (Btx), toxin which irreversibly binds to the receptor subunits. Many AChRs clusters were present on the myotubes incubated with the chicken neuronal agrin fragment, no clusters were present on the myotubes following the incubation with the muscle agrin fragment, and only some were induced by the *C. elegans* 2LamG agrin fragment (Fig. 18).

Since the concentration of *C. elegans* agrin was more than 100 times higher than the controls, and yet did not give significant clustering, we could conclude that *C. elegans* agrin does not actively cluster AChRs on vertebrate muscle fibers. *In vivo* expression pattern data together with the behavioral analysis of agrin mutants do not support its function at the NMJ. Therefore, we could conclude that AChR clustering at the NMJ in *C. elegans* probably does not depend on agrin.

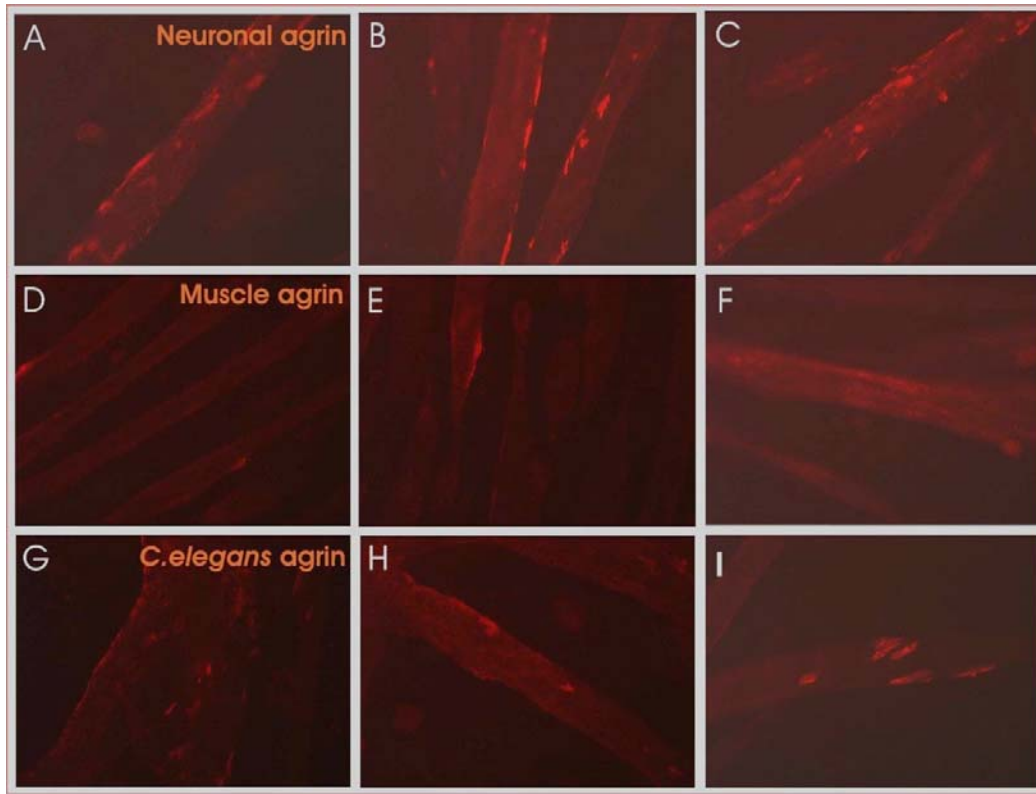


Fig 18. *In vitro* AChRs clustering assay on differentiated muscle myotubes. Clustered receptors were visualized by rhodamine-labelled bungarotoxin (Btx). **A-C**, Neuronal agrin served as positive control (at concentration 1 ng/μl) and the induced clusters can clearly be seen. **D-F**, Muscle agrin did not show clustering activity. **G-I**, Recombinant fragment of *C. elegans* agrin did not show a significant clustering activity, although some clusters could be detected (at concentration 125 ng/μl) (**I**).

III.2.3.2. Affinity chromatography experiments with *C. elegans* agrin fragment and worm lysates

[These experiments were carried out in collaboration with **Marianne Brown-Lüdi**, research associate in our laboratory]

With the objective to identify interacting partners of *C. elegans* agrin we analyzed proteins that bind to the 2LamG agrin recombinant fragment (see above). The purified 2LamG fragment was bound to CNBr-activated Sepharose beads and packed into a column following supplier's protocol (see Appendix). Worm lysates were prepared from large amounts of frozen worms and, after protein extraction the soluble fractions were passed over the affinity column. Following extensive washing, the proteins which interacted with agrin on the column, were eluted and the fractions analyzed by SDS PAGE followed by mass spectrometry (Fig.

19). An empty column, containing only CNBr-activated beads, served as a negative control. Several proteins did bind to the agrin column. Among them there were putative oxido-reductase metabolic enzymes involved in fatty acid oxidation (Q9BIC3_CAEEL and Q9GYT0_CAEEL) and YKA3_CAEEL from the thiolase family. A small quantity of a few known proteins was detected e.g. vitellogenin-6 (Q7KPP7_CAEEL), histone-4 (H4_CAEEL), and actin protein 4 isoform c (Q6A8K1_CAEEL). There was no clear connection between agrin and the identified proteins, therefore we did not consider the interactions specific and meaningful. Surprisingly, despite previously demonstrated interactions between agrin and α -dystroglycan (see Submitted manuscript), we could not confirm this by the affinity column experiment. A possible explanation is that dystroglycan is not soluble under the extraction conditions we used.

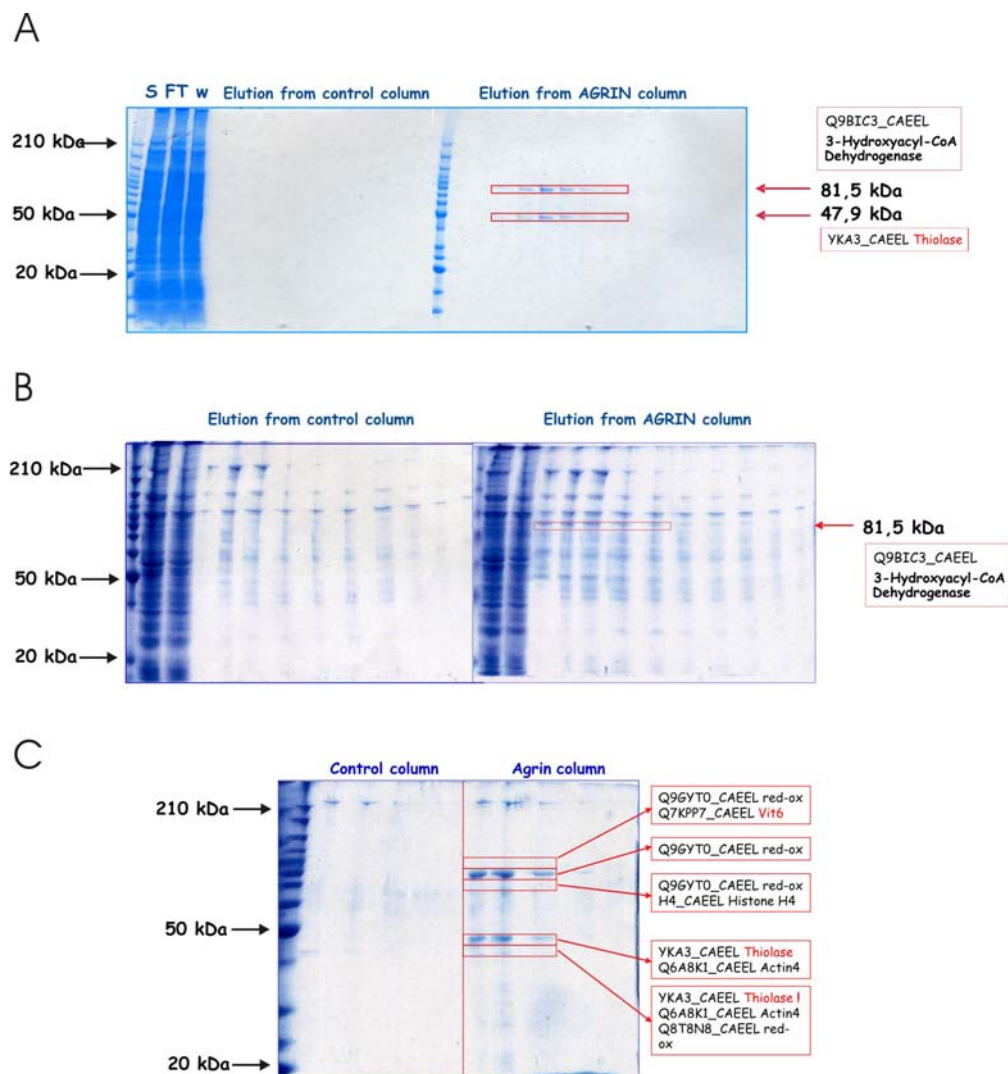


Fig 19. Putative agrin interacting partners were analyzed by affinity chromatography. The experiment was repeated three times with different buffers and conditions (A, B, C) and the proteins, which were selectively eluted only from the agrin column, were analyzed by mass-spectrometry.

III.2.4. Studies of agrin *in vivo* function in *C. elegans*

[The “*in vivo*” analysis was carried out in collaboration with Dr. Stefano Canevascini, post-doc in the laboratory]

III.2.4.1. Investigating possible agrin function at the NMJ

III.2.4.1.A. Pharmacological assays with levamisole and aldicarb

Based on the previously described and very well known agrin function at the NMJ (reviewed in [13]), we searched for similar functions in *C. elegans*. None of the agrin mutants, i.e. *agr-1(eg1770)*, *agr-1(eg153)*, *agr-1(tm2051)*, showed any locomotory defects that could be linked to defective NMJs. We have therefore used different pharmacological approaches to reveal possible agrin function at the synapse, and comparing the agrin mutants to the wild type worms.

Levamisole and aldicarb are nematocide drugs commonly used for this kind of experiments. Levamisole is an AChR agonist, which irreversibly binds to some of the receptor subunits and causes toxicity leading to the paralysis and death of wild type worms [86] [111]. Mutants with defects in AChR subunits or loss of receptor clustering at NMJ do not respond in this way and appear resistant to the drug, e.g. *lev-1(e211)* which we have used as a negative control. We tested agrin mutants for resistance to levamisole (Sigma) following a standard procedure (see Appendix - Experimental procedures). The agrin mutant *agr-1(eg1770)* showed a very weak resistance to the drug at low concentrations of 0.25mM, but not at a concentration of 0.5mM (Fig. 20). Taken together the fact that only a very low concentration of levamisole is tolerated and that the standard deviation values were high in our experiments, we could not claim that agrin plays an important role at the synapse.

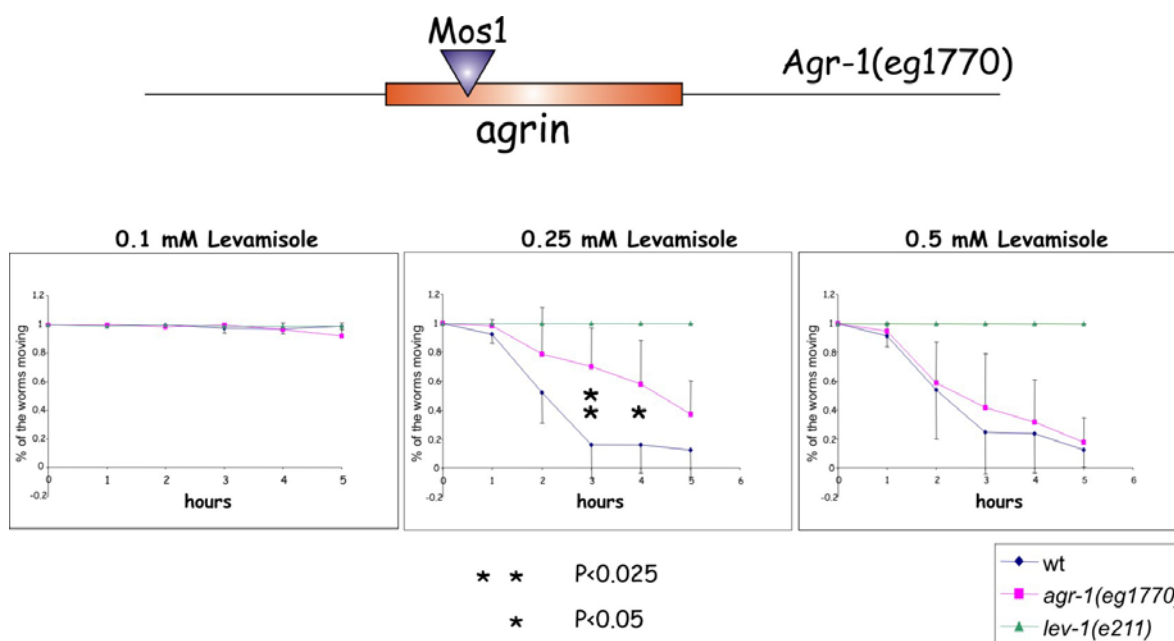


Fig 20. Levamisole resistance assays on *agr-1(eg1770)* worms at three different concentrations of the drug. **A**, schematic genomic representation of the *eg1770* agrin mutant, a Mos1 transposon is inserted in the *agrin* ORF. **B**, Agrin mutant shows a very weak resistance to 0.25mM levamisole, but not o 0.5mM levamisole.

In parallel with the levamisole assays, we have used aldicarb (ChemService) with the same goal. Aldicarb is an antagonist of acetylcholine-esterase (AChE), the enzyme that metabolizes ACh from synaptic cleft [88]. Under normal physiological situations, AChE removes the neurotransmitter and prevents its accumulation in the synapse after the signal is received by the muscle membrane. Aldicarb binds to the enzyme, thus preventing it from degrading ACh, which then over-accumulates in the synaptic cleft and causes toxicity, paralysis, and death. Many mutants with presynaptic (neuronal) and postsynaptic receptor defects exhibit resistance to aldicarb.

With the assumption that agrin might play a role in either of these NMJ aspects, we treated *agr-1(eg1770)* mutants with aldicarb and scored their viability (see Appendix - Experimental procedures). In addition to the single agrin mutation alone, we tested agrin triple mutants with dystrophin *dys-1(cx18)* and dystrobrevin *dyb-1(cx36)*, mutants that alone display hypersensitivity to ACh and aldicarb [147] [148]. Wild type and *agr-1(eg1770)* single mutant worms showed the same time-dependent sensitivity to aldicarb (Fig. 21). The dystrophin, dystrobrevin double mutant showed the expected hypersensitivity to the drug, while in combination with the agrin mutation the animals seemed more resistant. This result showed that absence of agrin “rescued” *dys-1 dyb-1* hypersensitivity, but due to the high standard deviations, it could not be concluded that AGR-1 definitely has a NMJ function. The experiment would have to be repeated on a larger sample by the genotype-blinded experimenter to avoid bias.

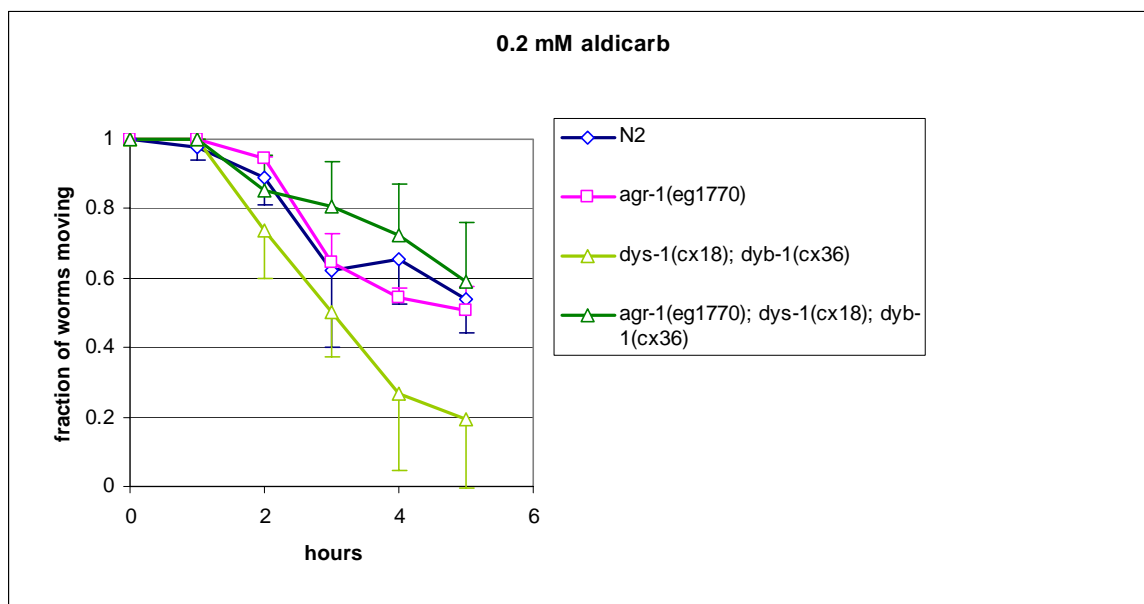


Fig 21. *In vivo* pharmacological assays on *C. elegans* with the AChE inhibitor aldicarb. Wild type, *agr-1(eg1770)*, *dys-1(cx18) dyb-1(cx36)* double mutants, and *dys-1(cx18) dyb-1(cx36); agr-1(eg1770)* triple mutants were tested on 0.2mM aldicarb. Agrin loss rescues aldicarb hypersensitivity of the *dys-1(cx18) dyb-1(cx36)* double mutants.

III.2.4.1.B. Investigating possible agrin genetic interactions with acetylcholine esterases (AChE)

In addition to pharmacological experiments towards elucidating possible agrin function at the NMJ, we undertook a genetic approach with the same goal. Since agrin mutants on their own did not display an obvious phenotype, we investigated possible genetic interactions in sensitized backgrounds. We chose to work with acetylcholine esterase (AChE) mutants. AChEs metabolize ACh at the synapse. There are four AChE genes in *C. elegans* but *ace-1* and *ace-2* exhibit 95% of the total esterase activity [179]. The *ace-1*; *ace-2* double mutant has an obvious uncoordinated phenotype due to the accumulation of ACh at the NMJs and concomitant toxicity. Our working hypothesis was the following: if agrin in *C. elegans* is involved in AChRs clustering in the worm, when mutated in the *ace-1*; *ace-2* background, it should rescue their *unc* phenotype, by reducing toxic signaling caused by the elevated ACh levels (Fig. 22).

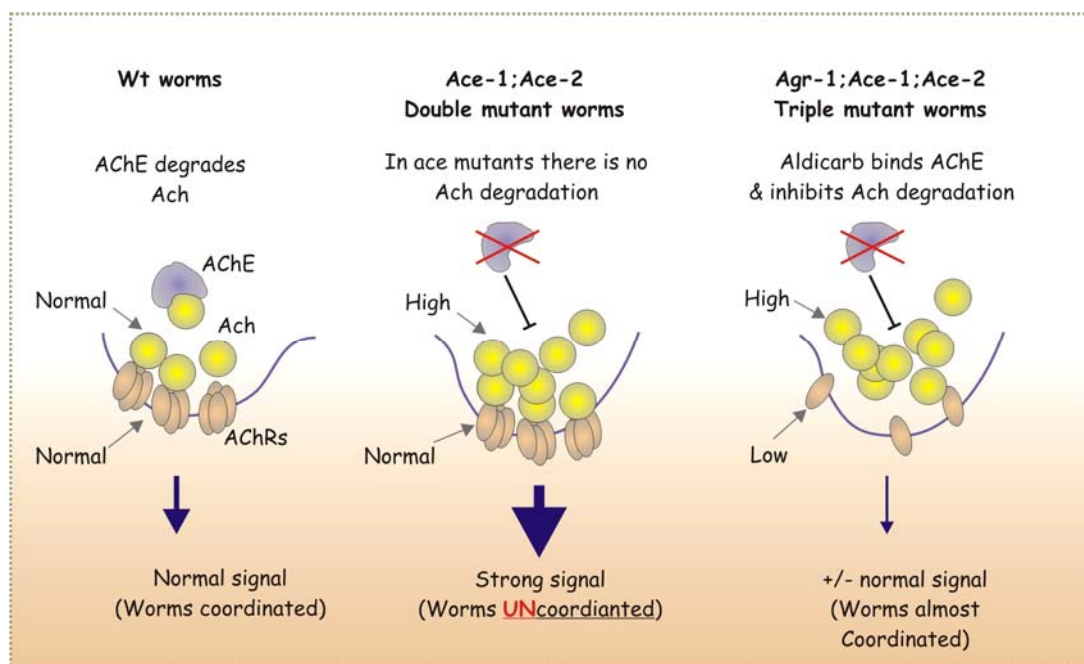


Fig 22. Schematic model of our working hypothesis that served as basis for genetic experiments with *agr-1* and *ace-1*; *ace-2* mutants. In case agrin in the worm had a function at the NMJ, we would expect it to rescue the *unc* phenotype of *ace-1*; *ace-2* mutants.

We crossed the *agr-1(eg1770)* mutant with the double mutant for the AChE genes *ace-1(p1000)*; *ace-2(g72)*, thus creating a triple mutant for three genes possibly interacting at the synapse. Cross progeny of the first generation (F₁) was heterozygous for all genes and the following generations had to be genotyped. Since the mutants carried different types of mutations, we applied different genotyping methods (Fig. 23), e.g. *ace-1*

had a point mutation which altered an existing restriction site and could be detected by restriction analysis of the PCR product that included the mutated nucleotide. As a result, mutant DNA subjected to *BsII* digestion was not cleaved while the wild type was cut in two fragments (Fig 24). The worm cloning procedure, lysis, and PCR are described in detail in the Appendix of the Thesis.

gene	Type of mutation	Screening
<i>Agr-1</i> (<i>Eg1770</i>)	Mos Insertion	PCR
<i>Ace-1</i> (<i>P1000</i>)	Point mutation	PCR + <i>BsII</i> digestion
<i>Ace-2</i> (<i>G72</i>)	Point mutation	PCR
<i>Ace-3</i>	Deletion	PCR
<i>Ace-4</i>	Deletion	
<i>Ace-1 ; Ace-2</i>		uncoordinated
<i>Ace-1 ; Ace-2 ; Ace-3</i>		lethal

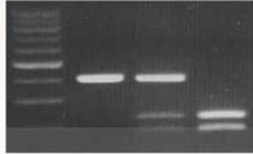
	0/0 0/+ +/+
Genotyping for <i>Ace-1</i>	

Fig 23. Mutants in *agr-1* and *ace* genes carry different types of mutations and therefore require specific screening approaches. The agarose gel in the lower panel shows, as an example, the controls for *ace-1* gene screening by PCR followed by digestion with *BsII*.

Since double mutants *ace-1;ace-2* had an *unc* phenotype, they could easily be identified in the progeny. *Unc* and non-*unc* animals of the third generation (F_3) were cloned and after having laid eggs the hermaphrodite parents were analysed by PCR for all three genes (Fig. 24). In case of *ace-1*, PCR was followed by *BsII* digestion and for *ace-2* by sequencing to confirm the point mutations. *Agr-1*(*eg1770*) was screened by PCR that distinguished between the wt and the mutant allele carrying the insertion of the Mos transposon. The screening procedures are described in the Appendix. All F_3 lines of *unc* animals were confirmed to be *agr-1*; *ace-1*; *ace2* homozygous mutants (Fig. 24A). Out of 9 tested non-*unc* animals, seven were heterozygous for both *ace-1* and *ace-2*, two were homozygous mutant for *agr-1* and *ace-1*, but the sequencing showed that these last two animals were heterozygous for *ace-2* (Fig. 24B, lines 2 & 4). The fact that the agrin mutation did not rescue the *unc* phenotype of the *ace-1*; *ace-2* worms suggests that agrin does not have an obvious function in clustering AChRs at NMJ.

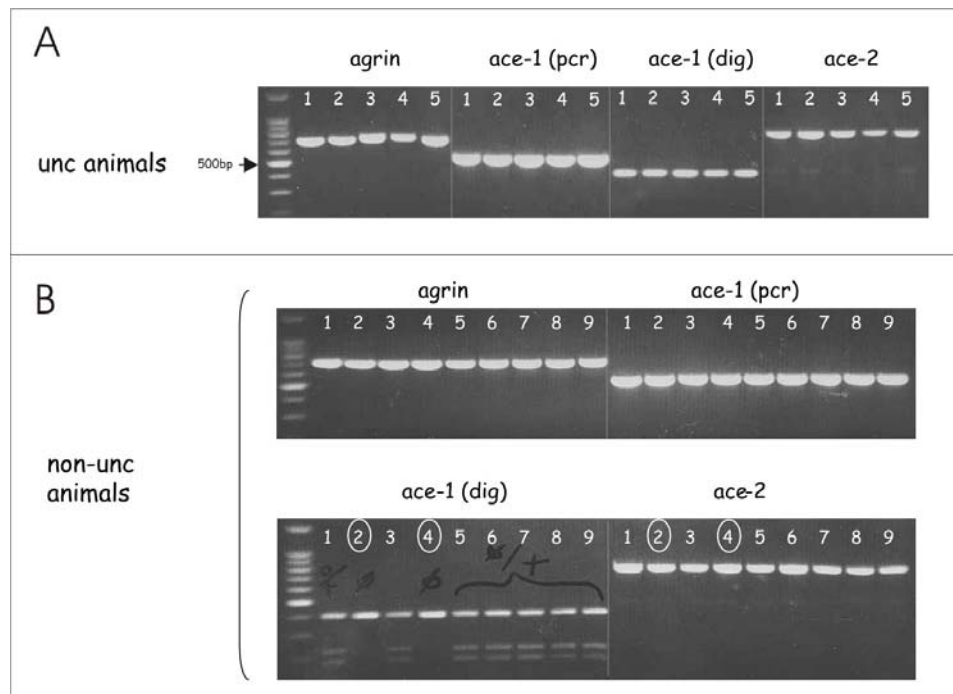


Fig 24. Genotyping of mutant uncoordinated (*unc*) and coordinated (*non-unc*) worms in the *agr-1* cross with *ace-1;ace-2*. Uncoordinated (*unc*) animals were homozygous mutants for all the genes, but *non-unc* were either homozygous mutants (0/0) for *ace-1* and heterozygotes (0/+) for *ace-2* (lines 2 & 4), or heterozygous for both *ace-1* and *ace-2*.

III.2.4.2. Analysis of putative agrin function in *C. elegans* muscle

III.2.4.2.A. Genetic approach to test possible agrin interactions with components of the dystrophin-glycoprotein complex (DGC)

In vertebrates agrin expressed by skeletal muscles and binds to α -dystroglycan as part of a large dystrophin glycoprotein complex (DGC), reviewed in [13]. It was shown that an agrin mini-gene consisting of the secretion signal, laminin-binding NtA domain, first follistatin-like domain, and the C-terminal three lamG domains, can rescue the dystrophic phenotype of laminin α 2-deficient mice by linking basement membrane to α -dystroglycan [66]. Based on the described function in vertebrates, we considered potentially similar role in *C. elegans*. Despite the fact that we did not detect any agrin expression in the body wall muscles, neither by reporter fusion analysis nor by immunofluorescence, undetectable levels could still be present and interact with other DGC components.

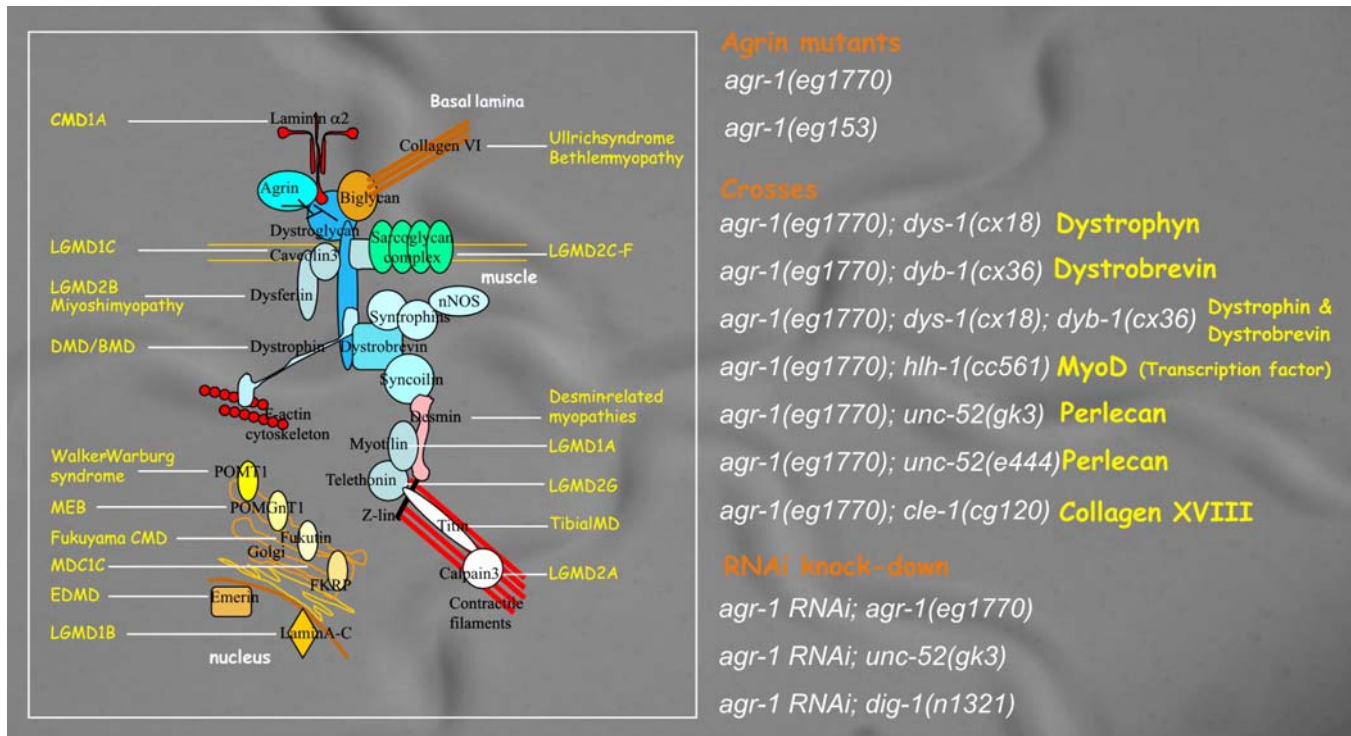


Fig 25. Schematic representation of the dystrophin-glycoprotein complex (DGC) and related proteins. Many muscular dystrophies are caused by mutations in these proteins (left panel). The right panel lists all the mutants and RNAi experiments we have used to investigate possible genetic interactions between agrin and known muscle-related proteins involved in providing muscular integrity.

We have created many double mutants with agrin and different components of the DGC (Fig 25). In addition, we knocked down agrin, by double stranded RNAi (dsRNAi), in perlecan *unc-52(gk3)*, *dig-1(n1321)*(dig = displaced gonad), and agrin mutant backgrounds *agr-1(eg1770)*. The procedure of dsRNA production and the “knock-down” experiment are explained in the Appendix.

Our rationale was that, if the tested genes had functional interactions, the combined loss would cause stronger defects. Therefore, we investigated possible morphological or behavioral defects, in double mutants and the dsRNA treated “knock-down” worms. We looked at muscle integrity by anti-myosin immunostaining, observed locomotion and viability, but could not detect any aggravated defects when compared to the single-gene mutants. Therefore, we could conclude that *C. elegans* agrin does not play a role in muscle function and stability.

III.2.4.2.B. Addressing possible genetic interaction between agrin and dystroglycan in *C. elegans*

In *C. elegans* three genes encode dystroglycan-like proteins: *dgn-1*, *dgn-2*, and *dgn-3* [158]. In terms of sequence and structural organization, *dgn-1* shares the highest similarity to the vertebrate protein. Nevertheless, there are significant differences to the vertebrates: firstly, *dgn-1* is not post-translationally cleaved into α and β subunits and the expression pattern seems to be quite different. No *dgn-1* expression was detected in body wall muscles. *Dgn-2* and *dgn-3* are expressed in a few neural and epithelial cells and null mutants in these genes do not show significant defects (James Kramer, unpublished results). The *dgn-1* gene is mostly expressed in the epithelia and some neurons and the mutant is sterile due to severe defects in gonad development.

We have previously demonstrated that a recombinant fragment of *C. elegans* agrin can bind to vertebrate dystroglycan (see section III.1. Submitted manuscript). Based on this result we decided to investigate possible genetic interaction between the two proteins. We crossed the *agr-1(eg1770)* mutant with either the triple mutant *dgn-1(cg121) dgn-2(ok209) dgn-3(tm1092)* or with the double mutant *dgn-2(ok209) dgn-3(tm1092)*, kindly provided to us by Prof. James Kramer, Northwestern University, Chicago. Since the triple mutant *dgn-1(cg121) dgn-2(ok209) dgn-3(tm1092)* is sterile, it was balanced by a wild type copy of the gene on an extrachromosomal array *cgEx308[dgn1(+), dgn-1::GFP, pRF4]* together with copies of *gfp* expressed under the control of the *dgn-1* promoter, and the *rol-6^{mut}* gene (pRF4) that causes a roller phenotype.

The strategy for the crosses is presented in the Fig 26 and Fig 29. At the beginning of both crosses *agr-1(eg1770)* males were crossed with *dgn* mutant hermaphrodites. Several worms from the progeny of each generation were cloned, let to lay eggs, and genotyped by PCR. The detailed protocols are found in the Appendix.

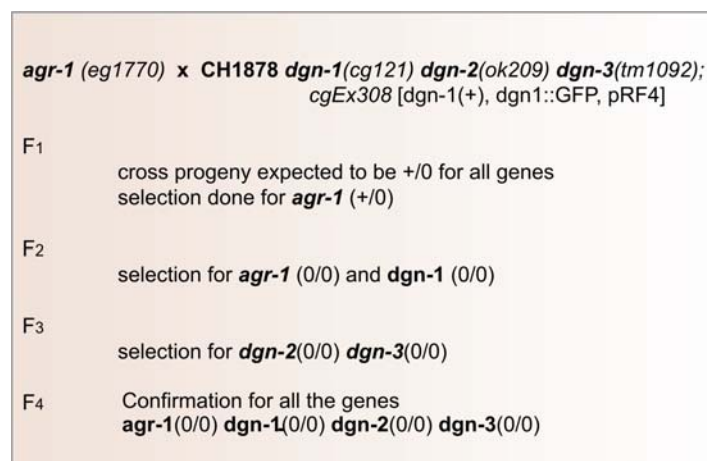


Fig 26. Scheme showing the strategy for the isolation of the quadruple mutant *agr-1(eg1770); dgn-1(cg121) dgn-2(ok209) dgn-3(tm1092)*.

Since the triple mutant *dgn-1(cg121) dgn-2(ok209) dgn-3(tm1092)* had to be rescued by a wild type *dgn-1* copy of the gene on an extrachromosomal array, the wild type gene was always amplified by PCR, therefore adding to the screening complexity. Since only the rescued animals (carrying the extrachromosomal array) could have progeny, we had to select them and clone the GFP-expressing rollers. The *dgn-1* deletion allele could be detected as a shorter band by PCR, but a large band, due to the wild type copy of *dgn-1* from the rescuing array, was always present on the agarose gel, making it impossible to distinguish between heterozygous animals and homozygous mutants which have the array. The final mutant lines were genotyped for all four genes (Fig. 27), and their non-rescued (non-green, non-rolling) progeny analysed for the *dgn-1* gene to avoid the confusion with extrachromosomal array (Fig. 28).

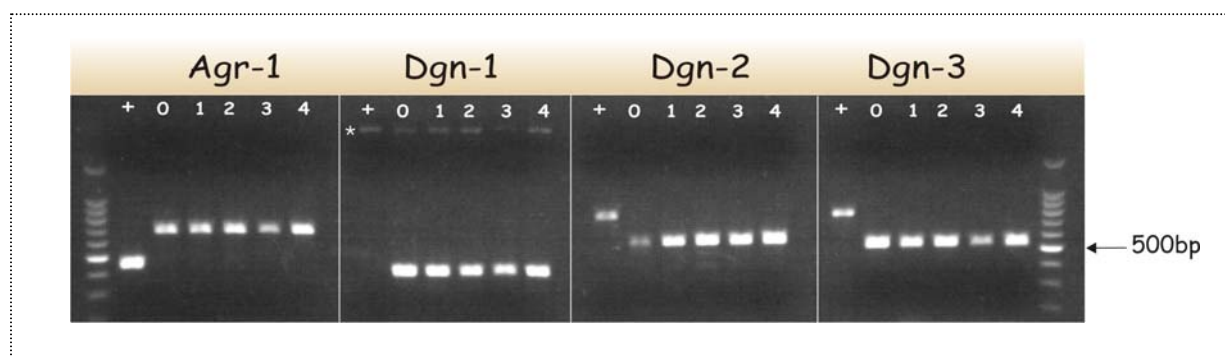


Fig 27. Final confirmation of the quadruple mutants *agr-1(eg1770); dgn-1(cg121) dgn-2(ok209) dgn-3(tm1092)*. “+” is the wild type control, “0” is the mutant positive control, and 1-4 are four independent lines of quadruple mutants. Asterisk denotes the wild type *dgn-1* band of almost 3000 bp. (Lanes with the same numbers correspond to the same worm lysed and genotyped)

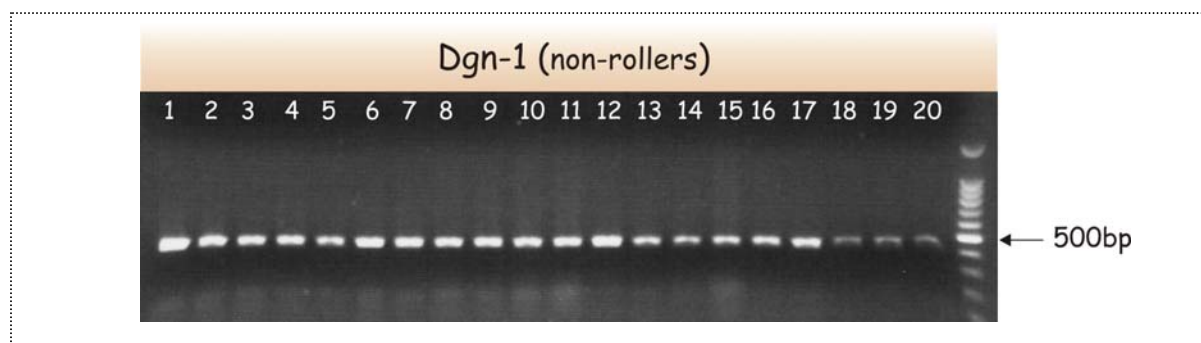


Fig 28. PCR of 20 cloned non-rolling worms *agr-1(eg1770); dgn-1(cg121) dgn-2(ok209) dgn-3(tm1092)*. None of the worms without extrachromosomal array had a wild type copy of the gene therefore they were all homozygous mutants for *dgn-1(cg121)*.

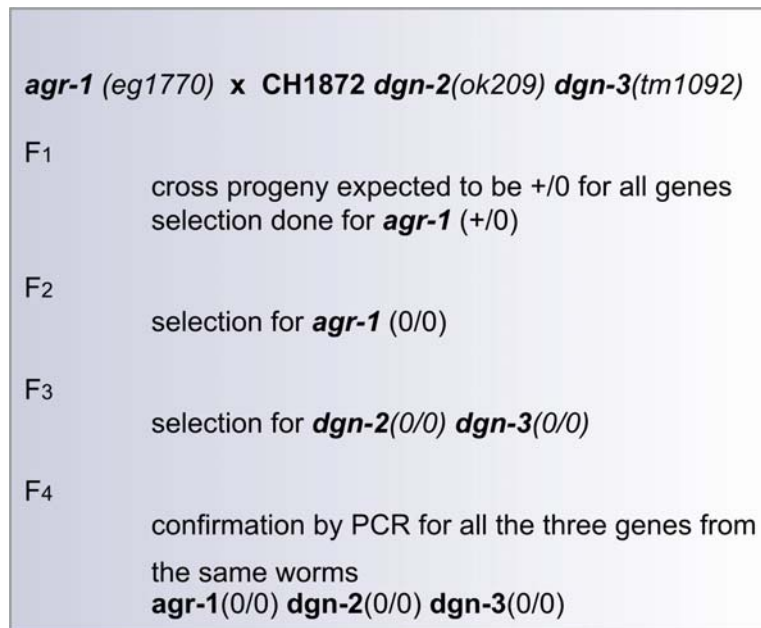


Fig 29. Scheme of the genotyping strategy for the *agr-1*(*eg1770*) cross with *dgn-2*(*ok209*) *dgn-3*(*tm1092*)

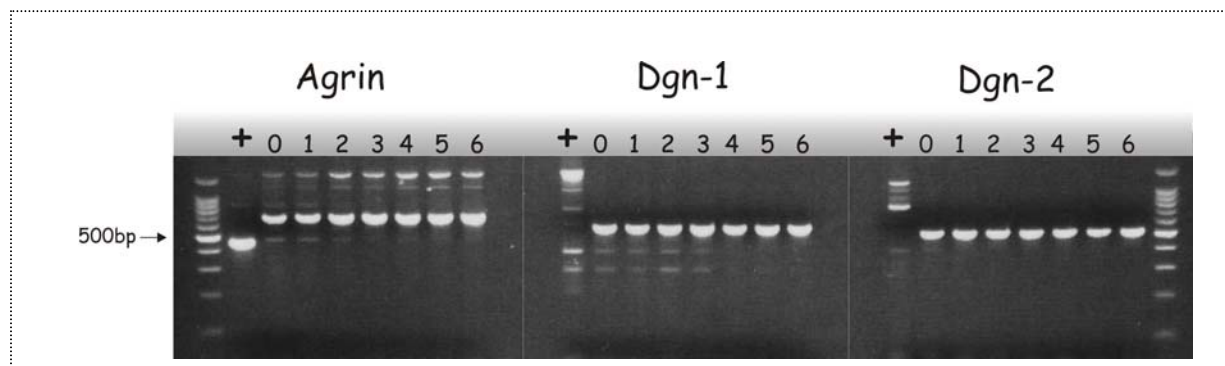


Fig 30. Final confirmation of the triple mutants *agr-1*(*eg1770*); *dgn-2*(*ok209*) *dgn-3*(*tm1092*). “+” is the wild type control, “0” is mutant positive control, and 1-6 are six independent lines of triple mutants.

The successful cross between *agr-1*(*eg1770*) and *dgn-2*(*ok209*) *dgn-3*(*tm1092*) was confirmed by PCR for all the three genes from the same worms (Fig. 30). *Dgn-2* and *dgn-3* are primarily expressed in the pharynx of the worms, but without any significant impairment in the mutants (J. Kramer, unpublished data). We expected that the triple mutant with *agr-1* might cause some visible defects in pharynx morphology or

function, in case they genetically interact. To our disappointment no obvious phenotype could be observed in the triple mutant strain.

III.2.4.3. Testing a possible role of agrin in pharynx-related functions

III.2.4.3.A. Pumping phenotype

The pharynx is a muscular pump important for ingesting food, and transporting it to the gut of the worm. Based on specific agrin immunostaining in the basal lamina of the pharynx, which was selectively absent in the agrin mutants, we hypothesized that agrin could have a pharynx-related function.

We chose to look at the pharynx pumping-rate and morphology and to follow them through the aging process (young to old adult worms) to see whether differences could be detected between the wild type and the agrin mutants *eg1770*, *eg153* and *tm2051* (Fig. 31) [120]. Based on a recent report [171], we expected that if agrin mutant had a lower pumping rate, pharyngeal morphology would be better preserved as the worms get older. In our experiments we did not observe any significant difference neither in the pumping rate (Fig. 30) nor in pharynx morphology (results not shown) between the wild type and the mutant worms.

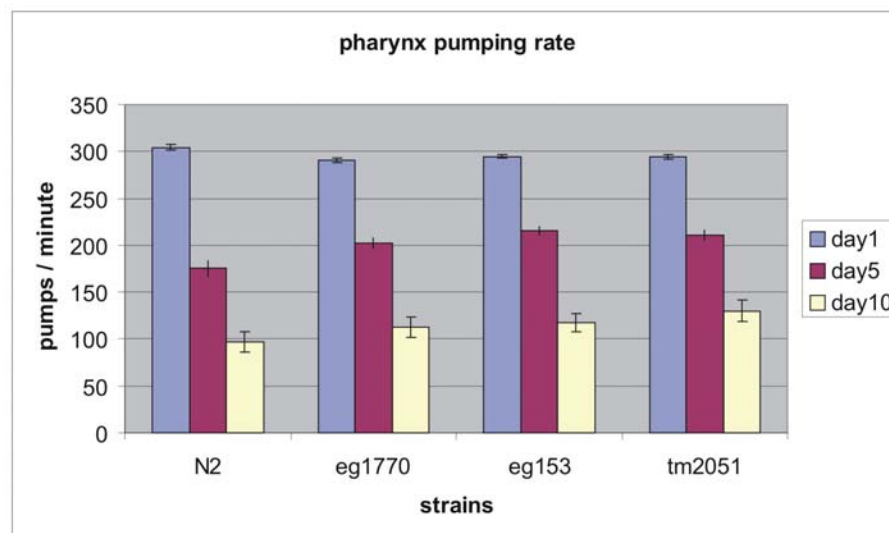


Fig 31. Pharyngeal pumping experiment. The wild type worms (N₂ strain) and three agrin mutants (*eg1770*, *eg153* and *tm2051*) (n=50) were followed during 10 days and the pumping-rate was measured at 5 day-intervals.

III.2.4.3.B. Feeding phenotype – large bacteria

It was reported previously that large-sized bacterial strains are eaten less efficiently by worms [120]. Therefore, we tested whether agrin mutants showed any difference in growth rate when fed with the large-sized bacterial strain *Bacillus megatherium* as compared to the *E. coli* strain commonly used for feeding (Fig. 32). The *Bacillus megatherium* strain was kindly provided to us by the Bacillus Genetic Stock Centre, Dept. of Biochemistry, Ohio State University.

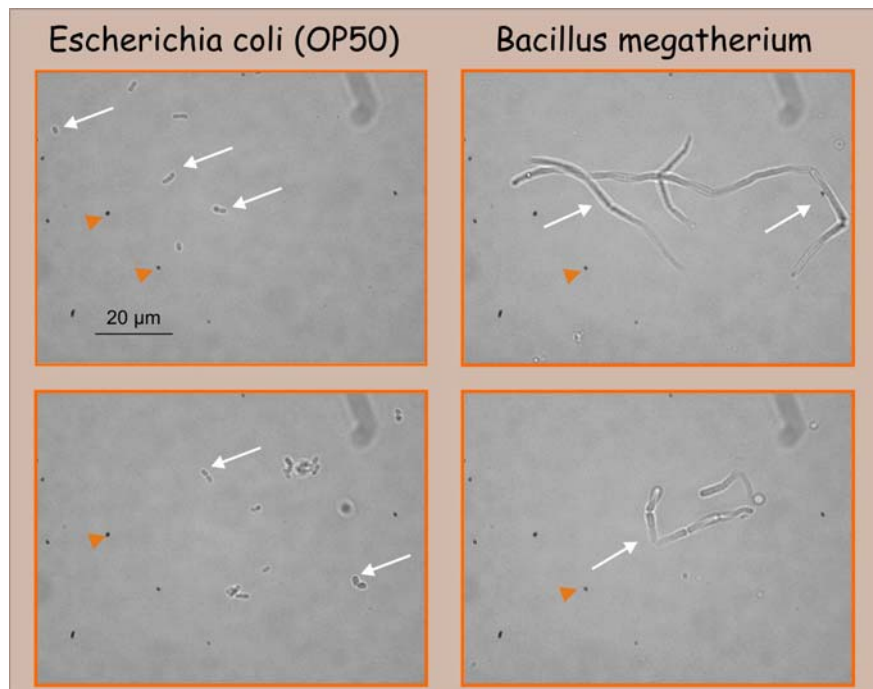


Fig 32. Bacterial strains used for *C. elegans* feeding experiment. White arrows show the bacteria and orange arrowheads point to the impurities on the specimen.

The experiment was done following the protocol in the publication by Avery et al [120]. Starved synchronized L1 worms were grown on lawns of the two bacterial strains at 18°C and observed every 1-3 hours until approximately 50% of the animals reached the adult stage. The inverse of time (in days) it took hermaphrodites to become adults was plotted as growth rate (Fig. 33). Adults were recognized by the appearance of a mature vulva, and by the production of eggs.

There was no significant difference in growth rates of agrin mutants fed on the two different bacterial strains, except for a slight difference between *agr-1(eg1770)* and wild type on the *E. coli*. Worm strains *eat-4(ky5)* and *eat-5(ad464)* served as positive control and showed greater sensitivity to large the bacterial strain as observed by their slower growth rate.

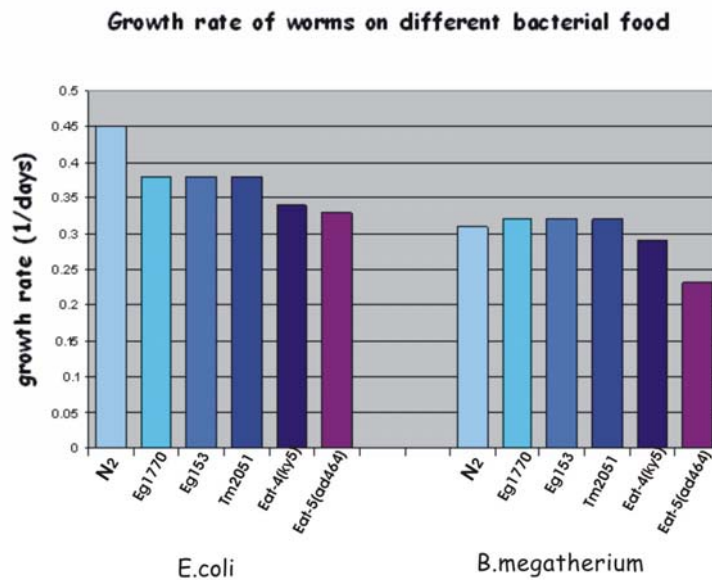


Fig 33. Growth rate of worms fed on two different bacterial strains: *E. coli* or *B. megatherium*. N2 is the wild type; *eg1770*, *eg153*, and *tm2051* are agrin mutants; *eat-4(ky5)* and *eat-5(ad464)* are strains that served as positive control.

III.2.4.4. Search for phenotypes related to IL1 head neurons

We found agrin expression in the worm only in four head neurons and some pharyngeal cells, by reporter gene fusions. The four neurons were identified as the mechanosensory IL1 neurons, based on their structure, also suggested to carry out interneuron and motor neuron functions [98], (Driscoll M & Kaplan J, chapter in [88]). There are in total six IL1 neurons, distributed in three pairs: dorsal, lateral, and ventral [93], but, interestingly, agrin seems to be expressed only in the dorsal and ventral pairs. IL1 neurons mediate an aversive head-withdrawal reflex when worms are touched on either the dorsal or ventral sides of their nose [98]. The laboratory experiment is usually done with an eyelash and is commonly called the “eyelash test” [88]. We tried to set up the “eyelash test” in our laboratory, but touching the small area of the nose of a 1mm-long moving worm under the dissecting microscope turned out to be too difficult and not satisfactorily reproducible.

To investigate whether agrin has a function in IL1 neuron-mediated mechanosensation, we chose instead to perform a technically easier “nose touch” experiment. The experiment was done by putting an eyelash on the worms crawling path and scoring their avoidance of the obstacle when they collided with it. Agrin mutants *agr-1(eg1770)* were compared to wild type worms as a positive control. As negative controls we tested *che-3(p801)* mutants that have impaired perception of the light nose touch due to a mutation in dynein

heavy chain (DHC), 1b isoform, that affects the structural integrity of sensory cilia. In order to avoid the bias, the experimenter did not know the genotype of the tested strains during the experiment. Agrin mutant strains *agr-1(eg1770)* and *agr-1(tm2051)* were as sensitive to the stimulus as the wild type worms (Fig. 34), but the standard deviations were very high, probably due to the high degree of experimental mistakes.

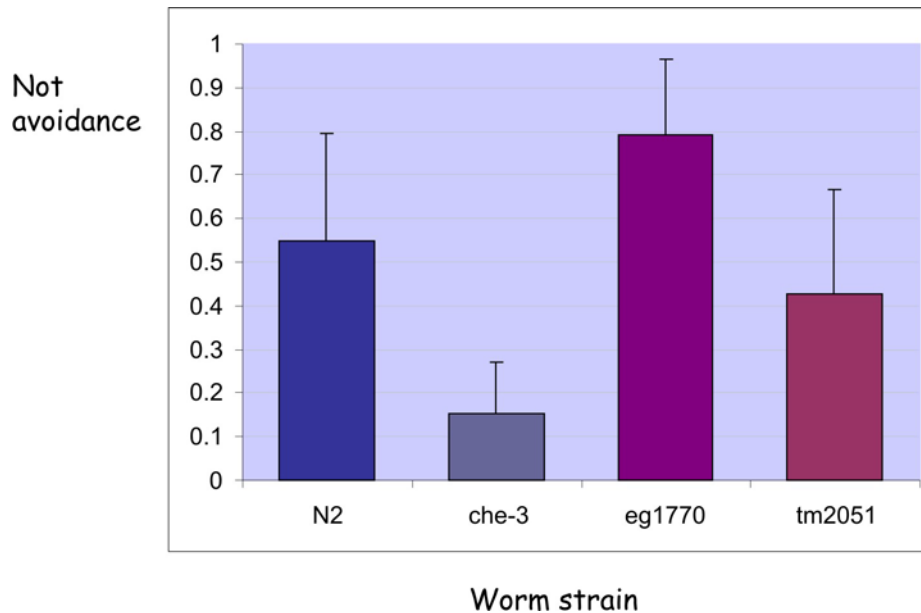


Fig 34. Nose-touch (Not) avoidance scored by the “nose touch” test.

We were as well interested to see if loss of agrin had an impact on the morphology of IL1 neurons. With this goal we created transgenic strains expressing the YFP reporter under the control of the agrin promoter (*Ex[agr-1::YFP]*) in *agr-1(eg1770)* mutants (Fig. 35). Transgenic lines were analyzed by confocal microscopy (with falsely-coloured YFP as green) and did not display any noticeable dendritic defects.

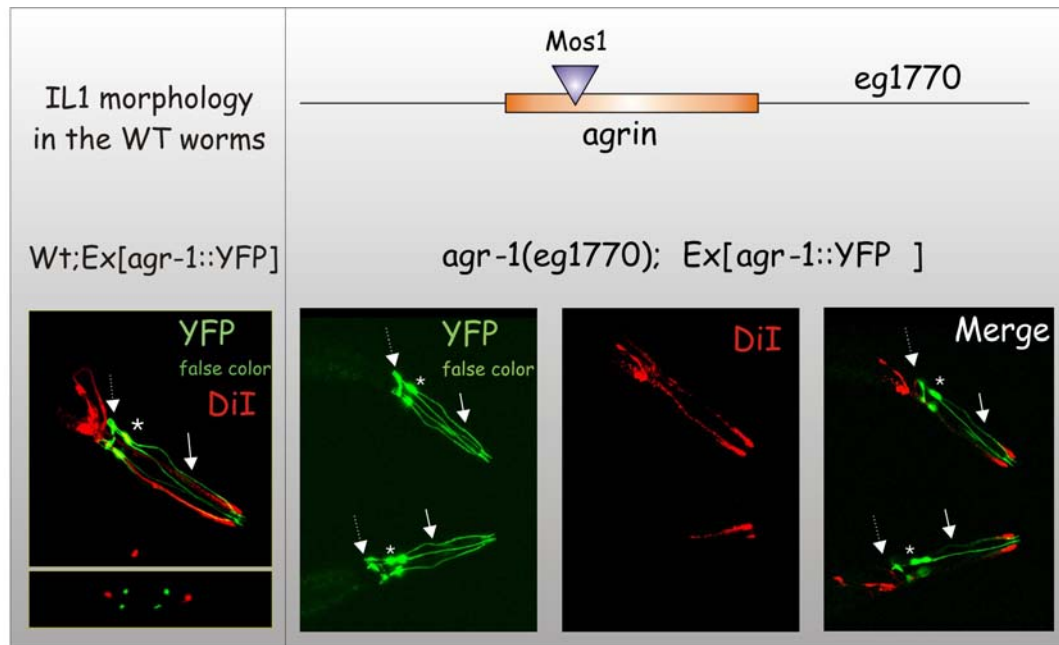


Fig 35. Morphology of IL1 neurons in the wild type and the *agr-1(eg1770)* worms. Mos1 transposon is inserted in the agrin ORF. Full-line arrows point to the dendrites of IL1 neurons, dashed arrows show the axons in the nerve ring, and asterisks label the cell bodies.

III.2.4.5. Genetic approach to test possible agrin interactions with different signaling pathways involved in gonad development

Gonad development in *C. elegans* involves a precise combination of multiple guidance and signaling systems [88]. Distal tip cells (DTCs) are the “leading edge” of the developing gonad which sense environmental cues for proper pathfinding and thereby determine the gonad morphology. Impairment in several signaling mechanisms causes defective gonad migration which is easily visible under a microscope. The observation that agrin is expressed in the DTCs at the time of migration (see Submitted manuscript), prompted us investigate whether it may play an active role in this process.

We took advantage of the powerful *C. elegans* model system for genetic studies. We investigated whether agrin has a significant impact on gonad migration and whether it can modulate the signaling pathways previously shown to play a role in this process. The approach and the strains used are based on a publication by the Culotti’s lab [132]. All strains that we crossed with our *agr-1(eg1770)* strain were obtained from the Caenorhabditis Genetics Centre (CGC). The gonads were analyzed in the single mutant background and in double mutants. Defects were classified according to the morphology of the anterior and posterior arms, as

dorsalization, ventralization and various severe defects (Table 1). All defects were expressed as % of the total number of the gonads scored.

Table 1. Gonad migration defects caused by mutations in several signaling pathways in combination with *agr-1(eg1770)*. The most significant differences, due to agrin mutation in various backgrounds, are framed in red.

		anterior arm				posterior arm				
		Only Prominent defects	Dorsalization	Ventralization	Antero-Posterior migration defects	Only prominent defects	Dorsalization	Ventralization	Antero-Posterior Migration defects	n
Wnt	<i>agr-1(eg1770)</i>	6.5	0.9	4.7	0.0	3.7	0.0	2.8	0.9	107
	<i>egl-20(mu39)</i>	9.1	3.0	1.5	0.0	12.1	3.0	4.5	3.0	66
	<i>agr-1(eg1770); egl-20(mu39)</i>	10.5	3.9	6.6	0.0	2.6	0.0	7.9	0.0	76
TGF β	<i>unc-129(ev554)</i>	1.5	0.0	3.0	0.0	7.5	4.5	7.5	0.0	67
	<i>agr-1(eg1770); unc-129(ev554)</i>	1.6	0.0	1.6	0.0	3.2	1.6	6.5	0.0	62
FGF	<i>egl-17(e1313)</i>	11.8	0.0	3.9	9.8	3.9	0.0	7.8	0.0	51
	<i>agr-1(eg1770); egl-17(e1313)</i>	5.9	0.0	3.9	0.0	7.8	3.9	13.7	0.0	51
	<i>clr-1(e1745)</i>	4.5	0.0	1.5	1.5	6.0	1.5	0.0	1.5	67
	<i>agr-1(eg1770); clr-1(e1745)</i>	5.3	1.1	2.1	1.1	2.1	0.0	2.1	0.0	94
netrin	<i>unc-5(e152)</i>	16.0	2.0	18.0	0.0	52.0	0.0	58.0	0.0	50
	<i>agr-1(eg1770); unc-5(e152)</i>	11.6	0.8	9.1	0.0	49.6	0.8	49.6	0.0	121
Basement membrane	<i>cle-1(cg120)</i>	5.9	1.5	7.4	0.0	10.3	4.4	4.4	0.0	68
	<i>agr-1(eg1770); cle-1(cg120)</i>	11.1	3.9	4.6	0.0	9.8	2.6	6.5	0.7	153
Basement membrane	<i>nid-1(cg119)</i>	8.0	2.0	2.0	0.0	14.0	0.0	2.0	0.0	50
	<i>agr-1(eg1770); nid-1(cg119)</i>	5.2	1.7	5.2	0.0	0.0	0.0	3.4	0.0	58
	<i>unc-52(gk3)</i>	9.1	1.5	9.1	0.0	1.5	3.0	7.6	0.0	66
	<i>agr-1(eg1770); unc-52(gk3)</i>	0.0	1.9	1.9	0.0	3.7	3.7	0.0	0.0	54
	<i>unc-52(e1421)</i>	0.0	0.0	0.0	0.0	0.0	0.0	4.2	0.0	48
	<i>agr-1(eg1770); unc-52(e1421)</i>	1.1	1.1	0.0	0.0	3.4	1.1	0.0	2.3	87

All defects are expressed as % of the total number of worms analysed

We never observed differences higher than 15% between the single and the double mutants. Furthermore, in most cases where differences were observed the double mutants showed less defects. Since in this kind of experiment high variations are normal and a 15% difference is not considered significant [132]. Therefore, the results suggest that agrin does not play a major role in gonad migration in the worm.

III.2.4.6. Axon guidance experiments

[These experiments were done in collaboration with Dr. Christa Rhiner and Prof. Michael Hengartner, University of Zürich]

Proteins expressed in the basement membrane of the hypodermal ridge are known to have axon guidance properties [180]. Based on our detection of agrin in the hypodermal ridge by immunofluorescence, we investigated potential agrin involvement in axon guidance in *C. elegans*, in collaboration with Christa Rhiner from Michael Hengartner's lab in Zürich. Hengartner and collaborators have previously shown that the heparan sulphate proteoglycan syndecan plays a role in axon guidance in the worm [101]. They investigated transgenic worms expressing fluorescent markers in distinct subclasses of neurons. DD neurons are motoneurons and their cell bodies are distributed along the ventral nerve cord. In normal development the axons of DD neurons grow circumferentially and reach the dorsal nerve cord where they synapse to their targets. In syndecan *sdn-1(zh20)* mutants DD commissures are often defective. When the *sdn-1* mutation is present in the genetic background of mutants for heparan sulphate synthesis (*hse-5(tm472)* and *hst-6(ok273)*), the DD defects occur at much higher rate. This finding suggested that deficiency of another heparan sulphate proteoglycan might be responsible for it and agrin was a possible candidate.

We investigated transgenic strains expressing GFP in DD neurons (OxIs12 array) and examined young larvae for DD commissural defects. Of the six commissures, which form during embryonic development, the first commissure migrates circumferentially on the left side, the other five on the right, and can relatively easily be traced. The results are presented in the Fig. 36. The agrin single mutants (either *agr-1(eg1770)* or *agr-1(tm2051)*) did not show significant defects and an *agr-1(eg1770); sdn-1(zh20)* double mutants did not show a stronger phenotype than the *sdn-1(zh20)* single mutant, thus suggesting that agrin is not involved in commissural axon guidance.

Hengartner and collaborators scored other neuron-types for defects caused by *sdn-1* mutations [101]. Axons of the PVQ tail neurons in wild type animals run on both sides of the hypodermal ridge along the ventral midline of the worm (PVQL and PVQR, on the left and right side, respectively). Normally, they do not cross over, but in *sdn-1* mutants left and right fasciculation is lost and the axons do cross over the midline. We were interested to test whether the agrin mutation has an overt impact on the rate of the PVQ defects when present in a *sdn-1* mutant background. No significant difference could be observed neither in the *agr-1* single mutant nor in the double mutant *agr-1(tm2051); sdn-1(zh20)* (Fig. 37).

Based on the results of these neuron migration and pathfinding experiments, we could conclude that agrin does not have an important role in axon guidance in *C. elegans*.

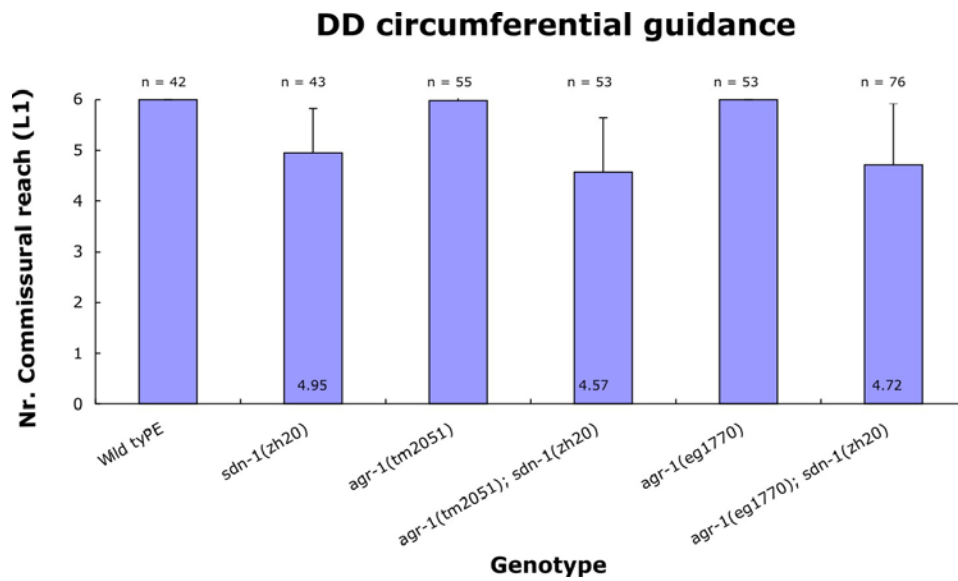


Fig 36. DD neuron circumferential guidance is not impaired due to agrin mutations. Number of successful commissural reach was scored in different mutant strains. Agrin mutation in *sdn-1* background does not cause reduction in commissural reach.

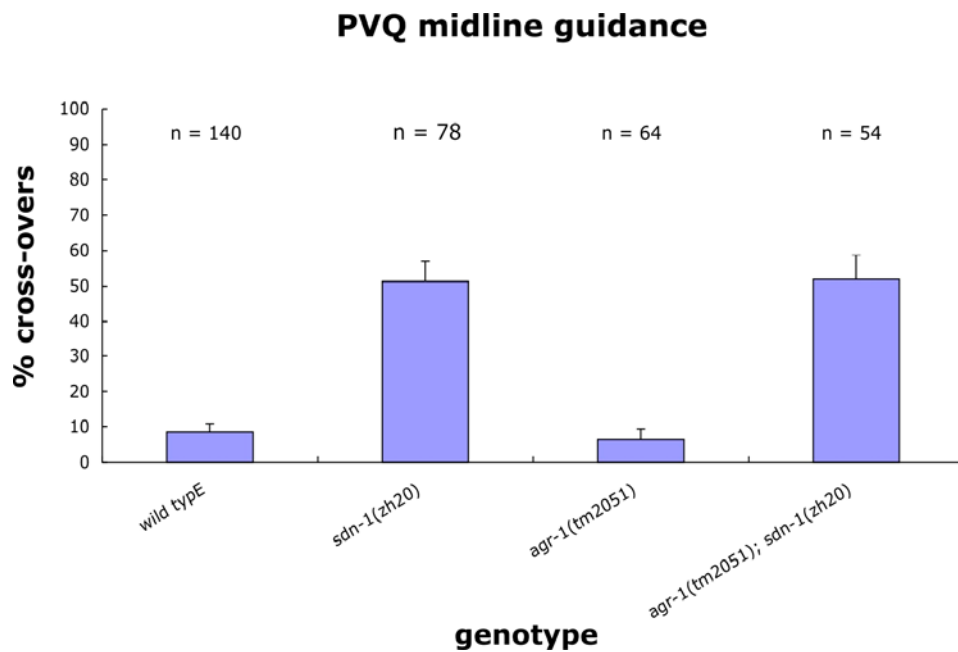


Fig 37. PVQ neuron guidance is not influenced by agrin mutation. Percent of PVQ cross-over was scored for agrin and syndecan mutants. No significant defect is caused by *agr-1*, either alone or with *sdn-1*.

IV. Discussion

The work presented in this thesis was done during four years, in the joint effort and true team work with several remarkable people: Dr. Stefano Canevascini and Gordon Lau under the supervision by Dr. Ruth Chiquet-Ehrismann. This discussion, therefore, refers to “our”, not “my” results.

IV.1. Our quest

We have decided to work on agrin in *C. elegans* with the goal to clone the gene and characterize this protein in the invertebrate model organism. Since a lot was already known about the functions of agrin in vertebrates, but nothing in simpler organisms, we aimed to learn more about this important protein and position it in the big picture through an evolutionary perspective.

Agtrin is crucial for the assembly and maintenance of the neuromuscular junction (NMJ) in vertebrates, (reviewed in [13,140]). Its null mutation in mice is lethal [38], but in man no known genetic disease has been assigned to any mutation in the agrin gene. The reason might be that agrin is essential already during very early embryonic development. Interestingly, despite more than two decades of extensive search for the direct signaling downstream of agrin, only some molecular players and fragments of the pathways have been elucidated so far. It is clear that agrin activates the muscle receptor tyrosine kinase MuSK, but only indirectly, through binding to a yet unidentified receptor. According to some speculations, the “hypothetical receptor” might as well be a modification of MuSK, its dimerization, or even a bigger complex with more components involved, reviewed in [13]. This big “question mark” in the studies of vertebrate agrin certainly was a part of our motivation for studying it in a simple model organism. We aimed to see if agrin function was conserved in *C. elegans* and to explore its great genetic potential to identify the missing links.

IV.2 The molecular structure of *C. elegans* agrin is significantly similar to the vertebrate homologue

We have cloned the *agr-1* cDNA on the basis of the prediction published by Dr. Harald Hutter [117] and the sequence from the Wormbase (<http://www.wormbase.org>), the online *C. elegans* database. Despite the original prediction of two open reading frames, we identified only one unique agrin sequence composed of the two predicted sequences. The size of the gene, comprising 4422 bp, was not surprising since all the known vertebrate orthologs are relatively large. In distinction to the vertebrate agrin, we could identify only one isoform containing a putative cleaved signal sequence, but without any potential domain similar to the laminin-binding NtA domain in vertebrates. Therefore worm agrin may not bind to laminin in the basement membrane (BM), or it could be binding to it through another domain. Other possibility is that it interacts with laminin indirectly, via other proteins. This, still open question, could be addressed in the future by

affinity studies with the N-terminal and central part of the molecule as “protein baits”. The overall protein structure of *C. elegans* agrin is relatively conserved compared to the vertebrates. The lack of serine-threonine (S/T) rich regions in the central part of the molecule could be affecting its glycosylation profile. Since in vertebrate agrin there are several confirmed attachment sites for the O-linked carbohydrate modifications, one would expect that *C. elegans* agrin does not carry such glycosaminoglycan chains (GAG) chains. Nevertheless, our analysis of the proteins in *C. elegans* lysates by western blotting, resulted in a band about the predicted size of agrin (160 kDa) plus a smear that could correspond to its glycosylation. Several potential attachment sites for N-linked glycosylation were identified by computer analysis of the protein sequence, but would have to be confirmed by the enzymatic treatment with specific deglycosylating enzymes.

Since in vertebrates the three lam G domains at the C-terminus play a very important part in the NMJ signaling [27], it was striking that *C. elegans* agrin actually has only two of them. BLAST searches with each of the domains separately indicated that the two AGR-1 lam G domains correspond to the second and the third domain of the vertebrate agrin homologues. We can postulate that the first lam G of vertebrate agrin was acquired later in evolution and brought a new interaction site to the molecule. Despite the fact that the AGR-1 lam G domains correspond to the vertebrate agrin lam G domains, no alternative splicing could be found at the conserved sites. Considering that the alternative splicing gives rise to the isoforms with significantly different AChRs clustering activity [23], we assumed that, if AGR-1 had an analogous function, the splicing inserts would be present. Contrary to the expected, we could neither identify any alternative splicing nor any potential exons in the genomic sequence in that region, that would encode the conserved amino acid inserts. We could conclude that AGR-1 exists in a single isoform, which probably corresponds to the vertebrate A₀B₀ splice variant. Another possibility, that can not be completely neglected, is that there might be another isoform, expressed at a significantly lower level, which was undetectable in the presence of the isoform that we identified.

IV.3. The *agr-1* expression pattern in the head neurons and pharyngeal epithelium was unexpected

Transgenic worm strains carrying reporter constructs, that encoded fluorescent proteins under varying portions of the *agr-1* promoter, revealed agrin expression in the buccal epithelium, IL1 head neurons, and the distal tip cells (DTCs) of developing gonads. To our surprise, no expression was detected in the body wall muscles or motoneurons innervating them, as would be expected by analogy to vertebrates. Nevertheless, we could not exclude the possibility that the expression level was under the detection threshold. The immunofluorescence data, with both monoclonal and polyclonal anti-agrin antibodies, supported the expression pattern analyzed by reporter genes. No staining was seen in the body wall muscles

and the motoneurons. Since the permeabilization and immunostaining of *C. elegans* often present substantial technical difficulties, we have used anti-myosin, anti-perlecan and anti-rim (pan-neuronal marker) antibodies as positive controls to verify the experimental success. AGR-1 was detected around the pharynx, presumably in its basement membrane, because the staining resembled the perlecan localization in that region. We propose that agrin is expressed by the epithelial cells and secreted and deposited in the pharyngeal basement membrane.

No immunostaining was detected in the four IL1 neurons. In case agrin is secreted from these cells, we would expect it to be localized in a small region at the tips of the dendrites (in the inner labial sensilla around worm's mouth) or at the axonal endings in the nerve ring. Such a low level of expression would require detection at a much better resolution, possibly even by electron microscopy. The IL1 neurons make synapses to the RMD motoneurons in the nerve ring, a neuropile around the worm's pharynx [93]. Additionally, the IL1 neuron were shown to synapse with RIP pharyngeal neurons, the only ones receiving extrapharyngeal input [93,119].

IV.4. Agrin function is dispensable for *C. elegans* under laboratory conditions

In order to investigate agrin function in the worm we analyzed several strains carrying mutations in the *agr-1* gene. Two mutants were molecular null because of the Mos1 transposon insertion (*eg1770*) and its imprecise excision (*eg153*) [170], while one strain had a short in-frame deletion of the exons 26 and 27 at the C-terminus (*tm2051*). Based on the well known function of agrin at the vertebrate NMJ, we expected that the *C. elegans agr-1* mutants would have locomotory defects due to defective NMJ synapses. Despite the unexpected expression pattern and immunostaining that never revealed any relation to body muscles and motoneurons, we studied typical functions that could be impaired due to NMJ defects. With that goal we performed locomotion studies on plates and in liquid (thrashing assay), pharmacological assays commonly used to score NMJ mutants (i.e. levamisole and aldicarb), and egg-laying experiments. We did observe a very mild resistance to levamisole at very low concentrations (i.e. 0.25 mM) which could be compared to the phenotype of the *lev-10* mutant, resistant at levamisole conc. ≤ 0.8 mM [112]. Nevertheless, the standard deviations were in our case too high to have a clear and reproducible result that would undoubtedly indicate an AGR-1 function at the NMJ analogous to vertebrates. In parallel with the *in vivo* experiments, we performed *in vitro* clustering assays with a recombinant AGR-1 fragment and mouse myotubes. Although some AChR clustering could be observed, it was induced only with the highest concentration of the fragment, at approximately 100 times more than the vertebrate A₄B₈ positive control. Taken together, these data indicate that in *C. elegans* agrin does not play a crucial role in AChR clustering at the NMJ. Interestingly, the phenotype of *lev-10* mutants could be rescued by overexpression of its extracellular

domain alone, suggesting that the AChR clustering mechanism in *C. elegans* might be regulated through a significantly different pathway than in vertebrates. These initial findings were quite surprising, and unexpected for us.

Another very important function of agrin in the entire muscle basement membrane is to provide structural support to the muscle by connecting the ECM to the cellular cytoskeleton through the DGC complex [65,66]. It is well known that mutations in components of this supramolecular complex lead to the outcome of muscular dystrophies (for a review, see [62]). Although we did not find any AGR-1 expression in *C. elegans* muscles, we considered that it could be below the detectable levels and investigated its impact on muscle structure. Since agrin alone did not cause any visible morphological changes in the muscles, as we could visualize by reporter constructs overexpressed under the muscle-specific promoters (*myoD*), we created several mutant strains with mutations in other BM and ECM proteins. None of the combinations caused morphological or behavioral defects that would suggest an AGR-1 function in the worms' muscles.

Since agrin was found to be expressed in four IL1 head neurons, we expected that its function might be related to them. There are three pairs of IL1 neurons, all polymodal by type: mechanosensory, interneurons and motoneurons, known to be involved in spontaneous foraging and the head withdrawal reflex as a response to light nose touch [98]. Worms in which the IL1 neurons were laser-ablated, forage abnormally slowly and show over-bending of the head. We scored the worms for the sensation of the light nose touch by the "eyelash test", but did not notice any resistance in *agr-1* mutants as compared to the wild type. We could conclude that agrin does not have a crucial role in the IL1 cells, although we could not exclude that it could have a sensory function compensated by another protein.

Based on the *agr-1* expression pattern in the distal tip cells of a developing gonad, we analyzed whether its mutation causes any gonad migration defects that could impact gonad morphology. It is known that several signaling pathways play a role in normal gonad development, for example: EGL-17/FGF; UNC-129/TGF- β ; and EGL-20/WNT [132]. We crossed *agr-1(egl1770)* mutants with several components of these pathways in order to investigate whether *agr-1* interacts genetically with any of them leading to a synergistic effect on gonad development. We did observe a slight reduction (up to 10%) of the defect rate in the double mutants as compared to the single mutants. It seemed that *agr-1* slightly suppressed the phenotypes caused by mutations in growth factor-like molecules, resembling the effects in the *perlecan/unc-52* mutants. Therefore, we could speculate that AGR-1 has a role in the gonad development, but to verify it, these experiments would have to be repeated with a larger population of worms and by a genotype-blinded analysis in order to ensure statistical significance and to avoid bias. Agrin might be important for gonad migration as one of many molecular components acting in parallel.

Previous studies have demonstrated that ECM and membrane-bound proteins provide signaling cues for axon guidance during development of the nervous system. Heparan sulphate proteoglycans (HSPGs) play an important part in neuronal development, reviewed in [181]. Since they are essential for many processes in the worm, their complete elimination is lethal [182], but partial depletion of only some HS-modifying enzymes (sulfotransferases and epimerases) cause distinct defects in axon guidance [180]. These data point to the importance of precise “sugar codes” that axons can follow as they grow and migrate. Null mutations in the *C. elegans* syndecan homologue SDN-1, cause severe defects in axon guidance of many neurons, such as HSN motoneurons, PVQ interneurons, and D-type motoneurons (fasciculation and circumferential growth) [101]. Interestingly, both of the *sdn-1* mutant alleles (*zh20* and *ok449*) in combination with HS-modifying enzymes, *hse-5(tm472)* and *hst-2(ok595)* cause axon guidance defects at a level significantly higher than the addition of single-mutant defects. These data suggested that the mutations in HS-modifying enzymes affect another HSPG. Since we did observe some AGR-1 immunostaining in the region of the ventral and dorsal midline, we suspected that it might play a role in the anteroposterior or circumferential axon guidance and represent the missing HSPG. We analyzed the *agr-1(eg1770); sdn-1(zh20)* double mutants, but did not detect any increase in guidance defects as compared to *sdn-1* alone. Therefore, we could conclude that AGR-1, although it might be modified by the HS-modifying enzymes, does not play an important role in axon guidance.

IV.5. Significance of the identified interacting partners

Agrin is a large protein, built of different structural modules that provide possibilities for interactions with many different proteins, reviewed in [13,67]. Since the interaction with α -dystroglycan (α -DG) is very well established, we investigated whether this interaction is conserved in *C. elegans* as well. The *in vitro* membrane overlay assays undoubtedly indicated that the recombinant AGR-1 fragment, comprising the two lamG domains, binds to the purified chicken α -DG [33]. This interesting result was supported by an experiment in COS cells transfected with the same AGR-1 recombinant fragment. The transfected cells exhibited the secreted agrin fragment on the cell surface, in a patchy pattern overlapping with endogenous α -DG, again pointing to an interaction despite the cross-species barriers. Therefore, we speculated that they might interact physiologically *in vivo*. To further investigate this interesting observation, we crossed *agr-1(eg1770)* mutants with the *dgn-1(cg121) dgn-2(ok209) dgn-3(tm1092)* and *dgn-2(ok209) dgn-3(tm1092)* [158]. Contrary to the expected, no obvious phenotypic defects could be seen in the combined mutant strains. The *dgn-1(cg121)* mutation alone causes severe gonad defects and sterility, but even analysed as a rescued heterozygote (with a wild type gene on an extrachromosomal array) the *agr-1(eg1770)* mutation did not affect the phenotype. The *dgn-2* and *dgn-3* are expressed in pharynx and some head neurons, but mutants do not have significant phenotypes. Since the expression pattern resembles the *agr-1* pattern, we expected that

a triple mutant *agr-1(eg1770) dgn-2(ok209) dgn-3(tm1092)* might have stronger pharynx-related defects. Although we did not see a strong defect due to a possible genetic interaction, it would need to be analyzed in more detail as it might anyway be present. We consider it as a high probability that AGR-1 and DGN genes do interact *in vivo*, but its significance has to be challenged by finding the appropriate narrow and more specific experimental niche.

Our attempts to identify interaction partners for AGR-1 did not focus only on α -DG. We tried to learn more about other putative interacting factors by affinity chromatography experiments. We could identify several proteins that selectively bound to the AGR-1 recombinant fragment (of two lamG domains), while they did not bind to a control column that served as a negative control. To our disappointment, the mass-spectroscopy analysis revealed that the interacting proteins were mostly enzymes of the oxido-reductase family, involved in fatty acid metabolism. Despite all efforts, we could not make a logical connection that would support the physiological relevance of these interactions. Another unexpected fact is that, under the experimental set-up that we used, we could not even confirm the interaction with α -DG. A possible explanation could be that the recombinant AGR-1 fragment, which we used, did not have the correct sugar modification that would mimic the three dimensional structure of the endogenous protein and allow a physiological interaction with the endogenous α -DG.

IV.6. Open questions and possible directions

Our studies furnished us with interesting insight into the first invertebrate agrin homologue identified so far. We demonstrated successfully its expression in the worm, revealed its unexpected expression pattern and dissected its possible roles. It is clear that AGR-1 is dispensable for *C. elegans* because the *agr-1* mutants grow, move and reproduce normally. Although none of the functional studies that we undertook clearly led us to the AGR-1 function, they left substantial basis for possible further experiments. Since negative results in experimental research are never completely informative, several of the experimental directions that we explored can be pursued further.

The expression of *agr-1* in the IL1 heads neurons suggests its role related to those cells. As mentioned earlier, AGR-1 is probably secreted by the dendrites and/or axons, and stays integrated in the basement membrane. Despite the computer prediction of the secretion signal and the cleavage site, there is a possibility that in IL1 neurons it stays anchored to the cell membrane. The dendrites of the IL1 neurons have endings enclosed within the inner labial sensilla cuticle and pass through channels lined with extracellular material [183]. Sublocalization of AGR-1 could be investigated by electron microscopy of the labial sensilla ECM. If secreted and localized there, agrin could be involved in the fine sensory input. On the other hand,

the IL1 axons, projecting into the nerve ring, synapse to the RMD motoneurons, thereby forming neuronal circuits for head-withdrawal reflex and spontaneous foraging [98,184]. Since the RMD neurons express glutamate receptor *glr-1*, which if mutated causes defects in the mentioned functions, it would be interesting to see if it interacts genetically with *agr-1* mutation. The experimental procedure for scoring head withdrawal phenotypes is technically demanding, therefore the experimentation would have to be improved.

The IL1 neurons, together with IL2, URA, and RME, synapse to the RIP pharyngeal neurons [93], the only ones that make synaptic connections outside of the pharynx (for a review, see [119]). Therefore, AGR-1 expressed by the IL1 neurons might be involved in the pharyngeal pumping in combination with many other inputs. One way to investigate this possible role of AGR-1 is to record the electrophysiological profile by a special procedure developed in the Leon Avery's lab, called "electropharyngeogram" (EPG) [185]. Despite our inability to see the defects in the pharyngeal movements, the fine changes might be recorded by the EPG and seen as an altered potential profile compared to the wild type. The experiment should as well be repeated at different temperatures, for example 15°C, 20°C and 25°C.

Another possible phenotype to be tested is the resistance to the potent nematocidal drug ivermectin. Ivermectin activates the glutamate-gated chloride channels, resulting in increased chloride permeability and toxicity. It was shown that a combination of mutations in three genes is needed to confer resistance to ivermectin: *glc-1*, *avr-15* and *avr-14* [186]. AGR-1 could possibly be involved in the ivermectin-induced toxicity and the mutation might induce slight resistance, either alone, or in combination with some of the above mentioned genes.

Since it was shown recently that agrin in vertebrates binds to the Na⁺/K⁺ ATPase in the CNS [75], which has an ortholog in *C. elegans* encoded by *eat-6* [122,187], it would be challenging to further investigate if *agr-1* genetically interacts with it. EAT-6 has been implicated in the feeding, since the mutants exhibit slow and delayed relaxation of the pharyngeal muscles. The defects are detectable by eye as well as recordable by the previously mentioned EPG method [185]. The possible interaction with *agr-1* could maybe be seen as an altered EPG pattern.

In addition to analyzing the *agr-1* mutants, we tried RNAi-mediated gene knock-down under muscle and neuron-specific promoters. Neurons in *C. elegans* are known to be difficult target for RNAi, therefore it might have been more useful to do the experiment in the sensitized strains, such as *rrf-1* [188] or *eri-1* [189]. Conversely, the AGR-1 overexpression specifically in the IL1 neurons would be an interesting approach complementary to the mutant analysis. Additionally, an overexpression of a "dominant-negative" mutant, i.e. missing the last two lamG domains, in the *agr-1* or wt background, would be an interesting experiment. In

such experimental setting it might be easier to see the defects, than in a complete null mutant where the loss of AGR-1 might be compensated for by other proteins.

In our attempts to identify more factors interacting with AGR-1, we focused only on the C-terminal interactions through the two lam G domains. It would be interesting to overexpress the full length protein and search for its interactions with endogenous *C. elegans* proteins. The reason why our affinity column experiments were not very successful might be due to the lack of other necessary domain in our AGR-1 fragment or the lack of appropriate glycosylation.

Although one part of our goal, to identify the missing links in the agrin-MuSK pathway, was not met, our work represents an original and interesting characterization of a novel protein. We successfully identified the expression and localization of agrin in *C. elegans* and undertook significant efforts to elucidate its function. Our results clearly demonstrate that between the worm and man there still is a large evolutionary gap. Seen from the evolutionary perspective, AGR-1 was probably a protein involved in sensory or structural functions, in parallel with several others. It should be noted that no agrin ortholog has been identified in *Drosophila melanogaster* and other proteins are involved in synapse development in insects. Since in vertebrate agrin the three lamG domains are functionally the most important part for AChRs clustering at the NMJ, the lack of one of these domains in AGR-1 supports the speculation for a completely different function. Through evolution, the ancestral AGR-1 molecule might have acquired the third lam G domain which developed a new interaction site and gave rise to the new, “evolutionarily younger”, function at the NMJ.

V. Appendix

V.1. Experimental procedures (*unpublished data*)

***In vitro* clustering assay of AChRs on muscle myotubes**

[*protocol and reagents were kindly provided by Prof. Markus Rüegg*]

▪ **Culture media**

The following media were used for the growth and differentiation of myoblasts:

Growth medium:

- DMEM high glucose with Pyruvate with Phenol red
- 20 % Foetal Calf Serum
- 1% L-Glutamin
- 1% Sodium Pyruvate
- 1% Penicillin -Streptomycin

Differentiation (fusion) medium:

- DMEM high glucose with Pyruvate with Phenol red
- 5 % Horse serum
- 1% L-Glutamin
- 1% Sodium Pyruvate
- 1% Penicillin -Streptomycin

▪ **Culturing and splitting myoblasts**

C₂C₁₂ myoblast cells were maintained at low density (not higher than 50% confluency), therefore the cultures had to be split every 2-3 days.

For the clustering assays, C₂C₁₂ myoblasts first had to be differentiated by fusion into myotubes. Before plating the cells, 3.5cm-tissue culture plates were coated with 0.03% gelatin and kept during at least 30min at 37°C. The rest of the liquid was aspirated and 30 000 cells were plated into each dish, in 2 ml of the normal growth medium.

▪ **Myoblast fusion**

To induce the fusion of C₂C₁₂ myoblasts, the growth medium was replaced by the differentiation medium when cells reached approximately 80% confluency (about 2 days after plating). The differentiation medium had to be exchanged for the fresh one 2 days later. In total 5 days after the first exchange to the differentiation medium, the formed syncytial myotubes and were ready for the clustering assay.

- **AChRs clustering assay**

In order to treat the myotubes and to perform the clustering assay, medium was removed and cells washed twice in PBS⁻. The concentration of the *C. elegans* recombinant agrin was estimated by the band intensity, as compared to the known dilutions of BSA, on the Coomassie stained SDS PAGE gel. The samples were prepared in serial dilutions as following: 1/2 (125 ng/μl) → 1/10 (25 ng/μl) → 1/50 (5 ng/μl) → 1/250 (1 ng/μl) → 1/500 (0.5 ng/μl), in differentiation medium. Purified recombinant fragments of chicken neuronal and muscle agrin were used as positive and negative control, respectively. The concentrations of both samples were about 0.5 μg/μl, and were diluted 500 times to the final concentration of 1 ng/μl.

The cells were incubated with agrin samples overnight, during approximately 16 hours at 37°C. The following morning, the media were removed, cells rinsed with PBS⁻ and incubated with fluorescently labeled α-bungarotoxin (α-Btx) during 30 min at 37°C, in the dark.

Myotubes were gently washed 3 times in PBS⁻ and fixed in 2% paraformaldehyde during 10 minutes at room temperature in the dark. Following washing with PBS⁻, a drop of mounting medium was added to the fixed cells and the samples analyzed under Zeiss Axioscope Bio microscope, with a rhodamine filter.

Affinity column experiments in search for potential agrin binding factors

The affinity column experiments were repeated three times following modifications of the following protocol.

- **Samples preparation**

Worms for the preparation of lysates were cultured as described in section III.1. After washing, they were frozen and stored at -80°C. For protein extraction, they were ground in liquid nitrogen in a porcelain mortar. Initial amounts of frozen worm material varied between experiments (Annex, Table 1). In the three successive experiments, different lysis /homogenization buffers were used for the preparation of the suspensions (Table 1). In the first experiment the pulverized samples were resuspended in equal volumes of ice-cold ddH₂O and lysed with the addition of an equal volume of 2x lysis buffer. In the second and third experiment the pulverized worms were resuspended in the homogenization buffers. Cocktails of protease inhibitors containing EDTA (Complete Mini, Roche) were added to the solutions. The suspensions were centrifuged at 10000 g for 20 minutes to separate soluble from insoluble fractions. Following the

centrifugation, pellets were resuspended in the lysis buffer and centrifuged again. In the first experiment lysis buffer contained protease inhibitors with EDTA, but in the next experiments we used only EDTA-free inhibitor cocktails. In the first experiment both supernatants were pooled (Table 1, shaded fields), filtered through a 1.2µm filter and split into two batches, one to be used on agrin-containing column and a negative control column. In the repeated experiments the first supernatants were discarded and only the second ones (without EDTA) were used for the column (Table 1, shaded fields).

- **Columns preparation**

Binding of the purified agrin fragment to the CNBr-activated SepharoseTM 4B resin beads (Amersham Pharmacia) was done following supplier's protocol. A control column was prepared without agrin and with blocking reagent only. Both columns contained equal volume of approx. 2.5 ml of packed beads. And they were kept in Column Storage buffer (Table 1) at 4°C. Before each experiment, they were equilibrated at room temperature and prewashed with PBS⁻, followed by resuspension buffer, wash buffer, elution buffer and the end resuspension buffer again. In each experiment a half of the lysate supernatant was loaded on the agrin-containing column and the other half to the control column. Binding of the lysate supernatants to the beads was done following the supplier's protocol.

- **Loading the samples and the analysis**

The samples were loaded at the flow rate of approx. 0.3 ml/min, the columns washed with 15 ml of Wash buffer (the composition varied between the experiments, see the table below) and the bound proteins eluted and collected in fractions of 0.5 ml with a fraction collector at a flow rate of approx. 1 ml/min. In the first experiment no absorbance was measured because of the presence of NP-40 detergent in the Elution buffer. Different fractions were analysed by SDS PAGE on Ready Tris-HCl 4-15% linear gradient gels (BioRad) and detected by Coomassie Blue staining (Pierce).

Table 1. Compositions of the buffers used in three independent affinity column experiments

Step	Experiment 1 (with 8 ml of worms)	Experiment 2 (with 4 ml of worms)	Experiment 3 (with 10 ml of worms)
1) Lysis Or Homogenization	<u>Lysis buffer 1</u> * (2x stock solution): Tris pH8 100mM NaCl 240 mM NP-40 1% MgCl ₂ 4 mM + Prot. Inh. w EDTA	<u>Homogenization buffer 2</u> (1x) PBS ⁻ + Prot. Inh. w EDTA 1mM (<i>supernatant discarded</i>)	<u>Homogenization buffer 2</u> (1x) Tris pH 7.4 50 mM NaCl 50 mM + Prot. Inh. w EDTA (<i>supernatant discarded</i>)
2) Pellet Resuspension Buffer (as Column Binding buffer)	<u>Lysis buffer 1:</u> <i>As above</i> (<i>supernatants pooled and loaded on the column</i>)	<u>Lysis buffer 2:</u> PBS ⁻ CaCl ₂ 2mM MgCl ₂ 1mM NP-40 1% + Prot. Inh. w/o EDTA (<i>supernatant loaded on the column</i>)	<u>Lysis buffer 3:</u> Tris pH 7.4 50 mM NaCl 50 mM CaCl ₂ 2mM MgCl ₂ 1mM 1% NP-40 + Prot. Inh. w/o EDTA (<i>supernatant loaded on the column</i>)
3) Column Washing	<u>Wash buffer 1:</u> As the Lysis buffer 1 (1x)	<u>Wash buffer 2:</u> As the Lysis buffer 2	<u>Wash buffer 3:</u> As the Lysis buffer 3
4) Column Elution	<u>Elution buffer 1:</u> As the Lysis buffer 1 (1x) with 1M NaCl	<u>Elution buffer 2:</u> PBS ⁻ NaCl 1.8 M EDTA 10mM	<u>Elution buffer 3:</u> Tris pH 7.4 50 mM NaCl 2M EDTA 10mM
Other solutions	<u>Column Storage buffer</u> (5x stock solution): Borate pH8 (NaOH) NaCl 0.75 M		

* the supernatants of the samples in the shaded fields were loaded on the column

***In vivo* pharmacological assays – levamisole & aldicarb**

▪ **Preparation of the plates:**

Aldicarb was dissolved in 70% EtOH for 100mM stock solution. Levamisole was dissolved in H₂O for 100mM stock solution. The nematode growth medium (NGM) was prepared following the standard procedure [163]. After autoclaving and cooling down to 60°C, aldicarb or levamisole were added to the NGM at the desired final concentrations. The solutions were mixed and immediately poured in small 6cm plates.

- **Pharmacological assays**

For each test 20 young adults were picked from a “non-starved” plate and laid in the centre of a test plate, with levamisole, aldicarb or an NGM plate without supplements. During the experiment, the plates were kept at 21°C. At one hour intervals the animals were scored for movement (survival). The worms that were not able to make a full bend after prodding their tails were considered as “dead”. The test was always repeated three times for each strain. Statistical analysis of the scores was done by the Student T test.

- **Mutant genotypes**

The genotype of the strains for the levamisole test:

N2 wt

agr-1(eg1770)

lev-1(e211)

The genotype of the strains for the aldicarb test:

N2 wt

agr-1(eg1770)

dyb-1(cx36) dys-1(cx18)

dyb-1(cx36) dys-1(cx18); agr-1(eg1770)

(two lines of the same genotype were tested in our experiments)

Analysis of putative agrin function in *C. elegans* by dsRNA “knock-down” experiments

- **Gene cloning and production of dsRNA**

Agrin cDNA was synthesized from total RNA (from mixed-stage population) by SuperScriptTM II Reverse Transcriptase kit (Invitrogen) following the supplier’s protocol. For the positive control, a fragment of myosin (*myo3*) cDNA was amplified. Primers and plasmids for the constructs are shown below.

For the amplification of agrin:

agr8: CAGTGTGTGGATCAGAAGGAACAG (*EcoRI*, added to the primer)

agr9: GCCTCCATCTCATTGTCATTCATC (*EcoRI* added to the primer)

The PCR product was digested with *EcoRI* restriction enzyme and cloned into *pBluescriptKSII*⁺ (construct P230). The construct was then linearized with *XhoI* or *SpeI* for the *in vitro* RNA synthesis with T7 and T3 polymerases, respectively.

For the amplification of myosin3:

myo3RNAi1 TTCTGCAGTAACGAGGATCTTGCTCGC (*PstI* added to the primer)

myo3RNAi2 CCACTAGTGGTACCTTCGAGAGAGCGGTTCAAATC (*SpeI* added to the primer)

The PCR product was digested with *PstI* and *SpeI* restriction enzymes and cloned into *pBluescriptKSII*⁺ (construct P227). The construct was then linearized with *PstI* for the RNA *in vitro* transcription with T7 Polymerase or *SpeI* linearized for the RNA *in vitro* transcription with T3 Polymerase.

The single-stranded RNA was produced by *in vitro* transcription using T7 RiboMAXTM Express RNAi System (Promega) following the supplier's protocol. The two complementary ssRNA were then annealed and EtOH precipitated/purified.

▪ **Microinjection of the dsRNA into gonads of worms**

In vitro transcribed and annealed dsRNA was resuspended in 1x injection buffer: 20mM K₃PO₄, 20g/L PEG6000, 3mM KCitrate, pH 7.5, at an approximate final concentration of 200ng/μl. The dsRNA was injected in the gonads of young adults using an Eppendorf Femto Jet system for microinjections under a Zeiss Axiovert 10 microscope. Only animals for which the gonad filling was observed under the microscope were kept. The worms were put back on NGM plates, with *E. coli* (*OP50*) bacteria, to recover for 8 hours and then subsequently only healthy animals were transferred to a new NGM plate (one animal per plate).

▪ **Analysis of the phenotypes**

The progeny of the microinjected animals was scored for the following phenotypes: embryonic lethality (dead eggs), larval lethality, morphological defects, locomotion, and response to touch (nose and tail).

Crosses

▪ **Agrin mutant (*agr-1*) with Acetylcholine esterase (AChE) mutants (*ace-1*;*ace-2*)**

The agrin mutant strain *agr-1(eg1770)* was left for 4 hours at 30°C to raise the relative number of male progeny. Males from the progeny were crossed back to the *agr-1(eg1770)* hermaphrodites in order to maintain them. *Agr-1(eg1770)* males from the next generation were crossed with *ace-1(p1000)*; *ace-2(g72)* double-mutant hermaphrodites, by putting them on an empty NGM plate with only a little bacteria in the middle of the plate. Three days later, 12 coordinated worms were cloned from the progeny. Since homozygous *ace-1(p1000)*; *ace-2(g72)* mutants are uncoordinated (unc), all cross-progeny with *agr-1* was expected to be heterozygous, i.e. coordinated. Each cloned worm was grown on a separate plate, left to lay eggs, and then picked for genotyping. The worms were frozen, each in one tube, in 2μl of the lysis buffer (10mM Tris pH8.0, 50mM KCl, 0.5% NP-40, 0.5% Tween-20, 0.01% gelatine, 0.1 mg/ml Proteinase K),

thawed and then lysed at 60°C during 1 hour followed by 15 min at 94°C for the inactivation of the Proteinase K enzyme. PCR was done directly by the addition of 18µl of a master mix. If PCRs for more genes had to be done from the same worm, the worm was lysed in bigger volume of the lysis buffer and the lysate aliquoted for the separate PCRs.

<u>Master mix:</u>	final concentration	
ddH ₂ O		13.3 µl
HiFi reaction buffer (10x, company)	1x	2 µl
dNTP mix (each dNTP 10mM, Eppendorf)	2mM	0.4 µl
Primers mix (2mM each)	200nM	2 µl
<u>Expand HiFi polymerase, Roche (3.5U/µl)</u>	<u>1U/mix</u>	<u>0.3 µl</u>
Total mix volume		18 µl
Total reaction volume		20 µl

Primers for genotyping the *agr-1(egl770)* allele were the following:

Agr6 TGGGCATACACATTTGGGTTTTCCG

AgrMos GCTGCCTGCAAGGAACAAAAGG

Mos2 GCATTCGGTGCGATTCCGCAG

Primers for genotyping the *ace-1(p1000)* allele were the following:

Ace1-5' GCTGTTGATCTCATTACCTC

Ace1-3' TCACGTCATTCTAATCTGTTGC

Primers for genotyping the *ace-2(g72)* allele were the following:

Ace2-5' GATTAGTTTATGACAAAAATTG

Ace2-3' GTCGAAATTGATGCAAATGG

The PCR amplification was done in 35 cycles, with the annealing temperature set to 55°C for all three genes, and the elongation time to 1min 30sec. The sizes of the resulting PCR products were the following: for agrin Wt allele 400 bp and *agr-1(egl770)* 700 bp. *Ace-1(p1000)* and *ace-2(g72)* carry point mutations, therefore mutant alleles could not be distinguished from the Wt by size on the agarose gel, for *ace-1* the PCR product was 400 bp long, for *ace-2* 800 bp. Since the point mutation in *ace-1(p1000)* causes an alteration of a restriction site, we analysed the PCR product by digestion with the *BsII* restriction enzyme. The fragment was purified from the PCR mix with the PCR Purification Kit from Qiagen, following the supplier's protocol, and digested with *BsII* (5U) during 1h 30min at 55°C. The digestion of the *ace-1* wild type allele gave fragments of 230 bp, 180 bp, and 80 bp, and for the mutant 410bp and 80bp. PCR products of the *ace-2* allele had to be gel-purified and sequenced.

- **Agirin mutant (*agr-1*) with Dystroglycan mutants (*dgn-1;dgn-2;dgn-3*)**

The general procedure of the cross was done as described above, with additional modifications. Since *dgn-1; dgn-2; dgn-3* triple mutants are sterile, they were balanced with the wild type copy of the *dgn-1* gene on an extrachromosomal array, which as well carried the roller gene *rol-6^{mut}* (pRF4) and the *pdgn-1::gfp* as markers. Therefore, only green roller worms were fertile. During the cross we cloned only the green rollers and genotyped them. For *dgn-1* two different PCRs were done in order to distinguish between the wt and mutant alleles in heterozygotes.

Primers for genotyping *dgn-1(cg121)* allele were the following:

Dgn 1 A CTAGTGACAACCGCCATTTCCG [*upstream of the deletion region*]

Dgn 1 B GAATTCCAAAGGCTCAGAGAGC [*downstream of the deletion region*]

With *dgn 1A* & *dgn 1B* primers, deletion mutant *dgn-1* allele gives a band of 397 bp and the wild type 2792 bp (not distinguishing between genomic DNA and the extrachromosomal array)

Dgn 1 D AATGTGAAAGGTAGTGCCAGGC [*downstream of the deletion region*]

Dgn 1 E TTCCCACCAACACAATCCCAC [*within the deletion region*]

With *dgn 1D* & *dgn 1E* primers, only wild type allele on genomic DNA gives a band of 1000 bp.

Primers for genotyping *dgn-2(ok209)* allele were the following:

Dgn 2 A CATCCATTCGGCAGATGCTACC [*upstream of the deletion region*]

Dgn 2 B AAGAGACGCAGACTTCTCAACC [*downstream of the deletion region*]

Dgn 2 C GATTTTGACATCACCGGCTTGG [*within the deletion region*]

Deletion mutant allele *dgn-2* with primers *dgn2A* and *dgn2B* gives a band of 577 bp.

Wild type *dgn-2* gene with primers *dgn2A* and *dgn2C* gives a band of 805 bp.

Primers for genotyping *dgn-3(tm1092)* allele were the following:

Dgn 3 A CATCTCCTCAGTCAATGGTTCG [*upstream of the deletion region*]

Dgn 3 B AGCCTAAGCCTAAACTGGAACC [*downstream of the deletion region*]

Dgn 3 C GGTACACTATTGTGAGATGGGC [*within the deletion region*]

Deletion mutant allele *dgn-3* with primers *dgn3A* and *dgn3B* gives a band of 526 bp.

Wild type *dgn-3* gene with primers *dgn3A* and *dgn3C* gives a band of 796 bp.

The lysis and the PCRs were done as described above. PCRs were done with Taq polymerase from Roche.

V.2. List of Abbreviations

<i>agr</i>	agrin gene in <i>C. elegans</i>
ACh	neurotransmitter acetylcholine
AChE	acetylcholine esterase
AChR	acetylcholine receptor
APC	antigen presenting cell
α -DG	α -dystroglycan
BL	basal lamina
BM	basement membrane
bp	base pairs
CK2	casein kinase 2
CNS	central nervous system
DGC	dystrophyn-associated glycoprotein complex
DTC	distal tip cell
Dvl	dishevelled
ECM	extracellular matrix
EGF	epidermal growth factor
FS	follistatin-like domains
GAG	glycosaminoglycan
HS	heparan-sulphate
lamG	laminin globular doman
LE	laminin EGF-like domains
<i>lev</i>	levamisole-resistant mutants
LN	long amino (N)-terminal agrin isoform
MASC	myotube-associated specificity component
MuSK	muscle-specific kinase
<i>myd</i>	myodystrophy mouse model
NMJ	neuromuscular junction
NKA	Na ⁺ /K ⁺ -ATPase
NtA	amino (N)-terminal agrin domain
PAK	p21-activated kinase
<i>pat</i>	mutants with paralysis at two-fold embryonic stage
PCP	planar cell polarity pathway
PTB	phosphotyrosine-binding

RATL	rapsyn-associated transmembrane linker
SEA	sperm protein, enterokinase, and agrin domain
SN	short amino (N)-terminal agrin isoform
S/T	serine/threonine-rich regions
TM	transmembrane [agrin isoform]
TN-C	tenascin C
<i>Unc</i>	uncoordinated mutants

V.3. References

- 1 Godfrey EW, Nitkin RM, Wallace BG, Rubin LL and McMahan UJ (1984) Components of Torpedo electric organ and muscle that cause aggregation of acetylcholine receptors on cultured muscle cells. *J Cell Biol* 99(2): 615-627.
- 2 Nitkin RM, Smith MA, Magill C, Fallon JR, Yao YM et al. (1987) Identification of agrin, a synaptic organizing protein from Torpedo electric organ. *J Cell Biol* 105(6 Pt 1): 2471-2478.
- 3 Magill-Solc C and McMahan UJ (1988) Motor neurons contain agrin-like molecules. *J Cell Biol* 107(5): 1825-1833.
- 4 Magill-Solc C and McMahan UJ (1990) Agrin-like molecules in motor neurons. *J Physiol (Paris)* 84(1): 78-81.
- 5 Magill-Solc C and McMahan UJ (1990) Synthesis and transport of agrin-like molecules in motor neurons. *J Exp Biol* 153: 1-10.
- 6 McMahan UJ (1990) The agrin hypothesis. *Cold Spring Harb Symp Quant Biol* 55: 407-418.
- 7 Reist NE, Werle MJ and McMahan UJ (1992) Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. *Neuron* 8(5): 865-868.
- 8 Rupp F, Payan DG, Magill-Solc C, Cowan DM and Scheller RH (1991) Structure and expression of a rat agrin. *Neuron* 6(5): 811-823.
- 9 Tsim KW, Ruegg MA, Escher G, Kroger S and McMahan UJ (1992) cDNA that encodes active agrin. *Neuron* 8(4): 677-689.
- 10 Denzer AJ, Gesemann M, Schumacher B and Ruegg MA (1995) An amino-terminal extension is required for the secretion of chick agrin and its binding to extracellular matrix. *J Cell Biol* 131(6 Pt 1): 1547-1560.
- 11 Smith MA, C. Magill-Solc, et. al. (1992) Isolation and characterization of a cDNA that encodes an agrin homolog in the marine ray. *Molecular and Cellular Neuroscience* 3(5): 406-417.
- 12 Groffen AJ, Buskens CA, van Kuppevelt TH, Veerkamp JH, Monnens LA et al. (1998) Primary structure and high expression of human agrin in basement membranes of adult lung and kidney. *Eur J Biochem* 254(1): 123-128.
- 13 Bezakova G and Ruegg MA (2003) New insights into the roles of agrin. *Nat Rev Mol Cell Biol* 4(4): 295-308.
- 14 Tsen G, Halfter W, Kroger S and Cole GJ (1995) Agrin is a heparan sulfate proteoglycan. *J Biol Chem* 270(7): 3392-3399.
- 15 Winzen U, Cole GJ and Halfter W (2003) Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters. *J Biol Chem* 278(32): 30106-30114.

- 16 Ferns M, Hoch W, Campanelli JT, Rupp F, Hall ZW et al. (1992) RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. *Neuron* 8(6): 1079-1086.
- 17 Denzer AJ, Brandenberger R, Gesemann M, Chiquet M and Ruegg MA (1997) Agrin binds to the nerve-muscle basal lamina via laminin. *J Cell Biol* 137(3): 671-683.
- 18 Kammerer RA, Schulthess T, Landwehr R, Schumacher B, Lustig A et al. (1999) Interaction of agrin with laminin requires a coiled-coil conformation of the agrin-binding site within the laminin gamma1 chain. *Embo J* 18(23): 6762-6770.
- 19 Neumann FR, Bittcher G, Annies M, Schumacher B, Kroger S et al. (2001) An alternative amino-terminus expressed in the central nervous system converts agrin to a type II transmembrane protein. *Mol Cell Neurosci* 17(1): 208-225.
- 20 Burgess RW, Dickman DK, Nunez L, Glass DJ and Sanes JR (2002) Mapping sites responsible for interactions of agrin with neurons. *J Neurochem* 83(2): 271-284.
- 21 Burgess RW, Skarnes WC and Sanes JR (2000) Agrin isoforms with distinct amino termini: differential expression, localization, and function. *J Cell Biol* 151(1): 41-52.
- 22 Rupp F, Ozcelik T, Linial M, Peterson K, Francke U et al. (1992) Structure and chromosomal localization of the mammalian agrin gene. *J Neurosci* 12(9): 3535-3544.
- 23 Ruegg MA, Tsim KW, Horton SE, Kroger S, Escher G et al. (1992) The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. *Neuron* 8(4): 691-699.
- 24 Gesemann M, Cavalli V, Denzer AJ, Brancaccio A, Schumacher B et al. (1996) Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. *Neuron* 16(4): 755-767.
- 25 Burgess RW, Nguyen QT, Son YJ, Lichtman JW and Sanes JR (1999) Alternatively spliced isoforms of nerve- and muscle-derived agrin: their roles at the neuromuscular junction. *Neuron* 23(1): 33-44.
- 26 Ferns MJ, Campanelli JT, Hoch W, Scheller RH and Hall Z (1993) The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. *Neuron* 11(3): 491-502.
- 27 Gesemann M, Denzer AJ and Ruegg MA (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *J Cell Biol* 128(4): 625-636.
- 28 Hoch W, Ferns M, Campanelli JT, Hall ZW and Scheller RH (1993) Developmental regulation of highly active alternatively spliced forms of agrin. *Neuron* 11(3): 479-490.
- 29 Sanes JR and Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci* 2(11): 791-805.
- 30 Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K et al. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* 15(3): 573-584.
- 31 Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J et al. (1996) Agrin acts via a MuSK receptor complex. *Cell* 85(4): 513-523.

- 32 Herbst R, Avetisova E and Burden SJ (2002) Restoration of synapse formation in Musk mutant mice expressing a Musk/Trk chimeric receptor. *Development* 129(23): 5449-5460.
- 33 Scotton P, Bleckmann D, Stebler M, Sciandra F, Brancaccio A et al. (2006) Activation of MuSK and binding to dystroglycan is regulated by alternative mRNA splicing of agrin. *J Biol Chem*.
- 34 Frail DE, McLaughlin LL, Mudd J and Merlie JP (1988) Identification of the mouse muscle 43,000-dalton acetylcholine receptor-associated protein (RAPsyn) by cDNA cloning. *J Biol Chem* 263(30): 15602-15607.
- 35 Apel ED, Glass DJ, Moscoso LM, Yancopoulos GD and Sanes JR (1997) Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. *Neuron* 18(4): 623-635.
- 36 Mittaud P, Marangi PA, Erb-Vogtli S and Fuhrer C (2001) Agrin-induced activation of acetylcholine receptor-bound Src family kinases requires Rapsyn and correlates with acetylcholine receptor clustering. *J Biol Chem* 276(17): 14505-14513.
- 37 Wallace BG, Qu Z and Haganir RL (1991) Agrin induces phosphorylation of the nicotinic acetylcholine receptor. *Neuron* 6(6): 869-878.
- 38 Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH et al. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85(4): 525-535.
- 39 DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT et al. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* 85(4): 501-512.
- 40 Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC et al. (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* 377(6546): 232-236.
- 41 Jones G, Meier T, Lichtsteiner M, Witzemann V, Sakmann B et al. (1997) Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. *Proc Natl Acad Sci U S A* 94(6): 2654-2659.
- 42 Meier T, Hauser DM, Chiquet M, Landmann L, Ruegg MA et al. (1997) Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. *J Neurosci* 17(17): 6534-6544.
- 43 Luo ZG, Wang Q, Zhou JZ, Wang J, Luo Z et al. (2002) Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. *Neuron* 35(3): 489-505.
- 44 Okada K, Inoue A, Okada M, Murata Y, Kakuta S et al. (2006) The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. *Science* 312(5781): 1802-1805.
- 45 Cheusova T, Khan MA, Schubert SW, Gavin AC, Buchou T et al. (2006) Casein kinase 2-dependent serine phosphorylation of MuSK regulates acetylcholine receptor aggregation at the neuromuscular junction. *Genes Dev* 20(13): 1800-1816.
- 46 Cohen MW, Jacobson C, Godfrey EW, Campbell KP and Carbonetto S (1995) Distribution of alpha-dystroglycan during embryonic nerve-muscle synaptogenesis. *J Cell Biol* 129(4): 1093-1101.
- 47 Gee SH, Montanaro F, Lindenbaum MH and Carbonetto S (1994) Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* 77(5): 675-686.

- 48 Campanelli JT, Roberds SL, Campbell KP and Scheller RH (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell* 77(5): 663-674.
- 49 Cote PD, Moukhles H, Lindenbaum M and Carbonetto S (1999) Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. *Nat Genet* 23(3): 338-342.
- 50 Meier T, Gesemann M, Cavalli V, Ruegg MA and Wallace BG (1996) AChR phosphorylation and aggregation induced by an agrin fragment that lacks the binding domain for alpha-dystroglycan. *Embo J* 15(11): 2625-2631.
- 51 Jacobson C, Montanaro F, Lindenbaum M, Carbonetto S and Ferns M (1998) alpha-Dystroglycan functions in acetylcholine receptor aggregation but is not a coreceptor for agrin-MuSK signaling. *J Neurosci* 18(16): 6340-6348.
- 52 Grady RM, Zhou H, Cunningham JM, Henry MD, Campbell KP et al. (2000) Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin-glycoprotein complex. *Neuron* 25(2): 279-293.
- 53 Jacobson C, Cote PD, Rossi SG, Rotundo RL and Carbonetto S (2001) The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. *J Cell Biol* 152(3): 435-450.
- 54 Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR et al. (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* 410(6832): 1057-1064.
- 55 Yang X, Arber S, William C, Li L, Tanabe Y et al. (2001) Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* 30(2): 399-410.
- 56 Misgeld T, Kummer TT, Lichtman JW and Sanes JR (2005) Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. *Proc Natl Acad Sci U S A* 102(31): 11088-11093.
- 57 Ervasti JM and Campbell KP (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J Cell Biol* 122(4): 809-823.
- 58 Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW et al. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* 355(6362): 696-702.
- 59 Tremblay MR and Carbonetto S (2006) An extracellular pathway for dystroglycan function in acetylcholine receptor aggregation and laminin deposition in skeletal myotubes. *J Biol Chem* 281(19): 13365-13373.
- 60 Michele DE and Campbell KP (2003) Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. *J Biol Chem* 278(18): 15457-15460.
- 61 Meier T and Ruegg MA (2000) The Role of Dystroglycan and Its Ligands in Physiology and Disease. *News Physiol Sci* 15: 255-259.
- 62 Durbeej M and Campbell KP (2002) Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models. *Curr Opin Genet Dev* 12(3): 349-361.

- 63 Batchelor CL and Winder SJ (2006) Sparks, signals and shock absorbers: how dystrophin loss causes muscular dystrophy. *Trends Cell Biol* 16(4): 198-205.
- 64 Michele DE, Barresi R, Kanagawa M, Saito F, Cohn RD et al. (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* 418(6896): 417-422.
- 65 Bezakova G and Lomo T (2001) Muscle activity and muscle agrin regulate the organization of cytoskeletal proteins and attached acetylcholine receptor (AChR) aggregates in skeletal muscle fibers. *J Cell Biol* 153(7): 1453-1463.
- 66 Moll J, Barzaghi P, Lin S, Bezakova G, Lochmuller H et al. (2001) An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy. *Nature* 413(6853): 302-307.
- 67 Smith MA and Hilgenberg LG (2002) Agrin in the CNS: a protein in search of a function? *Neuroreport* 13(12): 1485-1495.
- 68 Campagna JA, Ruegg MA and Bixby JL (1995) Agrin is a differentiation-inducing "stop signal" for motoneurons in vitro. *Neuron* 15(6): 1365-1374.
- 69 Chang D, Woo JS, Campanelli J, Scheller RH and Ignatius MJ (1997) Agrin inhibits neurite outgrowth but promotes attachment of embryonic motor and sensory neurons. *Dev Biol* 181(1): 21-35.
- 70 Halfter W, Schurer B, Yip J, Yip L, Tsen G et al. (1997) Distribution and substrate properties of agrin, a heparan sulfate proteoglycan of developing axonal pathways. *J Comp Neurol* 383(1): 1-17.
- 71 Mantych KB and Ferreira A (2001) Agrin differentially regulates the rates of axonal and dendritic elongation in cultured hippocampal neurons. *J Neurosci* 21(17): 6802-6809.
- 72 Bixby JL, Baerwald-De la Torre K, Wang C, Rathjen FG and Ruegg MA (2002) A neuronal inhibitory domain in the N-terminal half of agrin. *J Neurobiol* 50(2): 164-179.
- 73 Hilgenberg LG, Hoover CL and Smith MA (1999) Evidence of an agrin receptor in cortical neurons. *J Neurosci* 19(17): 7384-7393.
- 74 Hilgenberg LG, Ho KD, Lee D, O'Dowd DK and Smith MA (2002) Agrin regulates neuronal responses to excitatory neurotransmitters in vitro and in vivo. *Mol Cell Neurosci* 19(1): 97-110.
- 75 Hilgenberg LG, Su H, Gu H, O'Dowd DK and Smith MA (2006) Alpha3Na⁺/K⁺-ATPase is a neuronal receptor for agrin. *Cell* 125(2): 359-369.
- 76 Hoover CL, Hilgenberg LG and Smith MA (2003) The COOH-terminal domain of agrin signals via a synaptic receptor in central nervous system neurons. *J Cell Biol* 161(5): 923-932.
- 77 Ross ST and Soltesz I (2001) Long-term plasticity in interneurons of the dentate gyrus. *Proc Natl Acad Sci U S A* 98(15): 8874-8879.
- 78 Godfrey EW (1991) Comparison of agrin-like proteins from the extracellular matrix of chicken kidney and muscle with neural agrin, a synapse organizing protein. *Exp Cell Res* 195(1): 99-109.
- 79 Groffen AJ, Ruegg MA, Dijkman H, van de Velden TJ, Buskens CA et al. (1998) Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *J Histochem Cytochem* 46(1): 19-27.

- 80 Gesemann M, Brancaccio A, Schumacher B and Ruegg MA (1998) Agrin is a high-affinity binding protein of dystroglycan in non-muscle tissue. *J Biol Chem* 273(1): 600-605.
- 81 Raats CJ, van den Born J, Bakker MA, Oppers-Walgreen B, Pisa BJ et al. (2000) Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies. *Am J Pathol* 156(5): 1749-1765.
- 82 van den Hoven MJ, Rops AL, Bakker MA, Aten J, Rutjes N et al. (2006) Increased expression of heparanase in overt diabetic nephropathy. *Kidney Int.*
- 83 Khan AA, Bose C, Yam LS, Soloski MJ and Rupp F (2001) Physiological regulation of the immunological synapse by agrin. *Science* 292(5522): 1681-1686.
- 84 Zhang J, Wang Y, Chu Y, Su L, Gong Y et al. (2006) Agrin is involved in lymphocytes activation that is mediated by alpha-dystroglycan. *Faseb J* 20(1): 50-58.
- 85 White J (1988) The Anatomy. In: Wood WB, editor. *The Nematode Caenorhabditis elegans*: Cold Spring Harbor Laboratory.
- 86 Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77(1): 71-94.
- 87 Sulston JE and Horvitz HR (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol* 56(1): 110-156.
- 88 Riddle DL, Blumenthal T, Meyer BJ and Priess JR (1997) Introduction to *C. elegans*. In: Riddle DL, Blumenthal T, Meyer BJ and Priess JR, editors. *C elegans II*: Cold Spring Harbor Laboratory Press.
- 89 Jones AK, Buckingham SD and Sattelle DB (2005) Chemistry-to-gene screens in *Caenorhabditis elegans*. *Nat Rev Drug Discov* 4(4): 321-330.
- 90 Zwaal RR, Broeks A, van Meurs J, Groenen JT and Plasterk RH (1993) Target-selected gene inactivation in *Caenorhabditis elegans* by using a frozen transposon insertion mutant bank. *Proc Natl Acad Sci U S A* 90(16): 7431-7435.
- 91 Kaletta T and Hengartner MO (2006) Finding function in novel targets: *C. elegans* as a model organism. *Nat Rev Drug Discov* 5(5): 387-398.
- 92 Hobert O (2005) Specification of the Nervous System. In: *Wormbook*. The *C. elegans* Research Community. Available: <http://www.wormbook.org>. Published August 8, 2005.
- 93 White JG, Southgate E, Thomson JN and S. B (1986) The structure of the nervous system of the nematode *C. elegans*. *Philos Trans R Soc Lond B Biol Sci* 314: 1-340.
- 94 Durbin RM (1987) Studies on the development and organisation of the nervous system of *Caenorhabditis elegans* [PhD]. Cambridge, England: University of Cambridge
- 95 Bargmann CI and Kaplan JM (1998) Signal transduction in the *Caenorhabditis elegans* nervous system. *Annu Rev Neurosci* 21: 279-308.
- 96 Bargmann CI and Mori I (1997) Chemotaxis and Thermotaxis. In: Riddle DL, Blumenthal T, Meyer BJ and Priess JR, editors. *C elegans II*: Cold Spring Harbor Laboratory Press.

- 97 Kaplan JM and Horvitz HR (1993) A dual mechanosensory and chemosensory neuron in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 90(6): 2227-2231.
- 98 Hart AC, Sims S and Kaplan JM (1995) Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* 378(6552): 82-85.
- 99 Chalfie M and Sulston J (1981) Developmental genetics of the mechanosensory neurons of *Caenorhabditis elegans*. *Dev Biol* 82(2): 358-370.
- 100 Hobert O and Bulow H (2003) Development and maintenance of neuronal architecture at the ventral midline of *C. elegans*. *Curr Opin Neurobiol* 13(1): 70-78.
- 101 Rhiner C, Gysi S, Frohli E, Hengartner MO and Hajnal A (2005) Syndecan regulates cell migration and axon guidance in *C. elegans*. *Development* 132(20): 4621-4633.
- 102 Moerman DG and Fire A (1997) Muscle: Structure, Function, and Development. In: Riddle DL, Blumenthal T, Meyer BJ and Priess JR, editors. *C elegans II*: Cold Spring Harbor Laboratory Press.
- 103 Moerman DG and Williams BD (2006) Sarcomere assembly in *C. elegans* muscle. *Wormbook The C. elegans Research Community*.
- 104 Francis R and Waterston RH (1991) Muscle cell attachment in *Caenorhabditis elegans*. *J Cell Biol* 114(3): 465-479.
- 105 Hresko MC, Williams BD and Waterston RH (1994) Assembly of body wall muscle and muscle cell attachment structures in *Caenorhabditis elegans*. *J Cell Biol* 124(4): 491-506.
- 106 Bercher M, Wahl J, Vogel BE, Lu C, Hedgecock EM et al. (2001) *mua-3*, a gene required for mechanical tissue integrity in *Caenorhabditis elegans*, encodes a novel transmembrane protein of epithelial attachment complexes. *J Cell Biol* 154(2): 415-426.
- 107 Hresko MC, Schriefer LA, Shrimankar P and Waterston RH (1999) Myotactin, a novel hypodermal protein involved in muscle-cell adhesion in *Caenorhabditis elegans*. *J Cell Biol* 146(3): 659-672.
- 108 Dixon SJ, Alexander M, Fernandes R, Ricker N and Roy PJ (2006) FGF negatively regulates muscle membrane extension in *Caenorhabditis elegans*. *Development* 133(7): 1263-1275.
- 109 Dixon SJ and Roy PJ (2005) Muscle arm development in *Caenorhabditis elegans*. *Development* 132(13): 3079-3092.
- 110 Lewis JA, Wu CH, Berg H and Levine JH (1980) The genetics of levamisole resistance in the nematode *Caenorhabditis elegans*. *Genetics* 95(4): 905-928.
- 111 Fleming JT, Squire MD, Barnes TM, Tornoe C, Matsuda K et al. (1997) *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. *J Neurosci* 17(15): 5843-5857.
- 112 Gally C, Eimer S, Richmond JE and Bessereau JL (2004) A transmembrane protein required for acetylcholine receptor clustering in *Caenorhabditis elegans*. *Nature* 431(7008): 578-582.
- 113 Koga M, Take-uchi M, Tameishi T and Ohshima Y (1999) Control of DAF-7 TGF-(α) expression and neuronal process development by a receptor tyrosine kinase KIN-8 in *Caenorhabditis elegans*. *Development* 126(23): 5387-5398.

- 114 Forrester WC, Dell M, Perens E and Garriga G (1999) A *C. elegans* Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. *Nature* 400(6747): 881-885.
- 115 Francis MM, Evans SP, Jensen M, Madsen DM, Mancuso J et al. (2005) The Ror receptor tyrosine kinase CAM-1 is required for ACR-16-mediated synaptic transmission at the *C. elegans* neuromuscular junction. *Neuron* 46(4): 581-594.
- 116 Ackley BD, Kang SH, Crew JR, Suh C, Jin Y et al. (2003) The basement membrane components nidogen and type XVIII collagen regulate organization of neuromuscular junctions in *Caenorhabditis elegans*. *J Neurosci* 23(9): 3577-3587.
- 117 Hutter H, Vogel BE, Plenefisch JD, Norris CR, Proenca RB et al. (2000) Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes. *Science* 287(5455): 989-994.
- 118 Albertson DG and Thomson JN (1976) The pharynx of *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci* 275(938): 299-325.
- 119 Franks CJ, Holden-Dye L, Bull K, Luedtke S and Walker RJ (2006) Anatomy, physiology and pharmacology of *Caenorhabditis elegans* pharynx: a model to define gene function in a simple neural system. *Invert Neurosci* 6(3): 105-122.
- 120 Avery L and Shtonda BB (2003) Food transport in the *C. elegans* pharynx. *J Exp Biol* 206(Pt 14): 2441-2457.
- 121 Avery L and Horvitz HR (1989) Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron* 3(4): 473-485.
- 122 Avery L (1993) The genetics of feeding in *Caenorhabditis elegans*. *Genetics* 133(4): 897-917.
- 123 McKay JP, Raizen DM, Gottschalk A, Schafer WR and Avery L (2004) *eat-2* and *eat-18* are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. *Genetics* 166(1): 161-169.
- 124 Lee RY, Sawin ER, Chalfie M, Horvitz HR and Avery L (1999) *EAT-4*, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in *Caenorhabditis elegans*. *J Neurosci* 19(1): 159-167.
- 125 Starich TA, Lee RY, Panzarella C, Avery L and Shaw JE (1996) *eat-5* and *unc-7* represent a multigene family in *Caenorhabditis elegans* involved in cell-cell coupling. *J Cell Biol* 134(2): 537-548.
- 126 Kramer JM (2005) Basement membranes. In: *Wormbook*. The *C. elegans* Research Community. Available: <http://www.wormbook.org>. Published September 1, 2005.
- 127 Huang CC, Hall DH, Hedgecock EM, Kao G, Karantza V et al. (2003) Laminin alpha subunits and their role in *C. elegans* development. *Development* 130(14): 3343-3358.
- 128 Zhu X, Joh K, Hedgecock EM and Hori K (1999) Identification of *epi-1* locus as a laminin alpha chain gene in the nematode *Caenorhabditis elegans* and characterization of *epi-1* mutant alleles. *DNA Seq* 10(4-5): 207-217.
- 129 Forrester WC and Garriga G (1997) Genes necessary for *C. elegans* cell and growth cone migrations. *Development* 124(9): 1831-1843.

- 130 Rogalski TM, Williams BD, Mullen GP and Moerman DG (1993) Products of the *unc-52* gene in *Caenorhabditis elegans* are homologous to the core protein of the mammalian basement membrane heparan sulfate proteoglycan. *Genes Dev* 7(8): 1471-1484.
- 131 Mullen GP, Rogalski TM, Bush JA, Gorji PR and Moerman DG (1999) Complex patterns of alternative splicing mediate the spatial and temporal distribution of perlecan/UNC-52 in *Caenorhabditis elegans*. *Mol Biol Cell* 10(10): 3205-3221.
- 132 Merz DC, Alves G, Kawano T, Zheng H and Culotti JG (2003) UNC-52/perlecan affects gonadal leader cell migrations in *C. elegans* hermaphrodites through alterations in growth factor signaling. *Dev Biol* 256(1): 173-186.
- 133 Kang SH and Kramer JM (2000) Nidogen is nonessential and not required for normal type IV collagen localization in *Caenorhabditis elegans*. *Mol Biol Cell* 11(11): 3911-3923.
- 134 Guo XD, Johnson JJ and Kramer JM (1991) Embryonic lethality caused by mutations in basement membrane collagen of *C. elegans*. *Nature* 349(6311): 707-709.
- 135 Baum PD and Garriga G (1997) Neuronal migrations and axon fasciculation are disrupted in *ina-1* integrin mutants. *Neuron* 19(1): 51-62.
- 136 Williams BD and Waterston RH (1994) Genes critical for muscle development and function in *Caenorhabditis elegans* identified through lethal mutations. *J Cell Biol* 124(4): 475-490.
- 137 Schwarzbauer JE and Spencer CS (1993) The *Caenorhabditis elegans* homologue of the extracellular calcium binding protein SPARC/osteonectin affects nematode body morphology and mobility. *Mol Biol Cell* 4(9): 941-952.
- 138 Hesselson D, Newman C, Kim KW and Kimble J (2004) GON-1 and fibulin have antagonistic roles in control of organ shape. *Curr Biol* 14(22): 2005-2010.
- 139 Vogel BE and Hedgecock EM (2001) Hemicentin, a conserved extracellular member of the immunoglobulin superfamily, organizes epithelial and other cell attachments into oriented line-shaped junctions. *Development* 128(6): 883-894.
- 140 Ruedg MA and Bixby JL (1998) Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction. *Trends Neurosci* 21(1): 22-27.
- 141 Martin PT (2003) Dystroglycan glycosylation and its role in matrix binding in skeletal muscle. *Glycobiology* 13(8): 55R-66R.
- 142 Millet AC and Ewbank JJ (2004) Immunity in *Caenorhabditis elegans*. *Curr Opin Immunol* 16(1): 4-9.
- 143 Jorgensen EM and Mango SE (2002) The art and design of genetic screens: *caenorhabditis elegans*. *Nat Rev Genet* 3(5): 356-369.
- 144 Letunic I, Copley RR, Pils B, Pinkert S, Schultz J et al. (2006) SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res* 34(Database issue): D257-260.
- 145 Bendtsen JD, Nielsen H, von Heijne G and Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340(4): 783-795.

- 146 Tsalik EL, Niacaris T, Wenick AS, Pau K, Avery L et al. (2003) LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the *C. elegans* nervous system. *Dev Biol* 263(1): 81-102.
- 147 Bessou C, Giugia JB, Franks CJ, Holden-Dye L and Segalat L (1998) Mutations in the *Caenorhabditis elegans* dystrophin-like gene *dys-1* lead to hyperactivity and suggest a link with cholinergic transmission. *Neurogenetics* 2(1): 61-72.
- 148 Gieseler K, Bessou C and Segalat L (1999) Dystrobrevin- and dystrophin-like mutants display similar phenotypes in the nematode *Caenorhabditis elegans*. *Neurogenetics* 2(2): 87-90.
- 149 Chen L, Krause M, Draper B, Weintraub H and Fire A (1992) Body-wall muscle formation in *Caenorhabditis elegans* embryos that lack the MyoD homolog *hlh-1*. *Science* 256(5054): 240-243.
- 150 Rogalski TM, Gilchrist EJ, Mullen GP and Moerman DG (1995) Mutations in the *unc-52* gene responsible for body wall muscle defects in adult *Caenorhabditis elegans* are located in alternatively spliced exons. *Genetics* 139(1): 159-169.
- 151 Strome S (1986) Fluorescence visualization of the distribution of microfilaments in gonads and early embryos of the nematode *Caenorhabditis elegans*. *J Cell Biol* 103(6 Pt 1): 2241-2252.
- 152 Campagnola PJ, Millard AC, Terasaki M, Hoppe PE, Malone CJ et al. (2002) Three-dimensional high-resolution second-harmonic generation imaging of endogenous structural proteins in biological tissues. *Biophys J* 82(1 Pt 1): 493-508.
- 153 Koushika SP, Richmond JE, Hadwiger G, Weimer RM, Jorgensen EM et al. (2001) A post-docking role for active zone protein Rim. *Nat Neurosci* 4(10): 997-1005.
- 154 Nguyen M, Alfonso A, Johnson CD and Rand JB (1995) *Caenorhabditis elegans* mutants resistant to inhibitors of acetylcholinesterase. *Genetics* 140(2): 527-535.
- 155 Avery L and Thomas JH (1997) Feeding and Defecation. In: Riddle DL, Blumenthal T, Meyer BJ and Priess JR, editors. *C. elegans II*: Cold Spring Harbor Laboratory press.
- 156 Schedl T (1997) Developmental Genetics of the Germ Line. In: Riddle DL, Blumenthal T, Meyer BJ and Priess JR, editors. *C. elegans II*: Cold Spring Harbor Laboratory Press. pp. 241.
- 157 Grewal PK and Hewitt JE (2003) Glycosylation defects: a new mechanism for muscular dystrophy? *Hum Mol Genet* 12 Spec No 2: R259-264.
- 158 Johnson RP, Kang SH and Kramer JM (2006) *C. elegans* dystroglycan DGN-1 functions in epithelia and neurons, but not muscle, and independently of dystrophin. *Development* 133(10): 1911-1921.
- 159 Lee M, Cram EJ, Shen B and Schwarzbauer JE (2001) Roles for beta(pat-3) integrins in development and function of *Caenorhabditis elegans* muscles and gonads. *J Biol Chem* 276(39): 36404-36410.
- 160 Brown NH (2000) Cell-cell adhesion via the ECM: integrin genetics in fly and worm. *Matrix Biol* 19(3): 191-201.
- 161 Krause M (1995) Techniques for analyzing transcription and translation In: Epstein HF and Shakes D, editors. *Caenorhabditis elegans: Modern Biological Analysis of an Organism*. San Diego, CA: Academic Press. pp. 515.

- 162 Sulston JE and Brenner S (1974) The DNA of *Caenorhabditis elegans*. *Genetics* 77(1): 95-104.
- 163 Epstein H and Shakes D (1995) *Caenorhabditis elegans: Modern Biological Analysis of an Organism.*: Academic Press, San Diego, CA.
- 164 Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD et al. (2003) ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* 31(13): 3784-3788.
- 165 Pagni M, Ioannidis V, Cerutti L, Zahn-Zabal M, Jongeneel CV et al. (2004) MyHits: a new interactive resource for protein annotation and domain identification. *Nucleic Acids Res* 32(Web Server issue): W332-335.
- 166 Clamp M, Cuff J, Searle SM and Barton GJ (2004) The Jalview Java alignment editor. *Bioinformatics* 20(3): 426-427.
- 167 Rice P, Longden I and Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 16(6): 276-277.
- 168 Sambrook J, Fritsch EF and Maniatis T (1989) *Molecular cloning: a laboratory manual*: Cold Spring Harbor Laboratory Press, New York.
- 169 Mello CC, Kramer JM, Stinchcomb D and Ambros V (1991) Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *Embo J* 10(12): 3959-3970.
- 170 Bessereau JL, Wright A, Williams DC, Schuske K, Davis MW et al. (2001) Mobilization of a *Drosophila* transposon in the *Caenorhabditis elegans* germ line. *Nature* 413(6851): 70-74.
- 171 Chow DK, Glenn CF, Johnston JL, Goldberg IG and Wolkow CA (2006) Sarcopenia in the *Caenorhabditis elegans* pharynx correlates with muscle contraction rate over lifespan. *Exp Gerontol* 41(3): 252-260.
- 172 Hedgecock EM, Culotti JG, Thomson JN and Perkins LA (1985) Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes. *Dev Biol* 111(1): 158-170.
- 173 Pearson CA, Pearson D, Shibahara S, Hofsteenge J and Chiquet-Ehrismann R (1988) Tenascin: cDNA cloning and induction by TGF-beta. *Embo J* 7(10): 2977-2982.
- 174 Fischer D, Chiquet-Ehrismann R, Bernasconi C and Chiquet M (1995) A single heparin binding region within the fibrinogen-like domain is functional in chick tenascin-C. *J Biol Chem* 270(7): 3378-3384.
- 175 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259): 680-685.
- 176 Finney M and Ruvkun G (1990) The unc-86 gene product couples cell lineage and cell identity in *C. elegans*. *Cell* 63(5): 895-905.
- 177 Miller DM, 3rd, Ortiz I, Berliner GC and Epstein HF (1983) Differential localization of two myosins within nematode thick filaments. *Cell* 34(2): 477-490.

- 178 Wallace BG (1986) Aggregating factor from Torpedo electric organ induces patches containing acetylcholine receptors, acetylcholinesterase, and butyrylcholinesterase on cultured myotubes. *J Cell Biol* 102(3): 783-794.
- 179 Culotti JG, Von Ehrenstein G, Culotti MR and Russell RL (1981) A second class of acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans*. *Genetics* 97(2): 281-305.
- 180 Bulow HE and Hobert O (2004) Differential sulfations and epimerization define heparan sulfate specificity in nervous system development. *Neuron* 41(5): 723-736.
- 181 Lee JS and Chien CB (2004) When sugars guide axons: insights from heparan sulphate proteoglycan mutants. *Nat Rev Genet* 5(12): 923-935.
- 182 Morio H, Honda Y, Toyoda H, Nakajima M, Kurosawa H et al. (2003) EXT gene family member rib-2 is essential for embryonic development and heparan sulfate biosynthesis in *Caenorhabditis elegans*. *Biochem Biophys Res Commun* 301(2): 317-323.
- 183 Ward S, Thomson N, White JG and Brenner S (1975) Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. *J Comp Neurol* 160(3): 313-337.
- 184 Driscoll M and Kaplan JM (1997) Mechanotransduction. In: Riddle DL, Blumenthal, T., Meyer, B.J., Priess, J.R., editor. *C elegans II*: Cold Spring Harbor Laboratory Press.
- 185 Avery L, Raizen D and Lockery S (1995) Electrophysiological methods. *Methods Cell Biol* 48: 251-269.
- 186 Dent JA, Smith MM, Vassilatis DK and Avery L (2000) The genetics of ivermectin resistance in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 97(6): 2674-2679.
- 187 Davis MW, Somerville D, Lee RY, Lockery S, Avery L et al. (1995) Mutations in the *Caenorhabditis elegans* Na,K-ATPase alpha-subunit gene, *eat-6*, disrupt excitable cell function. *J Neurosci* 15(12): 8408-8418.
- 188 Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV et al. (2003) Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. *PLoS Biol* 1(1): E12.
- 189 Kennedy S, Wang D and Ruvkun G (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* 427(6975): 645-649.