The Role of Transmembrane Agrin in Reorganizing the Cytoskeleton in Neurons and Non-Neuronal Cells

Inauguraldissertation

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

von
Godela Bittcher
aus Zürich

Basel, 2006

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

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Basel, den 16. 11. 2004

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Summary

The brain belongs to the most fascinating organs that developed in evolution. Its complexity is responsible for recording and organizing impressions from the environment, for our thoughts and feelings, for our personality. Knowledge of the mechanisms involved in the development of the brain, in thinking and transmission of neural signals is likely to also help our understanding of disease mechanisms underlying Alzheimer's and Parkinson's disease or neuromuscular diseases including muscular dystrophies.

The brain is a complex network of billions of neurons and supporting cells. Ramòn Y Cajal showed in the 19th century with the Golgi-technique that each neuron is a unit that communicates with other neurons by special contacts called synapses (Cajal, 1928). Most of our current knowledge of how synapses work and of how they develop derives from our profound understanding of the neuromuscular junction (NMJ), which is relatively simply structured and organized. The key regulator for the development and maintenance of the NMJ is the highly glycosylated heparansulfate proteoglycan (HSPG) agrin. Owing to alternative first exon usage, agrin can be expressed in a secreted form (SS-NtA-Agrin) or a transmembrane form (TM-Agrin). The amino-terminus of SS-NtA-Agrin binds to the extracellular matrix (ECM) via laminin. That of TM-Agrin consists of a short intracellular and a transmembrane region. TM-Agrin is preferentially expressed in the CNS, particularly by neurons of the brain (Neumann *et al*., 2001).

This thesis examines the function of TM-Agrin in non-neuronal and neuronal cells. Using transfection of cDNAs encoding full-length TM-Agrin, chimeric constructs and mutants thereof, I show that TM-Agrin has a strong effect on cell morphology. In particular, during my research, cells expressing TM-Agrin formed long and numerous actin-containing microprocesses. In the chimeric constructs I replaced the intracellular part, the extracellular part or the TM-domain of TM-Agrin with a corresponding part of another TM-protein. In the mutant the glycosaminoglycan (GAG)-attachment site between the 7th and 8th follistatin-like (FS) domain was mutated so that sugar chain could not attach. By this means I managed to elucidate that the described effect is dependent on the close association of the extracellular part of TM-Agrin with the membrane and, additionally, on the presence of the GAG-chain localized between the 7th and 8th FS domain.

To evaluate whether similar effects of TM-Agrin can also be observed in neuronal cells, we also transfected primary hippocampal mouse neurons. Indeed, transfected neurons

showed a curvy growth and developed microspikes on axons and dendrites indicating that TM-Agrin also affects neuritogenesis. To test whether these effects could be based on overexpression-induced self-dimerization of TM-Agrin, and whether TM-Agrin could directly activate a signalling cascade, we also used antibody-induced dimerization, a method that has been shown to allow activation of single transmembrane domain receptors (Heldin, 1995; Weiss and Schlessinger, 1998). Indeed anti-agrin antibodies induced dose- and time-dependent formation of microspikes on primary mouse hippocampal neurons, suggesting that TM-Agrin may have a function in inducing the reorganization of the actin-cytoskeleton and also in development of neurites and their outgrowth.

In the last part of the work we created a transgenic mouse in which the expression of a miniaturized version of mouse neural agrin could be temporally controlled. In Duchenne muscular dystrophy (DMD), dystrophin has mutated, which leads to fragility of muscle membranes to cause muscle wasting. It had been shown that overexpression of utrophin, an autosomal homologue of dystrophin can functionally compensate for the loss of dystrophin. With this mouse model we tested whether overexpression of agrin also causes upregulation of utrophin *in vivo*. This could be an appropriate way to ameliorate and eventually also cure DMD.

Abbreviations

A	alanine
aa	amino acid
AB	antibody
AChE	acetylcholine esterase
AChR	acetylcholine receptor
APC	antigen presenting cells
ASGP-R	asialoglycoprotein receptor
BAC	Bacterial Artificial Chromosome
BBB	blood-brain barrier
BSA	bovine serum albumin
CNS	central nervous system
DAP	dystrophin-associated proteins
DIV	days in vitro
DMD	Duchenne muscular dystrophy
E	embryonic day of development
ECD	extracellular domain
ECM	extracellular matrix
FCS	fetal calf serum
FS	follistatin like
GABDH	glyceraldehyde3-phosphate dehydrogenase
GAG	glycosaminoglycan
GAM	goat anti-mouse
GAR	goat anti-rabbit
HEK	human embryonic kidney cells
HoS	horse serum
HSPG	heparan sulphate proteoglycan
IS	immunological synapse
kb	kilobase
kDa	kilodalton
LG	laminin G-like

MHC-p	histocompatibility-peptide complexe
MuSK	rat homologue of the muscle specific kinase (nowadays used for all species)
NBM	neurobasal medium
NCAM	neural adhesion molecule
NGF	neural growth factor
NGS	normal goat serum
NMJ	neuromuscular junction
NP	nanopure
Nsk2	mouse homologue of the muscle specific kinase (old nomenclature)
NtA	N-terminal of agrin
oN	overnight
P/S	penicillin/streptomycin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PNS	peripheral nervous system
RT	room temperature
S	serine
S/T	serine-threonine rich
SG	serine-glycine
TM	transmembrane domain

Chapter I

General Introduction

The Synapse

The word ‘synapse’ derives from Greek ‘synaptein’ (‘syn’ meaning ‘together’ and ‘haptein’ meaning ‘fasten). First described for neurons, synapses form a highly specialized structure to guarantee a rapid and precise communication between neurons and their target cells. These junctions are responsible for the precise transformation of information from the pre- to the postsynaptic cell. The information is relayed by directed secretion of neurotransmitters. Most vertebrate synapses are activated by an action potential that lead to a subsequent Ca^{2+} influx in the presynaptic terminal. This results in fusion of vesicles with the nerve membrane and the neurotransmitter release into the synaptic cleft. The neurotransmitter diffuses and binds to specific receptors in the postsynaptic membrane. The postsynaptic membrane potential changes and can trigger the propagation of the action potential. During synaptogenesis, synapses form, mature, and stabilize and are also eliminated by a process that requires intimate communication between pre- and postsynaptic partners. Most of our understanding of synapse formation, maturation and stabilization comes from extensive studies on the neuromuscular junction (NMJ). The NMJ describes the close contact and interaction between motor neuron and the target muscle cell. Three most widely appreciated experimental advantages, its large size, its relative simplicity and unparalleled accessibility make this type of synapse a suitable model for understanding synapse development and function (Sanes and Lichtman, 1999).

Most of our knowledge and understanding about synapse formation, stabilization and function in the CNS derives from intensive studies of vertebrate NMJ. Recent advantages in methodologies, including real-time imaging of living neurons, allow a deeper insight into the molecular, cellular, and activity-dependent processes that guide synaptogenesis in the developing CNS. But nevertheless there are still many open questions.

The immune system also developed an elaborate system of cell-cell communication. The immunological synapse (IS) describes the direct contact between T-cells and the antigen presenting cells (APCs). Roaming through the body, APCs (e.g. dendritic cells, B cells or activated macrophages) capture and ingest infectious microbes. The pieces of their cut proteins (antigens) are displayed on their surfaces. These antigens, also called major

histocompatibility-peptide complexes (MHC-p), are inspected by the immune system's T lymphocytes, which decide whether to mount an antigen-specific immune response to the invader.

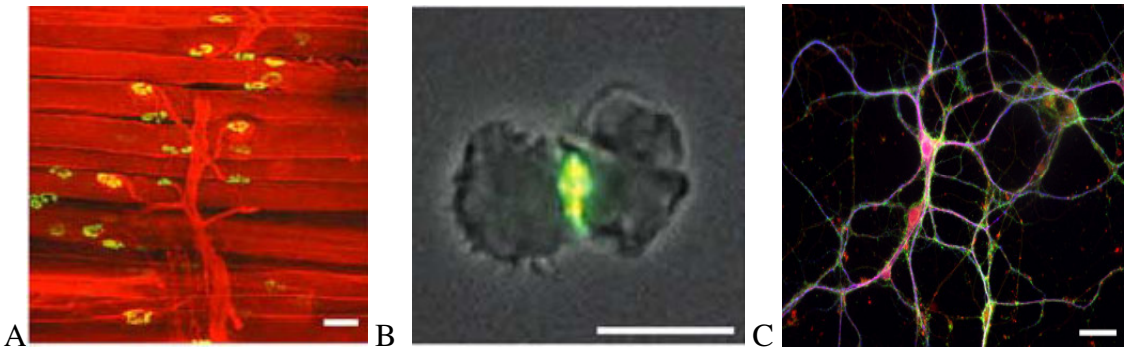

Fig. 1: Pictures NMJ, synapse in brain and immunological synapse

(A) A whole-mount view of neuromuscular synapses in rat soleus muscle. Nerve branches from presynaptic motor neurons and the postsynaptic muscle fibers were visualized with antibodies against laminin α2 (red). Postsynaptic AChRs (green or yellow) accumulate at the neuromuscular junctions. (B) At the immunological synapse, the T-cell receptor–CD3 complex (red) and agrin (green) aggregate at the site of contact between an antigen-presenting cell (APC) and an activated T cell (yellow). (C) Primary neurons stained at 20.day in vitro (DIV) for TM-Agrin (red), for the dendritic marker MAP2 (blue) and for the presynaptic marker Synaptophysin (green).
Scale bar in A is 50 µm, in B 10 µm and in C 50 µm. Note the marked differences in synapse number and size among the tissues.
(A and B from Bezakova *et al.*, 2003)

Agrin

During the last decades significant efforts have been made to understand the formation, structure and function of the postsynaptic apparatus. One of the important findings has been that the formation and stabilization of the postsynaptic structure is dependent on molecules provided by the axon (for review see Dennis, 1981). Later studies have shown that agrin is such a molecule. This protein was first discovered and purified from basal lamina containing extracts of the electric organ of the marine ray *Torpedo californica* (Godfrey *et al.*, 1984). Basal lamina-containing extracts showed AChR- and AChE-aggregating activity when added to myotubes (Godfrey *et al.*, 1984; Nitkin *et al.*, 1987; Magill-Solc and McMahan, 1988). This aggregating factor was then called “agrin”, which derives from the Greek ‘agrein’, meaning ‘assemble’. This highly glycosylated heparansulfate proteoglycan (HSPG) protein is a key organizer of postsynaptic structure of the NMJ (Ruegg and Bixby, 1998). Agrin also has an important role in presynaptic differentiation because it acts as a stop signal for motor neurons (Campagna *et al.*, 1995;

Campagna *et al.*, 1997). Later findings also provide evidence that the activity of agrin may not be restricted to the NMJ. The protein is also highly expressed in the CNS (Rupp *et al.*, 1991; Burgess *et al.*, 2000; Neumann *et al.*, 2001; Kroger und Schroder, 2002). Additionally, agrin was found at the immunological synapse (Khan *et al.*, 2001).

Thus agrin plays a crucial and well understood role at the NMJ, but its activities in the CNS might be of broader significance than previously assumed.

The Agrin Gene

Alternative splicing of the agrin cDNA generates agrin proteins that differ in their distribution and function (for review see Bezakova and Ruegg, 2003). The 3'end is very well characterized and highly conserved. Splice sites at the 3'end, called A and B (y and z in rodents), have a strong influence on the biological activity of agrin (Ruegg *et al.*, 1992; Ferns *et al.*, 1993). Splice site A contains 0 or 4 amino acids (aas); site B includes 0, 8, 11 or 19 aas (Ruegg *et al.*, 1992; Rupp *et al.*, 1992a). Only those splice variants containing amino acid inserts are active in inducing AChR clustering and are essential for the development of the NMJ. Inserts at B/z site are crucial for agrin-induced postsynapse-inducing activity in vivo (Ruegg *et al.*, 1992; Burgess *et al.*, 1999).

Isoforms with B/z inserts are expressed by motor neurons and are referred to as “active”. Those that lack aa inserts are expressed by the muscle fibers and are inactive in AChR clustering.

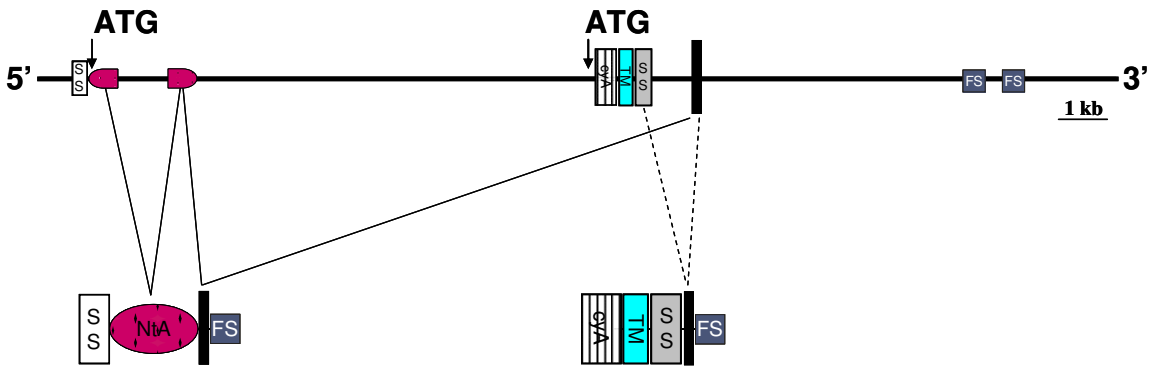

Fig. 2: 5'end of mouse agrin gene encodes two different amino-termini

Both agrin isoforms, the secreted protein SS-NtA-Agrin and the transmembrane protein typeII TM-Agrin, are expressed from the same gene. The sequence of NtA (N-terminus of agrin) is encoded by two exons and that of the N-terminus of TM-Agrin by only one. Alternative first exon usage results in the two different forms and is indicated by continuous line and broken line respectively.

In addition, alternative first exon usage results in the synthesis of two alternative amino-termini. The agrin gene therefore codes for a secreted protein (SS-NtA-Agrin) and for a typeII transmembrane protein (TM-Agrin; Burgess *et al*. 2000; Neumann *et al.*, 2001).

The Agrin Structure

The highly glycosylated heparansulfate proteoglycan agrin is differentially localized and strongly alters its function owing to alternative splicing and alternative first exon usage.

At the amino-terminus SS-NtA-Agrin includes a short signal sequence, which is cleaved so that the protein can be secreted. This domain is highly conserved among species. A start methionine is located in the KOZAK sequence (Kozak *et al*., 1986). The NtA domain contains a laminin binding site, which is crucial for the binding to the γ1-chains of laminins and thus for the immobilization of agrin in the basal lamina (Denzer *et al*., 1995; Denzer *et al*., 1997). A splice site at the 5'end that includes a 7-aa-long insert has also been found in chicken. This splice variant has a decreased affinity to laminin (Denzer *et al*., 1997).

TM-Agrin contains a short, not conserved cytoplasmic part and a transmembrane domain that is conserved among species and converts agrin to a typeII transmembrane protein and localizes the protein in an N_{cyto}/C_{exo} orientation in the plasma membrane (Neumann *et al*., 2001).

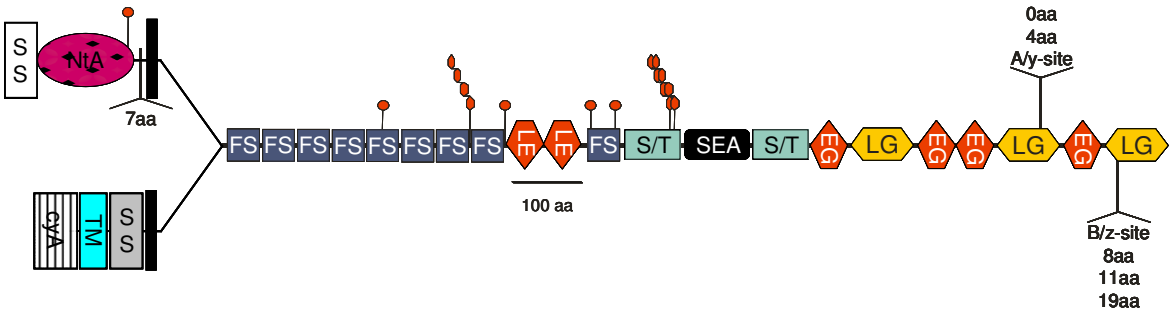

Fig. 3: Agrin domains of the secreted and of the transmembrane protein

When the protein is secreted, the signal sequence is cleaved. The SS-NtA-Agrin has a laminin-binding site which is essential for the binding of agrin to the basal lamina. This site is absent in TM-Agrin. This isoform is inserted in the plasmamembrane. It has a short cytoplasmic part and a transmembrane domain that shows high homology across species.

The following domains are attached to the amino-terminus:

Follistatin-like domains (FS)

A conserved glycosaminoglycan (GAG) chain attachment site is localized between the 7th and the 8th FS domain.

Laminin-EGF-like domains (LE)

One of such domains in the laminin γ1-chain binds nidogen. LE domains in agrin do not bind to nidogen.

Serine/Threonine-rich domains (S/T)

The first S/T domain carries 2 GAG chains.

Sea urchin, enterokinase, agrin domains (SEA)

This domain was first found in a sea urchin sperm protein, in enterokinase and in agrin.

EGF-like domains (EG)

These regions are characterized by a motif of 6 regularly spaced cysteine residues linked via disulfide bridges.

Laminin G-like domains (LG)

Two of these domains contain the A/y and B/z splice sites, which are characteristic for the nerve- or the muscle-derived agrin isoforms.

The agrin gene encodes in all species a protein with more than 2000 aas with a predicted mass of 225kDa. On SDS-PAGE the protein shows a diffuse band of about 400-600kDa. Further studies have shown that agrin is a heparan sulfate proteoglycan (Tsen *et al.*, 1995b). Glycosaminoglycan (GAG) attachment sites are localized to two locations within the agrin molecule. One site is localized between the 7th and 8th FS-domain. Three closely spaced serine-glycine (SG) clusters carry exclusively heparansulfate chains. The second GAG attachment site is positioned in the centrally located S/T-domain and contains a cluster of four closely packed SG consensus sequences. In contrast to the first GAG attachment site, the second carries mostly chondroitin sulfate side chains. Agrin exhibits

mixed glycosylation patterns so that agrin is not only a heparansulfate but also a chondroitin sulfate proteoglycan (Winzen *et al*., 2003).

Agrin at the Neuromuscular Junction

Several lines of evidence have led to the 'agrin hypothesis' (McMahan, 1990) that postulates that the heparansulfate proteoglycan agrin is synthesized in motor neurons and transported along the axons to the muscles where it is secreted into the synaptic cleft. There it binds to a still unknown agrin receptor on the myotube surface and causes acetylcholine receptors (AChRs), acetylcholinesterase (AChE) and proteins of the postsynaptic apparatus to aggregate. Agrin was then molecularly cloned from mammals and birds (Rupp *et al*., 1991; Tsim *et al*., 1992). The 'agrin-hypothesis' was confirmed by numerous independent groups and this gave rise to a much deeper understanding on mechanisms involved in formation and maintenance of NMJ (Gautam *et al*., 1996; DeChiara *et al*., 1996; Glass *et al*., 1996). The crucial role of agrin for synaptogenesis became obvious.

Agrin Affects the Pre- and Postsynaptic Differentiation at the NMJ

The NMJ is a precisely organized structure that is composed of the presynaptic nerve terminal of the motor neuron, the postsynaptic muscle cell and the Schwann cell.

Prior to innervation AChRs are freely diffusible and randomly distributed in the muscle cell plasma membrane and at a low density (~1000/ μm^2). The active isoform of agrin, also called neural agrin (for review see Ruegg and Bixby, 1998) contains an insert at the B/z splice site, is synthesized by motor neurons in the ventral horn of the spinal cord and then transported along the axons to the nerve terminal where it is secreted into the synaptic cleft. There it binds to laminin in the basal lamina (Denzer *et al*., 1997) and acts as a key regulator for the postsynaptic specialization. As soon as the growth cone of the developing motor neuron gets into contact with the muscle fiber, the distribution of AChR clusters begins to change dramatically. The synaptic density of the receptors increases to ~10000/μm^2, the extrasynaptic density decreases to <10/μm^2.

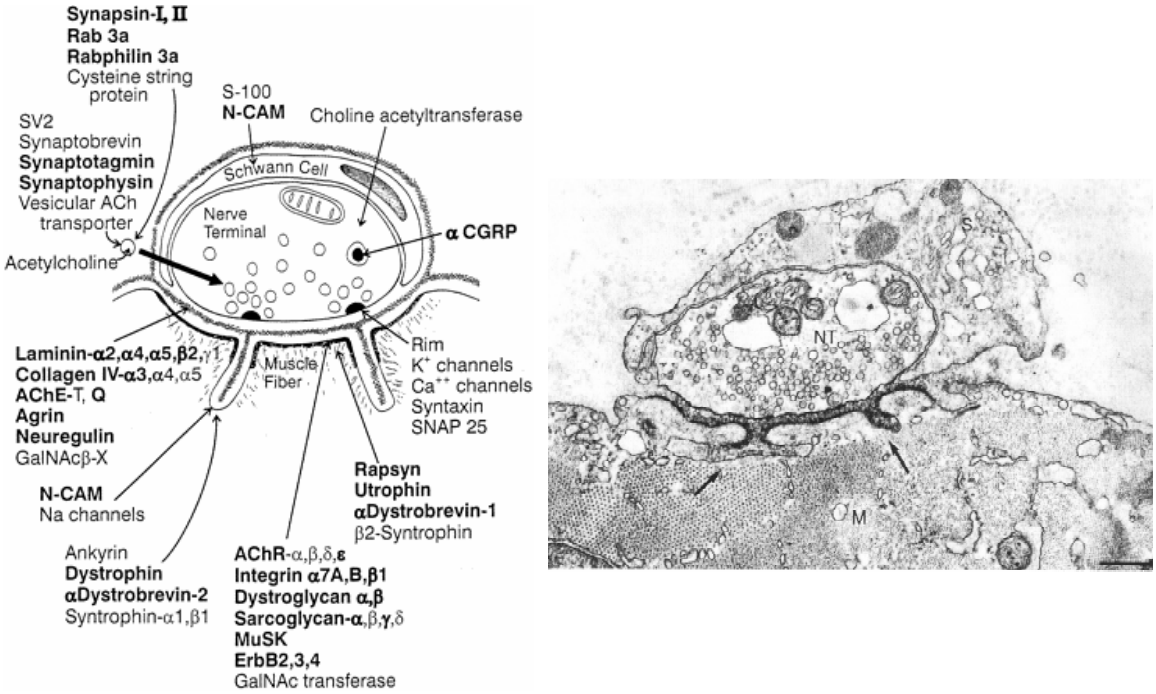

Fig. 4: The complexity of the NMJ

The NMJ consists of the postsynaptic nerve terminal of the motor neuron (NT), the postsynaptic muscle membrane (M) and the surrounding Schwann cell (S) (Burden *et al*., 1979; Sanes and Lichtman, 1999).

Neural agrin is active in aggregating AChRs, AChE and other proteins in the postsynapse. Agrin-deficient mice do not develop postsynaptic structures and die perinatally, because of respiratory failure (Gautam *et al.*, 1996; for review see Ruegg and Bixby, 1998). The number and density of AChR clusters and the transcription of synapse-specific genes is notably reduced. Many proteins have been identified to bind directly to agrin, but only the muscle-specific receptor tyrosine kinase MuSK is required for agrin activity (Gautam *et al.*, 1996). Mice lacking MuSK, which are similar to agrin-deficient mice fail, to induce postsynaptic specialization and die at birth (DeChiara *et al*., 1996).

The axon of the motor neuron does not stop growing and does not differentiate into a presynaptic terminal. Agrin influences the growth and differentiation of motor neurons, which means that agrin is also involved in presynaptic differentiation. These observations show that agrin is indispensable for the formation of both pre- and postsynaptic structures.

Agrin in the Central Nervous System

Most of our knowledge concerning the function of agrin derives from studies on the NMJ. Agrin is also highly expressed in the CNS. However its function in the CNS is mostly unknown. Owing to alternative mRNA splicing at the 5'end and alternate transcriptional start sites, two protein isoforms are characterized by their short or long amino-termini (Burgess *et al.*, 2000; Neumann *et al.*, 2001). The isoform with the long one (SS-NtA-Agrin) can bind to γ 1-chains of laminins in the ECM (Denzer *et al.*, 1997) and is present in muscle and other peripheral tissue. In contrast, the isoform characterized by the short amino-terminus (TM-Agrin), which lacks the laminin binding site, is a transmembrane protein typeII that is mainly expressed in the CNS. Owing to the high similarity of these two isoforms, they differ only in the amino-terminus. It is suggested that agrin might play a similar role in the CNS as at the NMJ (McMahan, 1990; Kirsch *et al.*, 1996; Kroger and Schroder, 2002). Within the CNS, agrin is expressed in both non-neuronal and neuronal cells. Glial cells exclusively express the agrin isoform B/z0 that is inactive in AChR aggregation whereas neurons and also blood vessels express both B/z0 and B/z+ isoforms respectively (Kroger and Schroder, 2002). Agrin seems to exhibit much broader functions than has been assumed so far, synaptic and non-synaptic functions. However, the role of agrin in the brain is controversial. Mice lacking agrin show no abnormalities in brain development (Gautam *et al.*, 1996). On the other hand, normal synaptic transmission is impaired when agrin expression is suppressed in cultured hippocampal neurons (Ferreira, 1999).

Non-Synaptic Functions of Agrin in the Central Nervous System

Agrin is highly expressed in the ventricular and subventricular zone of the early developing brain suggesting that agrin is responsible for neuro- and also gliogenesis (for review see Smith and Hilgenberg, 2002). The expression level decreases in adult brains (Stone and Nikolics, 1995; Cohen *et al.*, 1997a). Mice that lack agrin specifically in the brain survive, in contrast to homozygote agrin$^{-/-}$ mice, but they have smaller brains than wildtype-mice (Serpinskaya *et al.*, 1999). There are no consistent differences between the histology of mutant and wildtype mice brains.

Agrin may also play a central role in neurite outgrowth and axonogenesis as proteoglycans provide neurite outgrowth at an astrocyte boundary (Powell *et al.*, 1997). The function as a

'stop-signal' (Campagna *et al.*, 1995) is consistent with experiments where agrin is blocked and axonal and dendritic elongation persists (Mantych and Ferreira, 2001) and presynaptic differentiation is impaired and axonal arborization reduced (Bose *et al.*, 2000). This multifunctional protein is also upregulated in the brain microvascular basal lamina during the development of the blood-brain barrier, around the time the vasculature becomes impermeable. Thus, agrin may function as an important player in the formation and maintenance of cerebral microvascular impermeability. (BBB; Barber and Lieth, 1997; Kroger and Schroder, 2002) It can also bind to β-amyloid and therefore accumulates in the amyloid plaques in the brain of Alzheimer's patients (Cotman *et al*., 2000).

Synaptic Functions of Agrin in the Central Nervous System

The broad and predominantly neuronal distribution of agrin mRNAs in the CNS suggests that agrin may also play a role in the formation and maintenance of central synapses between neurons in the brain (O'Connor *et al.*, 1994; Ma *et al.,* 1994). It is also concentrated at the synapses of the retina (Ma *et al.,* 1994; Mann and Kroger, 1996). Agrin, which is shown to play an essential role in the formation and maintenance of the NMJ, is also assumed to be important in the function of neuronal synapses in the CNS (Mann and Kroger, 1996; Koulen *et al.*, 1999; Gingras and Ferns, 2001) because of higher concentration at synapses in the developing brain during the period of the synapse formation (Li *et al.*, 1997). Much of the knowledge about agrin function during synaptogenesis in the CNS derives from suppression experiments, where it has been shown that inhibition of agrin expression on cultured hippocampal neurons decreases the number of GABA receptor clusters (Ferreira, 1999). The observation that agrin plays a role in differentiation of neural tissue provides evidence for an agrin receptor whose characterization is a crucial step for the understanding of agrin in the brain.

Aim of Thesis

Agrin is widely expressed in non-neuronal as well as neuronal cells. Its function is well studied and understood in skeletal muscle where it plays a key role during the formation, maintenance and regeneration of the neuromuscular junction (for review see Ruegg and Bixby, 1998; Sanes and Lichtman, 2001). But only little is known about agrin's role in other tissues, in particular in the CNS (for review see Kroger and Schroder, 2002). There is

accumulation of evidence that agrin is not only a component of the extracellular matrix but that it can also be expressed as a transmembrane protein (Burgess *et al.*, 2000; Neumann *et al.*, 2001). The aim of this thesis has been to analyse and elucidate the effect of TM-Agrin expression on non-neuronal and neuronal cells and to get a deeper understanding of the role of agrin in the brain (Chapter II and III).

Agrin is the key neural factor that controls muscle postsynaptic differentiation, including the induction of synapse-specific gene transcription. In Duchenne muscular dystrophy, the x-linked gene encoding dystrophin has mutated. It has been shown that utrophin can functionally compensate dystrophin. Agrin is a potential factor to upregulate utrophin expression (Gramolini *et al*., 1998). Thus the second aim of the thesis has been to create a mouse model where expression of agrin can be temporally controlled to test whether such upregulation and the subsequent formation of postsynaptic-like structures could be a means to ameliorate the phenotype of mdx mice, a mouse model for Duchenne muscular dystrophy (Chapter IV).

Chapter II

An Alternative Amino-Terminus Expressed in the Central Nervous System Converts Agrin to a Type II Transmembrane Protein

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Published in Molecular and Cellular Neuroscience 17, 208-225 (2001)

Key words: neuromuscular junction; synapse; non-cleaved signal anchor; type II transmembrane protein; heparansulfate proteoglycan; retina

Summary

Agrin is a basal lamina-associated heparansulfate proteoglycan that is a key molecule in the formation of the vertebrate neuromuscular junction. The carboxy-terminal part of agrin is involved in its synaptogenic activity. The amino-terminal end of chick agrin consists of a signal sequence, required for the targeting of the protein to the secretory pathway, and the amino-terminal agrin (NtA) domain that binds to basal lamina-associated laminins. The cDNA encoding rat agrin lacks this NtA domain and instead codes for a shorter amino-terminal end. While the NtA domain is conserved in several species, including human, sequences homologous to the amino-terminus of rat agrin have not been described. In this work, we have characterized these amino-terminal sequences in mouse and chick. We show that they all serve as a nonleaded signal anchor (SA) that immobilizes the protein in an N_{cyto}/C_{exo} orientation in the plasma membrane. Like the secreted form, this transmembrane form of agrin is highly glycosylated indicative of a heparansulfate proteoglycan. The structure of the 5'end of the mouse agrin gene suggests that a distinct promoter drives expression of the transmembrane form. Agrin transcripts encoding this form are enriched in the embryonic brain, particularly in neurons. To our knowledge, this is the first example of a molecule that is synthesized both as a basal lamina and a plasma membrane protein.

Introduction

Several lines of evidence have indicated that the heparansulfate proteoglycan agrin is a key regulator of synaptogenesis at the developing and regenerating neuromuscular junction (NMJ; for review see Ruegg and Bixby, 1998). For example, agrin induces the formation of postsynaptic specializations including aggregates of acetylcholine receptors (AChRs) and other molecules on cultured myotubes *in vitro* (Nitkin *et al.,* 1987; Reist *et al.,* 1992). Targeted inactivation of agrin in mice results in grossly malformed NMJs, which display only very few pre- and postsynaptic specializations (Gautam *et al.,* 1996). Accordingly, these mice die at birth due to nonfunctional respiratory musculature (Gautam *et al.,* 1996). In addition, ectopic expression of agrin in extrajunctional regions of skeletal muscle fibers triggers the formation of postsynaptic specializations that includes the induction of endplate-specific gene transcription of the ε-subunit of the AChR, characteristic of the mature AChR (Jones *et al.*, 1997; Meier *et al.*, 1998b; Rimer *et al.*, 1997). This activation

of synaptic gene transcription is likely to involve the Ets-related transcription factor GABP (Schaeffer *et al.,* 1998; Briguet and Ruegg, 2000). In summary, agrin is necessary and sufficient for the induction of postsynaptic specializations at the NMJ.

Agrin cDNAs have been isolated from rat, chick, marine ray, and human (Rupp *et al.,* 1991; Tsim *et al.,* 1992; Smith *et al.,* 1992; Groffen *et al.,* 1998). In all species the majority of the sequence is highly homologous and the domain structure of the deduced proteins is conserved. In rat and chick, the only two species in which full-length cDNAs have been described, agrin has a predicted molecular weight of approximately 220kDa. *In vivo,* agrin is highly glycosylated and migrates on SDS–PAGE under reducing conditions with a molecular weight of approximately 500kDa. Since the molecular weight can be reduced to that of the core protein by digestion with heparitinases, agrin belongs to the family of heparansulfate proteoglycans (Tsen *et al.,* 1995; Denzer *et al.,* 1995). At least three glycosaminoglycan side chains have been observed using electron microscopy after rotary shadowing (Denzer *et al.,* 1998).

The agrin gene is subject to alternative mRNA splicing at several sites. Alternative exon usage at positions A and B (called y and z in rodents) at the 3'end of the cDNA has a strong influence on the biological activity of agrin. Only those isoforms are active at the NMJ *in vitro* and *in vivo* which have a peptide insert at both sites (Ruegg *et al.,* 1992; Ferns *et al.,* 1993; Gesemann *et al.,* 1995; Cohen *et al.,* 1997a; Burgess *et al.,* 1999). In contrast, agrin isoforms without a peptide insertion at these sites have no synaptogenic activity at the NMJ, although these isoforms might be involved in later steps of endplate maturation (Meier *et al.,* 1997; Cohen *et al.,* 1997a; Burgess *et al.,* 1999). Several other splicing sites have been identified, some of which appear to be species-specific and the biological role of these sites is entirely unclear (Rupp *et al.,* 1991; Tsen *et al.,* 1995b; Denzer *et al.,* 1995). Agrin transcripts are widely expressed throughout the peripheral and the central nervous system (O'Connor *et al.,* 1994; Stone and Nikolics, 1995; Kroger *et al.,* 1996; Cohen *et al.,* 1997b). Importantly, alternatively spliced transcripts encoding AChR aggregating and inactive versions of agrin are expressed in distinct cell types. For example neurons, including motor neurons, express agrin isoforms that are active in AChR aggregation while non neuronal cells, including muscle, kidney, lung, or glial cells, express isoforms that are inactive in this process (for review see Ruegg and Bixby, 1998).

Expression of agrin in the brain is particularly high during development and continues to be high in non-neuronal tissue like kidney and lung.

While the 3'end of the agrin gene is highly conserved between species and its function in postsynaptic differentiation is well established, the 5'end is less well characterized. In particular, a discrepancy exists between species with regard to the published 5'end sequences. The most 5'end of chick agrin contains a signal sequence and the NtA domain. The NtA domain confers binding of agrin to the γ1 chain of the laminins and is therefore important for immobilizing agrin in basal laminae (Denzer *et al.,* 1997, 1998; Kammerer *et al.,* 1999). This 5'end of agrin has been described in human, bovine, and mouse and its sequence is highly conserved across species (Denzer *et al.,* 1995; Groffen *et al.,* 1998). A different 5'end of agrin has been reported for rat agrin (Rupp *et al.,* 1991). There, the full-length cDNA codes for a shorter 5'end that is also supposed to encode a secreted form of agrin (Rupp *et al.,* 1991; Campanelli *et al.,* 1991). Because this form lacks the NtA domain, it is not clear how it binds to basal lamina. Moreover, this shorter version of agrin has not been identified in any other species. We therefore decided to analyze the 5'end of the agrin gene in more detail. In this study, we show that 5'ends homologous to the rat sequence also exist in mouse and chicken. We show that this form is generated by usage of an alternative first exon. We further show that this alternative amino-terminus does not serve as signal peptide but as a non-cleaved signal anchor, thus generating a type II transmembrane protein (Nilsson *et al.,* 1994). This transmembrane form of agrin is most likely regulated by an alternative promoter, is conserved between several species, and is primarily expressed in the central nervous system. Thus, the heparansulfate proteoglycan agrin also exists as a transmembrane molecule.

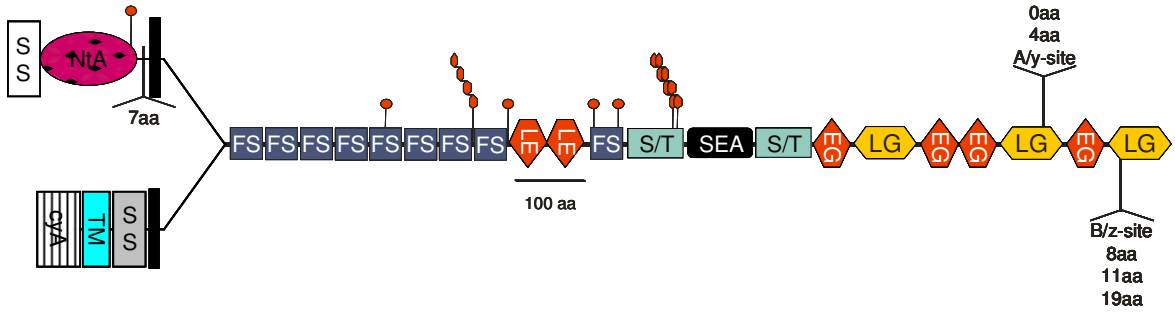
Results

The Two Different Amino-Termini are Conserved across Species

To investigate whether the amino-terminal end for rat agrin (Fig. 5A; Rupp *et al.,* 1991) is unique for this species, we performed reverse transcription followed by polymerase chain reaction (RT-PCR) on RNA isolated from different mouse tissues. To increase the specificity of the reaction, reverse transcription was initiated using a primer specific for mouse agrin. Subsequent PCR was performed using a nested agrin-specific antisense primer and a degenerate sense primer that was designed based on the published 5'sequence

of rat agrin. Using this strategy, PCR products were detected from RNA isolated from embryonic brain and spinal cord, kidney, and from the C2C12 muscle cell line. These PCR products were cloned and sequenced (Fig. 5B) confirming that mice also express agrin isoforms encoding the alternative amino-terminus described in rat. To further test the degree of conservation of this amino-terminus, we next performed primer extension analysis on RNA isolated from spinal cord and brain of 6-day-old chick embryos. Indeed, in both tissues, specific PCR products could be isolated that were derived from agrin mRNA encoding the alternative amino-terminus (Fig. 5B). The longest PCR product was 500bps long and contained 238bps of 5'untranslated region. As shown in Fig. 5B, the nucleotide sequences of mouse and rat agrin are highly homologous. In contrast, the sequence for chick agrin shows a low conservation in the 5'untranslated region and at the beginning of the protein-coding region. Indicative of initiation of translation at the ATG highlighted in Fig. 5B, the first in-frame stop codons in mouse and chicken are found 12 and 57 nucleotides upstream, respectively. In addition, the ATG is preceded by a Kozak consensus sequence in all species.

A



B

```
      1                                                                                                  100
  rat ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~T GCTTATCTCC TACCATGAAC CTGGAGGGCT GGGGAGAGGA
mouse ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~AGTGGATC CCCCGGGCTG CAGGAATTCT TCTTATCTCC TGCCATGAGC CTGGAGGGCT GGGGAGAGGG
chick GGGCTGGGAG GGTCTCTCCG GAGCACACTC AGACCCATGA TGGCAGATGT GGGGATGAAC TCTTGGTCCT GAAGGTGCCA CCAACCTGCC CTGCCCTCCT

      101                                                                                                151
  rat CCTGGTGCTC CCCTAGCCAT AACTGGCTAA ACTTTGAGAT CACAGCTG.C ATCTGCTGCT CCAGAGCACC CAGCATGGGG GGAGTAGCTC CGAAGAGGCC
mouse CCCGGTGCTC CCCTAGCCAC AACTGGCTAA ACTTTGCTAT CACAGCTGCC ATCTGCTGCT CCAGAGCACC CAGCATGGGG GGAGTAGCTC CGAAGAGGCC
chick GTTCCCTAGA GGATGGGAAC CTCTGGGAAA ACTGGGGTCT TGCTGCTGCC GAAAAGAGGG GAACGGAGGG CATCTGTGTG AGCCTCGTGG CCGCTCTGCC

      201                                                                                                300
  rat TAAGGGCACT CCATAAGAAC TCCCACACAC ACCCAGGAAT TGGGCTCGAG CTGTATGTAT CATGCCTCCT CTGCCACTGG AACACAGACC CAGGCAGGAG
mouse TAAAGGCCCT CCATAAGAAC GCCC.CACAC CCCCAGGGAC TGGGCTAGAG C....CGTAT CATGCCTCCT CTGCCACTGG AACACAGACC CAGGCAGCAG
chick TTGGATGGGT CACCAGCAGG TTGAACTTTG AGCACAGCAT GACGGCTTGC CAGTAC..CC CATGGCACCG GGGGCCTTGG AGCGGGACCG GCTGTACCAG
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      301                                                                                                  400
  rat CCTGGTGCCT CCATGCTGGT TCGATACTTC ATGATCCCCT GCAACATCTG CTTGATCCTG TTGGCCACTT CCACATTGGG CTTTGCGGTT CTGCTTTTCC
mouse CCTGGTGCCT CCGTGCTGGT TCGGTACTTC ATGATCCCCT GCAACATCTG CTTGATCCTC TTGGCTACTT CTACGTTGGG CTTTGCGGTG CTGCTTTTCC
chick CACAAGGTCT CCTTGGTGGT GCGCTACTTC ATGATCCCGT GCAACATTTG CCTCATCCTC CTGGCCACCT CCACGCTGGG ATTTGCTGTG CTGCTCTTCC

      401
  rat TCAGCAACTA
mouse TCAGCAACTG
chick TCAATAACTA
```

C

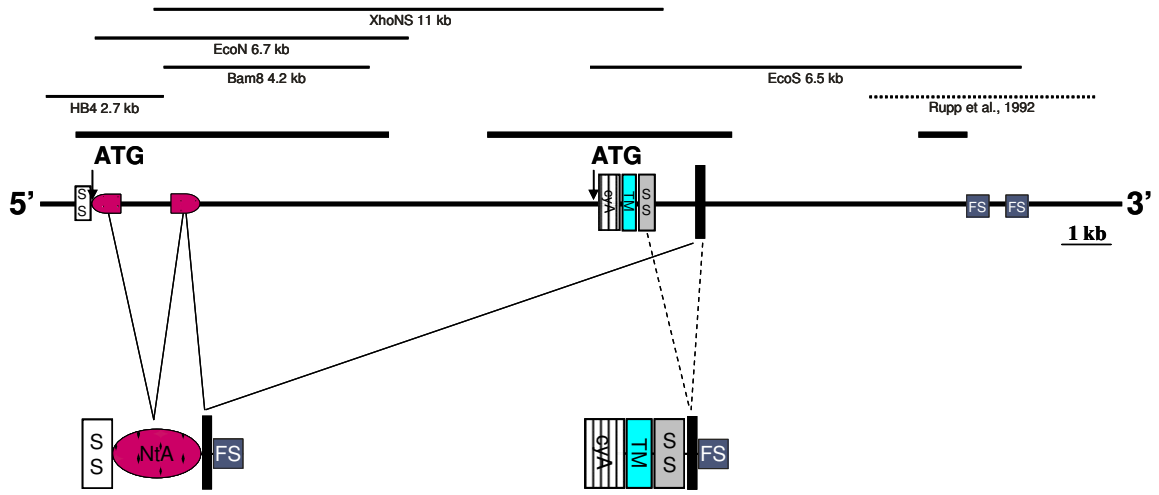

Fig. 5: Identification of alternative 5'ends homologous to rat agrin in mouse and chick

(A) Schematic representation of the protein structures as deduced from full-length cDNAs in chick and rat. The amino-terminus of chick agrin encodes a signal sequence (SS) followed by the NtA domain (Denzer *et al.,* 1995; Denzer *et al.*, 1997). Rat agrin encodes a shorter amino-terminal end (Rupp *et al.,* 1991) not described in any other species. The two proteins become highly homologous in a linker region (gray) that precedes the first follistatin-like domain (FS). For details on structural motifs of agrin see Ruegg and Bixby (1998). Sites of alternative mRNA splicing are indicated by ^. (B) Alignment of nucleotide sequences homologous to rat agrin. Putative sites of initiation of translation are indicated by boxes. (C) Genomic organization of the 5'end of mouse agrin. Name and size of DNA fragments used for the mapping are shown (lines). The genomic sequence published by Rupp *et al.* (1992) is indicated by a broken line. Regions that were fully sequenced are indicated by thick lines. The deduced structure of the mouse agrin gene is given at the bottom. Note that the signal sequence and the NtA domain are encoded by two exons. A large intron of ~8.6kbs precedes the exon encoding the sequence homologous to rat agrin. The two amino-termini converge at the linker region (gray). RNA splicing for the two different forms is indicated (broken line and dotted line, respectively).

The mouse agrin gene described so far ends at the exon encoding the first follistatin-like domain (Fig. 5C; Rupp *et al.,* 1992). To further compare the two amino-termini of agrin and to determine whether they derive from alternative mRNA splicing, we isolated the gene encoding the 5'end of the mouse agrin gene. To this end, bacterial artificial chromosome (BAC) DNA libraries were screened using a probe corresponding to the last 128bps encoding the secreted version of agrin (NtA domain) and one probe encoding the first 132bps of the alternative amino-terminus. Using this approach, we were able to

identify a single BAC clone that was positive for both probes. After isolation of DNA, the BAC inserts were further digested with appropriate restriction enzymes and the DNA fragments containing the different regions of the agrin gene were subcloned and their sequence was determined (see Experimental Methods). Digestion of DNA by restriction enzymes and partial sequencing of 5 subclones allowed compiling the entire 5'end of the mouse agrin gene (Fig. 5C). The most 5'localized clone, HB4, contains the 5'untranslated region preceding the amino-terminal part of agrin encoding the NtA domain. The overlapping clone, EcoN, comprises the first exon encoding the initiation methionine of the signal sequence and the amino-terminal part of the NtA domain. It overlaps with the largest, approximately 11-kb-long fragment, XhoNS, which encodes the second half of the NtA-domain and extends up to the alternative amino-terminus. Finally, the most 3'localized clone, EcoS, contains the entire 5'end of the alternative amino-terminus. At its 3'end, more than 1kbs are identical to the published sequence of the mouse agrin gene (Rupp *et al.,* 1992). Because we sequenced some of the fragments only partially, the sizes of the introns between exons encoding the NtA domain and the alternative amino-terminus, and between the linker region and the first follistatin like domain, are not known exactly. Based on restriction digests, we estimate them being 8.6 and 5.8kbs long, respectively (Fig. 5C). In summary, isolation of the mouse gene encoding agrin revealed that the signal sequence and the NtA-domain of agrin are encoded by two separate exons, while the alternative amino-terminus is encoded by a single exon. Moreover, the large distance between the exon coding for the second part of the NtA-domain and that coding for the alternative amino-terminus strongly suggests that expression of the alternative form of agrin is regulated by a separate promoter.

The Alternative Amino-Terminus Serves as Signal Anchor

Searching databases for protein sequences homologous to the alternative amino-terminus of rat agrin yielded one expressed sequence tag (EST), isolated from human B-cells derived from chronic lymphotic leukemia (Lennon *et al.,* 1996). Figure 6A shows the alignment of the deduced protein sequence from all four species. While the most amino-terminal ends are less conserved across species, a very high degree of conservation is observed further downstream. Interestingly, this highly conserved stretch is hydrophobic, indicative of a transmembrane segment.

A

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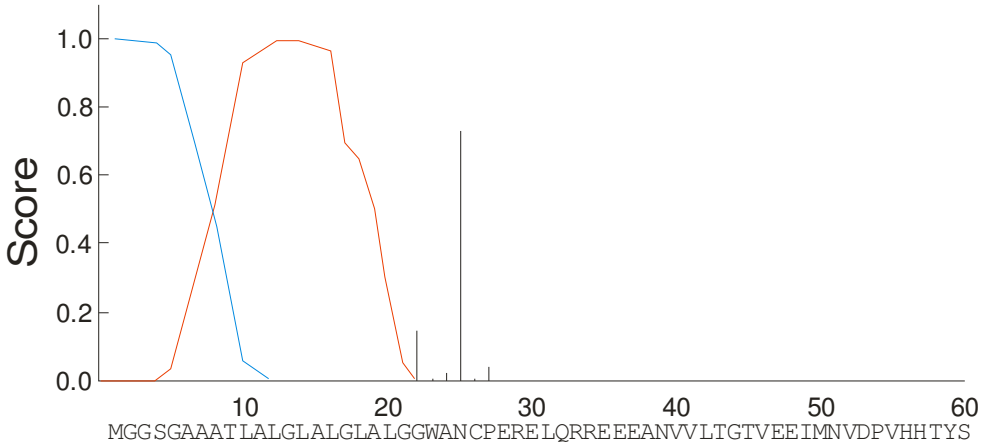
                1                                                    57
      rat  ~~~~~~~MPP LPLEHRPRQE PGASMLVRYF MIPCNICLIL LATSTLGFAV LLFLSNY
    mouse  ~~~~~~~MPP LPLEHRPRQQ PGASVLVRYF MIPCNICLIL LATSTLGFAV LLFLSNY
human_est  ~~~~~~~MPX LAVARDTRQP AGASLLVRGF MVPCNACLIL LATATLGFAV LLFLNNY
    chick  MTACQYPMAP GALERDRLYQ HKVSLVVRYF MIPCNICLIL LATSTLGFAV LLFLNNY
```

B



C

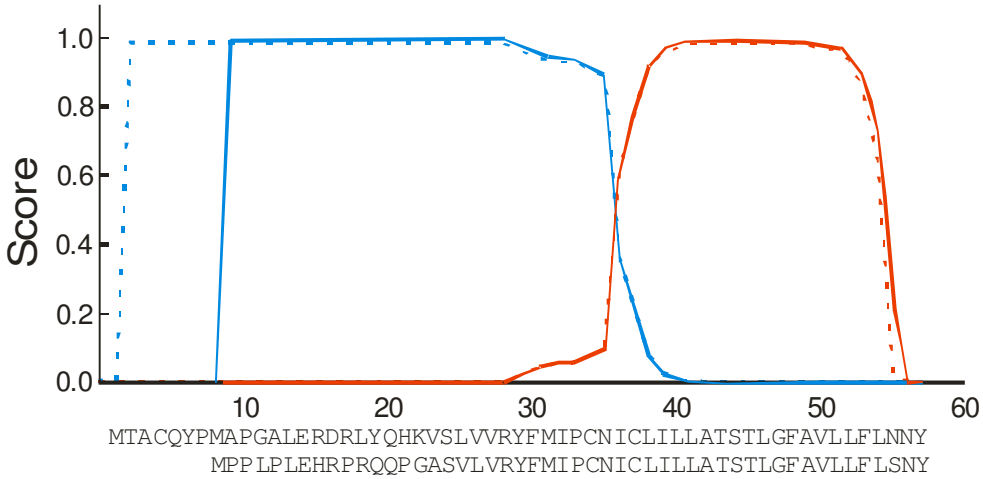


Fig. 6: The amino-terminus homologous to rat agrin is predicted to serve as a noncleaved signal anchor

(A) Alignment of deduced protein sequences from rat, mouse, a human expressed-sequence-tag (human-est), and chick. Conservation is low at the very amino-terminal end but becomes very high towards the carboxy-terminus. Identical amino acids are indicated (yellow). Note that the chick sequence is predicted to be 7 amino acids longer than the other sequences as the initiation site indicated in Fig. 5A is the first in-frame methionine. (B) The software SignalP-HMM (Nielsen and Krogh, 1998) predicts a cleaved signal peptide for the amino-terminus described in full-length chick agrin (Denzer *et al.,* 1995). This is indicated by the high score for the n-region (blue line) and the h-region (red line) at the very most amino-terminal end of the protein. Importantly, the program predicts a high probability for signal peptidase cleavage after the hydrophobic h-region (vertical bars). (C) SignalP-HMM predicts a noncleaved signal anchor for the alternative amino-terminal sequence from chick (dotted line) and mouse (solid line). The high scores for the h-region are shifted to the carboxy-terminal end and no signal peptidase cleavage site is predicted.

To characterize the sequences more fully, we analyzed all of them with the signal peptide prediction program SignalP V2.0 (Nielsen, 1997). To distinguish between cleaved signal peptides and non-cleaved signal anchors (SA), we used a modified version of the program making use of hidden Markov models (SignalP-HMM; Nielsen and Krogh, 1998).

The program predicted a cleaved signal peptide with a probability of 99.9% for the secreted form of chick agrin. As shown in Fig. 6B, such a targeting sequence is characterized by a short *n*-region (blue) followed by a short, hydrophobic *h*-region (red line). Most importantly, they show a high probability for cleavage by the signal peptidase after the hydrophobic region (vertical bars in Fig. 6B). Using the protein sequence of the alternative amino-termini of chick and mouse agrin, the SignalP-HMM program predicted non-cleaved SAs. These are characterized by a positively charged *n*-region at the amino-terminal end (blue line) followed by a hydrophobic region (*h*-region; red line) that is usually longer than classical signal peptides (Fig. 6C; Nilsson *et al.,* 1994). The predicted probabilities for non-cleaved SAs were above 90% in all species (mouse: 99.4%; human_est: 91.7%; chick: 98.5%).

The presence of a single non-cleaved SA at the amino-terminus results in a protein with a single transmembrane segment where the amino-terminus lies inside and the carboxy-terminal outside of the cell (N_{cyto}/C_{exo} orientation; type II transmembrane protein). To validate this prediction, we compared protein targeting and the orientation of different agrin constructs after transfection of COS-7 cells. To allow detection of the recombinant protein, the constructs were fused to human placental alkaline phosphatase (AP; Berger *et al.,* 1988) at their carboxy-terminal end (Fig. 7A). To determine the orientation of the recombinant protein, one construct was also tagged with a six fold repeated myc-epitope (Evan *et al.,* 1985). A similar construct derived from the secreted version of mouse agrin (SS-NtA-AP) served as the positive control. In a first set of experiments, transfected COS cells were stained alive at 4°C with an antibody directed against the AP fusion protein. As shown in Fig. 7B (upper panel), strong staining was observed when COS cells were transfected with constructs encoding the alternative amino-terminus (TM-FS-AP) and no specific staining could be seen with the control construct (SS-NtA-FS-AP). The latter construct could be visualized only if cells were permeabilized before the staining (Fig. 7B; lower left). Intracellular staining of TM-FS-AP was dominated by the staining of the cell membranes (Fig. 7B, lower right).

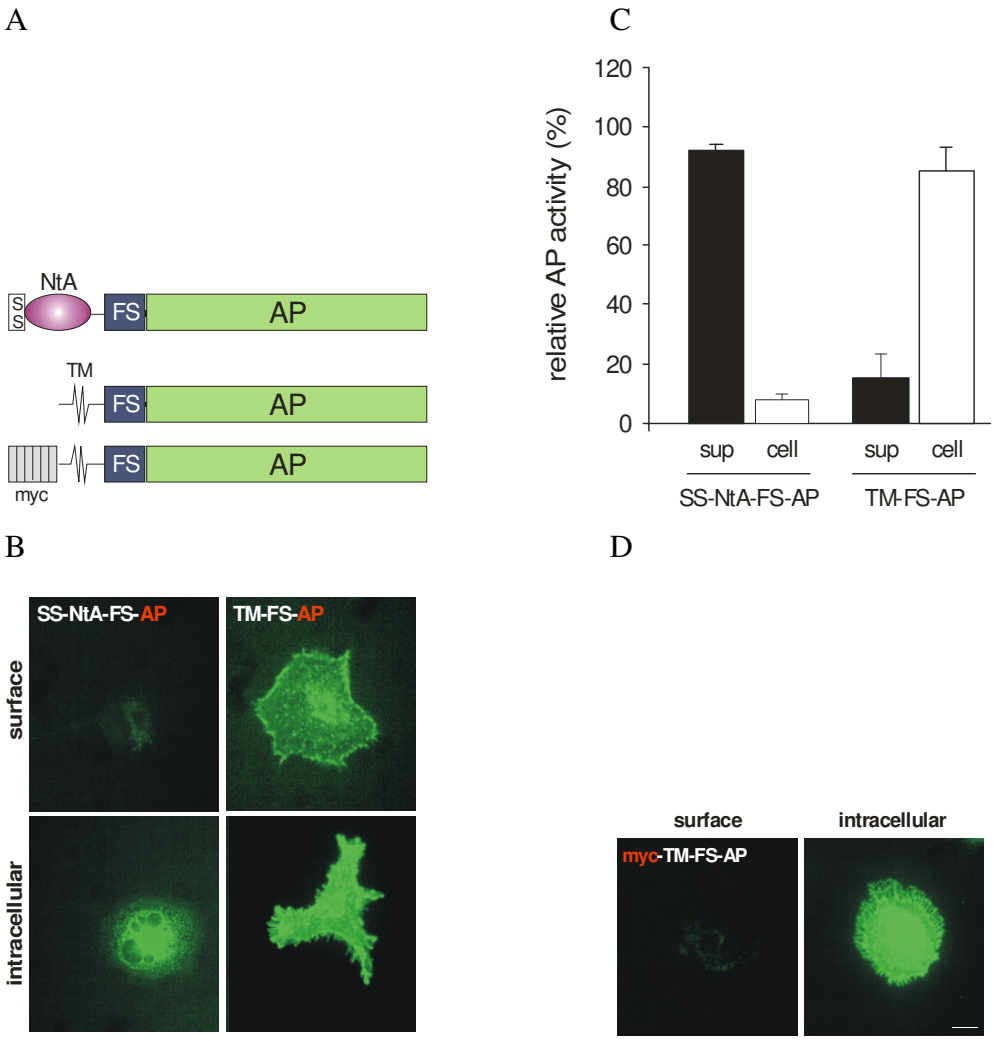


Fig. 7: Constructs encoding the new amino-terminus are inserted into the cell membrane of transfected COS cells

(A) Constructs used in this study encode the SS-NtA amino-terminus of mouse agrin, fused to placental alkaline phosphatase (AP) as a reporter (SS-NtA-FS-AP, top), the new amino-terminus predicted to be a transmembrane protein (TM-FS-AP, middle) and TM-FS-AP that was additionally tagged with six copies of a myc epitope (myc-TM-FS-AP, bottom). (B) COS cells transfected with SS-NtA-FS-AP (left column) or TM-FS-AP (right column) were stained with an anti-AP antibody. When the COS cells were stained alive (surface, top row), only COS cells expressing TM-FS-AP were positive. COS cells transfected with SS-NtA-AP showed immunoreactivity in the endoplasmatic reticulum and the Golgi apparatus after permeabilization (bottom row). (C) Quantification of AP activity in the conditioned medium (sup) and the cell lysate (cell). While the majority of the SS-NtA-FS-AP construct accumulates in the conditioned medium, most of the TM-FS-AP construct remains associated with COS cells. The values shown are the mean ±SEM from six independent experiments. (D) The amino-terminal myc tag cannot be stained in living COS cells (left), showing that the epitope is not accessible from the outside. Only after permeabilization of the cells with detergent, the transfected cells are brightly stained (right). Bar, 20µm.

As the presence of the AP fusion construct allowed quantification of the protein concentration in the different compartments, we measured the concentration of AP in the medium and in the COS cells 2 days after transfection. Again, the secreted version of agrin

served as the internal control. With this construct, more than 90% of the AP activity was detected in the medium (Fig. 7C, left column; sup) and only little AP activity was associated with the cells (Fig. 7C, left column; cell). In contrast, only approximately 15% of the total AP activity accumulated in the medium and 85% was found in the cell fraction, when COS cells were transfected with the construct encoding the alternative amino-terminus (Fig. 7C; right column). These results therefore support the conclusion that the alternative amino-terminus of agrin serves as a non-cleaved signal anchor.

To test the orientation of the protein in the plasma membrane, we next transfected a construct into COS cells that carried an additional myc-tag at its amino-terminal end (see Fig. 7A). In non-permeabilized cells, the anti-myc monoclonal antibody 9E10 (Evan *et al.,* 1985) did not stain any of the transfected cells (Fig. 7D, left panel). If COS cells were permeabilized before 9E10 was added, a strong cell membrane staining was observed (Fig. 7D, right panel). This staining pattern was indistinguishable from the intracellular staining with the anti-AP antibody (data not shown, but see Fig. 7B for comparison). The same N_{cyto}/C_{exo} orientation was also seen upon transfection of COS cells with the full-length cDNA encoding the chick version of the transmembrane form of agrin. There, antibodies raised against the N-terminal end did not stain transfected COS cells without permeabilization, while antibodies rose against the C-terminal part of agrin did stain (data not shown). Thus, the amino-terminus of this construct is localized to the cytoplasm of the transfected COS cells, which confirms the prediction that this alternative amino-terminus of agrin is sufficient to confer a type II transmembrane orientation to the protein. We therefore name this form of agrin transmembrane-Agrin (TM-Agrin). For clarity, we will call the secreted form of agrin in the following sections “SS-NtA-Agrin”, which is based on its domain structure at the amino-terminus.

The Transmembrane Form of Agrin is Highly Glycosylated and Induces AChR Aggregation

The secreted form of agrin (SS-NtA-Agrin) expressed in COS cells carries heparansulfate glycosaminoglycan side chains indicative of its being a heparansulfate proteoglycan (HSPG; Tsen *et al*., 1995b; Denzer *et al*., 1995). Moreover, agrin isolated from several tissues, including brain, has an appearance on SDS–PAGE reminiscent of a HSPG (Tsen *et al.*, 1995a; Denzer *et al.*, 1995; Gesemann *et al.*, 1998). To evaluate whether the

transmembrane form of agrin (TM-Agrin) undergoes the same posttranslational modifications as the secreted version, we assembled a cDNA encoding full-length chick TM-Agrin and expressed the protein in COS cells.

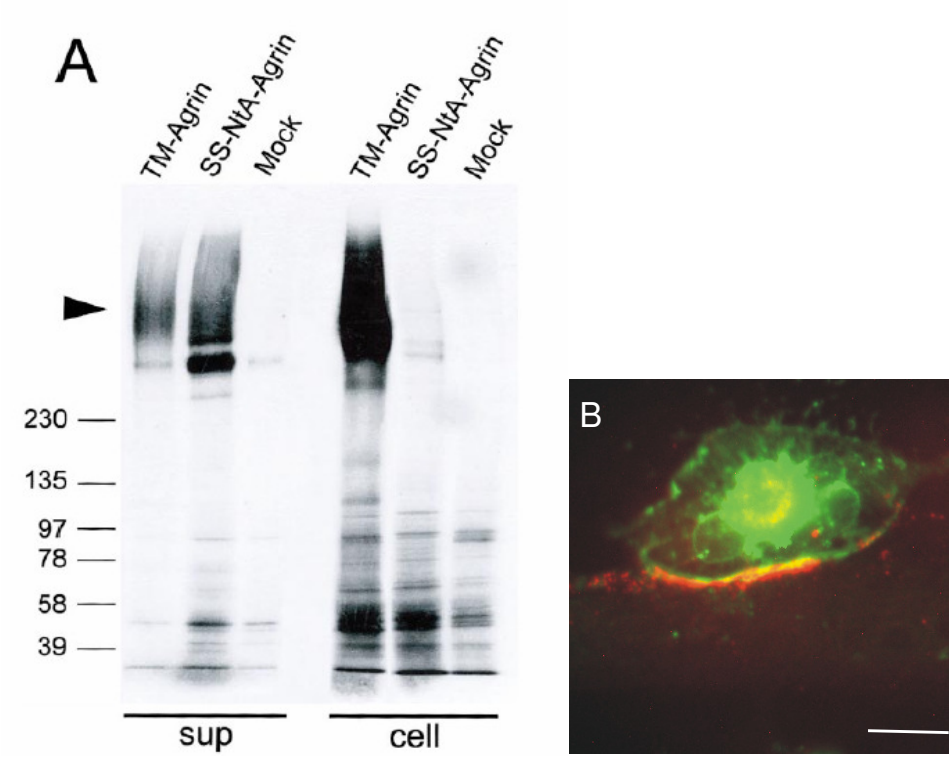


Fig. 8: The transmembrane form of agrin is highly glycosylated and induces the aggregation of AChR on cultured myotubes
(A) Agrin-like protein synthesized by COS cells transfected with full-length cDNAs encoding the transmembrane form of chick agrin (TM-Agrin), the secreted form of chick agrin (SS-NtA-Agrin) or with vector alone (Mock) were labeled with [$_{35}$S]methionine for 48h. The conditioned medium (sup) was collected and the cells were chased for 3h in the presence of non-labeled methionine. The COS cells were then lysed with detergents (cell). Agrin-like protein was immunoprecipitated with anti-agrin antibodies, separated on SDS–PAGE, and visualized by fluorography. Molecular weights of standard proteins are given in kDa. Arrow indicates agrin-like protein. The immunoreactive band below the smear (sup; SS-NtA-Agrin) was not observed consistently and may reflect incomplete glycosylation of the protein due to its overexpression. (B) Myotubes and TM-Agrin-transfected HEK293 cells were co-cultured for 24h and subsequently stained with anti-agrin antibodies (green) and with rhodamine-conjugated α-bungarotoxin (red) to visualize AChRs. Aggregation of AChRs at the site of myotube/HEK cell contact was observed, indicating that TM-Agrin is active in AChR clustering and that focal application of TM-Agrin induces the local aggregation of the AChR. Bar, 10μm.

Transfected COS cells were labeled with $_{35}$[S]methionine for 2 days. After harvesting the medium, COS cells were chased with non radioactive methionine for 3h. Agrin-like protein was immunoprecipitated from conditioned medium and the cell lysate, separated on SDS–PAGE, and processed for fluorography. As shown in Fig. 8A, the secreted form of agrin accumulated in the conditioned medium (SS-NtA-Agrin; sup) and migrated on SDS–PAGE with an apparent M_r of more than 400 kDa. In addition, only little agrin-like protein

was found in the cell lysate (SS-NtA-Agrin; cell). In strong contrast, in COS cells transfected with TM-Agrin, only little protein was found in the supernatant (TM-Agrin; sup) and the majority of the protein was associated with the cell lysate (TM-Agrin; cell). Importantly, the transmembrane form of agrin displayed a similar M_r on SDS–PAGE like the secreted form, indicating that it is also a HSPG.

The transmembrane form of agrin was also capable of inducing AChR clustering when transfected human embryonic kidney HEK293 cells were co-cultured with chick muscle cells (Fig. 8B). AChR were predominantly clustered at the site of direct contact between HEK 293cell and the myotubes. This is independent evidence that TM-Agrin is inserted in the membrane of HEK293 cells in a N_{cyto}/C_{exo} orientation because the C-terminal part of agrin is required to induce AChR aggregation on cultured muscle cells (Nitkin *et al.*, 1987; Tsim *et al.*, 1992; Ferns *et al.*, 1993; Gesemann *et al.*, 1995).

Transcripts Encoding the Transmembrane Form of Agrin are Enriched in the CNS

The structure of the agrin gene indicates that the secreted and the transmembrane form use distinct promoters (Fig. 5C). This suggested to us that the two forms might be differentially expressed. To address this question, we used real-time quantitative PCR (Head *et al.*, 1996) with primers and probes specific for the first FS-like domain (pan-Agrin), the transmembrane form (TM-Agrin) and the secreted form (SS-NtA-Agrin). To normalize the amount of mRNA for each tissue, primers and probes specific for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were used. Because the total amount of agrin mRNA expressed in each tissue must correspond to the sum of transcripts encoding the transmembrane and the secreted form of agrin, we first determined standard curves for the PCR reaction for probes specific for each form. To this end, we generated a construct that contained all three regions of agrin (SS-NtA, TM, and FS). Using different concentrations of this construct, we were able to determine the amplification efficiency for each set of agrin primers (see Experimental Methods). The amount of mRNA determined for the different primer sets was then adjusted based on the amplification efficiency.

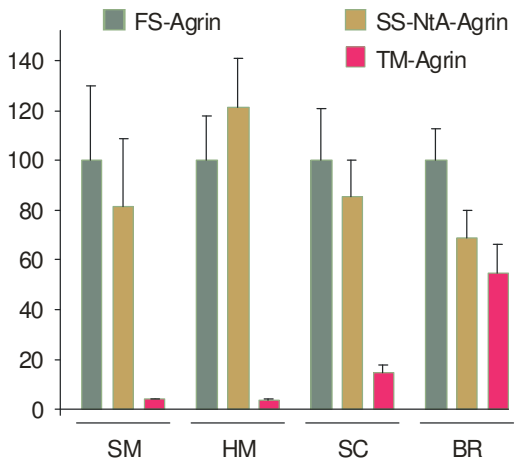
When we measured the relative amount of agrin mRNA encoding all agrin isoforms (pan-agrin), we found that a similar amount of agrin mRNA was expressed in each tissue (data not shown). This value was set to 100% and the relative contribution of TM-Agrin and SS-

NtA-Agrin was calculated using the amplification efficiency determined with the standard curves (see above).

The mRNA of E11 skeletal (SM) heart muscle (HM), spinal cord (SC) and brain (BR) was extracted. As shown in Fig. 9A, the contribution of individual agrin forms varied considerably between the different tissues. No or very little mRNA coding for TM-Agrin was detected in E11 skeletal and heart muscle and most, if not all agrin transcripts encoded SS-NtA-Agrin. In contrast, approximately 15% of the agrin mRNA expressed in spinal cord (SC) corresponded to TM-Agrin and the highest percentage of mRNA encoding TM-Agrin was detected in brain (BR). In brain, this form of agrin contributed almost 50% of the entire agrin mRNA. These experiments, thus, indicate that the transmembrane form of agrin is not expressed in peripheral, non neuronal cells and that its contribution is highest in the brain, suggesting that TM-Agrin may be most strongly expressed in CNS neurons or glial cells.

To validate this further, we performed RT-PCR on mRNA preparations of the retina from several embryonic stages. We chose the chick retina, since agrin in the retina has been extensively characterized and since the retina does not contain blood vessels, which have agrin immunoreactivity associated (Magill-Solc and McMahan, 1988) and which could therefore interfere with the analysis of neural agrin. As shown in Fig. 9B, transcripts coding for SS-NtA-Agrin (upper panel) and TM-Agrin (lower panel) were detected in E8 to E16 retina. The presence of transcripts coding for TM-Agrin at stages before retinal synapses develop, which in chick occurs around E12 (Hering and Kroger, 1996), indicates that TM-Agrin might have functions in the retina during very early stages of development.

A



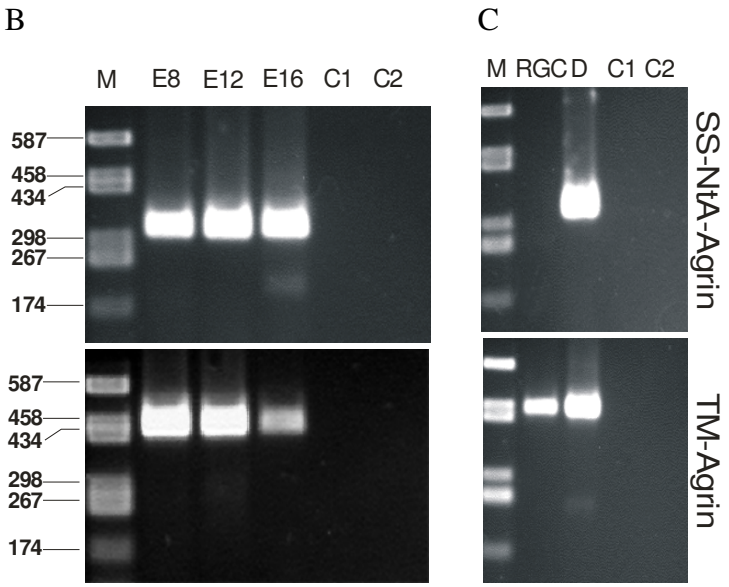


Fig. 9: Transcripts encoding the transmembrane form of agrin are enriched in the brain

(A) Quantitative real-time PCR of E11 chick skeletal muscle (SM), heart muscle (HM), spinal cord (SC), and brain (BR) using primers and probes specific for the first follistatin-like domain (pan-Agrin), the secreted form (SS-NtA-Agrin), and the Transmembrane from (TM-Agrin). TM-Agrin was not detected in skeletal muscle and heart but was clearly detected in spinal cord and brain with the highest levels in brain. Values given represent the mean ±SEM of three independent experiments. (B) mRNA from chick retinae of different developmental stages (E8, E12, E16) was analyzed for expression of SS-NtA-Agrin (upper panels) and of TM-Agrin (lower panels). At all stages, both forms of agrin could be identified, demonstrating the expression of TM-Agrin before, during and after synaptogenesis in the retina. (C) E6 retinal ganglion cells, which were purified by immunopanning (lane RGC), and retinal cell dissociate (lane D) were analyzed for the expression of SS-NtA-Agrin and TM-Agrin. While the retinal cell dissociate contained both forms of agrin, RGCs express only TM-Agrin.

To analyze in more detail which cells of the retina express the transmembrane form of agrin, we purified retinal ganglion cells (RGC) from E6 chick retina by immunopanning (Barres *et al.,* 1988). This procedure resulted in a cellular fraction in which RGCs represented more than 97% of all cells (data not shown). In RGCs (lane RGC; Fig. 9C), we could not detect a PCR product specific for SS-NtA-Agrin while a strong band was seen in the retinal cell dissociate (lane D; Fig. 9C). In contrast, both cell fractions gave rise to PCR products specific for TM-Agrin (lower panel; Fig. 9C). The controls of the first and second (nested) PCR, as detailed under Experimental Methods, were negative (lanes C1and C2 in Figs. 9B and 9C), demonstrating the specificity and the absence of a contamination in both PCR reactions. Sequencing of obtained PCR products showed that they were indeed derived from SS-NtA-Agrin and TM-Agrin, respectively (data not shown). We conclude that E6 retinal ganglion cells synthesize transcripts encoding the transmembrane form of agrin but not the secreted form.

To compare the localization of the transmembrane form of agrin with the secreted form of agrin, we analyzed their distribution in the E6 chick retina, using anti-peptide antisera generated against the specific forms of agrin. Cryostat sections stained with a panspecific antiserum against agrin are shown in Fig. 10A. As has been described previously (Kroger and Mann, 1996; Kroger *et al.*, 1997), agrin-like immunoreactivity is associated with the inner limiting membrane (ILM; arrows in Figs. 10A–10C), a basal lamina that separates the neural retina from the vitreous humor (Halfter *et al.,* 1987; Kroger and Mann, 1996). This antiserum also stained the axons of retinal ganglion cells (asterisks in Figs. 10A–10C), which grow fasciculated centripetally within the optic fiber layer (OFL in Figs. 10A–10C) of the retina to the optic nerve head. In contrast, antibodies specific for the cytoplasmic part of the transmembrane form of agrin exclusively stained the axon fascicles of retinal ganglion cells, but did not label the ILM (Fig.10B). Conversely, anti-peptide antisera against the NtA domain of agrin exclusively labeled the basal lamina but not the ganglion cell axons in the OFL (Fig. 10C). These results demonstrate a differential distribution of SS-NtA-Agrin and TM-Agrin in the E6 retina, with the transmembrane form of agrin being cell-associated and the secreted form being associated with the extracellular matrix.

Discussion

Our study provides strong evidence that agrin is not only a component of the extracellular matrix but that it is also expressed as a cell surface protein. We show that this form is a type II transmembrane protein where the amino-terminus is localized in the cytoplasm and the carboxy-terminus is exposed to the outside of the cell. This transmembrane form is generated by the usage of an alternative amino-terminal end whose sequence encoding the segment important for the incorporation into the plasma membrane is identical in chick, mouse, and rat.

We also provide evidence that the transmembrane form of agrin is a HSPG and that it is preferentially expressed in the CNS. In developing chick retina, we show that the transmembrane form of agrin is expressed by retinal ganglion neurons while the basal lamina-bound form is specifically associated with inner limiting membrane.

The Role of the Transmembrane Form at the NMJ

The usage of the alternative amino-terminal signal anchor eliminates agrin binding site to the laminins. This raises the question whether the transmembrane form of agrin plays a role in the formation of the NMJ. The current evidence indicates that this is not the case. For example, RT-PCR has shown that embryonic day 6 chick motor neurons express high amounts of the secreted form of agrin (Denzer *et al.,* 1995). Importantly, agrin-mutant mice in which the secreted form has been selectively inactivated show the same phenotype as mice that do not express any agrin at all (R. W. Burgess. Skarnes, and J. R. Sanes, *Abstract to Soc. Neurosci. Meeting,* 1999).

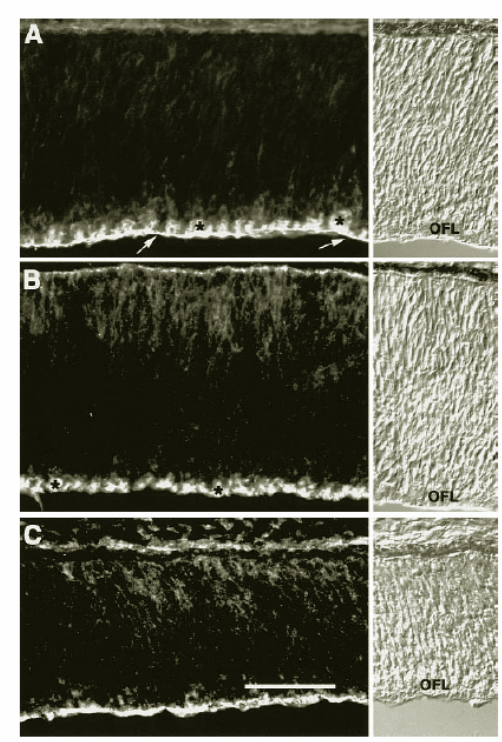


Fig. 10: Differential expression of the secreted and the transmembrane form of agrin in the retina

Cryostat sections from E6 chick retinae were stained with a pan-specific rabbit anti-agrin antiserum (A), with a sheep anti-TM-Agrin antiserum (B) or an antiserum reacting specifically with the NtA domain (C). The pan-specific antiserum stained the inner limiting membrane (ILM; arrows in A and C) and the ganglion cell axons in the optic fiber layer (OFL; asterisks in A and B). In contrast, the antiserum against the transmembrane form of agrin detected immunoreactivity only in the OFL, while the ILM was not stained. Conversely, the antiserum against the NtA domain specifically labeled the ILM, but showed no immunoreactivity in the OFL. Bar, 50µm.

Moreover, transgenic expression of a cDNA construct encoding the secreted form of chick neural agrin in motor neurons is sufficient to rescue the NMJ phenotype of agrin-deficient

mice (I. Ksiazek and MA. Ruegg., unpublished observation). These observations, together with our finding that the expression of the transmembrane form is highest in the brain suggest that this agrin form does not play a critical role in the formation of the NMJ. Because embryonic muscle is also devoid of the transmembrane form (Fig. 9B), the NMJ is likely to contain no agrin protein encoding this isoform.

Potential Role of the Transmembrane Form of Agrin in the CNS

Several lines of evidence suggest that agrin plays a role during brain development. In particular, there is circumstantial evidence that agrin may play a role during synaptogenesis in the CNS. For example, agrin transcripts are highly expressed in the developing brain and mRNA expressed by neurons codes for agrin splice variants that are active in aggregating AChRs in muscle cells (for review see Ruegg and Bixby, 1998). In addition, the protein is concentrated in the synaptic cleft of inter neural synapses in the retina (Koulen *et al.,* 1999). Agrin also induces cellular responses in CNS neurons, including phosphorylation of CREB (Ji *et al.,* 1998) and expression of the immediate early gene c-fos (Hilgenberg *et al.,* 1999). Moreover, antisense experiments on cultured hippocampal neurons have recently shown impairment of dendritic growth and of the numbers of synaptic contacts (Ferreira, 1999). Our results presented here add further evidence that agrin may play a role in the brain. As a transmembrane molecule, agrin could exert its function by being presented on the cell surface of neurons. Thus, eliciting an agrin-mediated response does not require its immobilization in a basal lamina, but instead cell–cell contact appears to be sufficient. Moreover, the transmembrane form of agrin would be mobile within the lipid bilayer whereas basal lamina associated agrin is highly immobile.

The homology of agrin with the neurexins (i.e., presence of laminin-G-like domains) together with the synaptic localization of agrin suggests that it may have a similar function as the neurexins. The neurexins have recently been shown to be involved in the formation of presynaptic specializations in pontine or granular neurons *in vitro* (Scheiffele *et al.,* 2000). A very similar result has been obtained when the transmembrane form of rat agrin is presented to chick ciliary ganglia neurons (Campagna *et al.,* 1995) and sensory dorsal root ganglia neurons (Chang *et al.,* 1997). Thus, the transmembrane form of agrin may also serve as an adhesion molecule involved in modulating neurite outgrowth and presynaptic

differentiation in the CNS. Our finding that the transmembrane form of agrin is highly glycosylated indicative of a HSPG, further suggests a potentially similar function to the syndecans, a family of cell surface HSPGs (for review see Bernfield *et al.,* 1999).

Like agrin, syndecan-2 is enriched at neuron–neuron synapses (Hsueh *et al.,* 1998). Moreover, syndecan-2 associates with the cytoplasmic adapter molecule CASK/Lin-2 (Hsueh *et al.,* 1998), which is thought to be important for localizing syndecan-2 to synapses. Interestingly, CASK/Lin-2 also associates with the neurexins (Hata *et al.,* 1996). Thus, extracellular adhesion molecules thought to be involved in synaptic function are structurally related to agrin, the first bona fide inducer of postsynaptic structures at the NMJ. Although this correlation between syndecans, neurexins and agrin is only based on structural similarities, it raises the possibility that neuron-neuron synapses may use several different molecules related to agrin. This may also explain why agrin-deficient mice do not show a CNS phenotype (Serpinskaya *et al.,* 1999; Li *et al.,* 1999) as both the syndecans and the neurexins may compensate for the loss of agrin in this tissue.

A Role of TM-Agrin as Receptor

We show here that TM-Agrin is associated with the fiber tract of chick retinal ganglion cells at the time of active axonal elongation (Fig. 10). Thus, it seems possible that this form of agrin is involved in regulating axonal growth or pathfinding. Moreover, cell surface-bound HSPGs, such as syndecans, have been shown to be important in the presentation of growth factors to their cognate receptors. This is particularly striking for the family of heparin-binding growth factors, like fibroblast growth factor (FGF) and HB-GAM/pleiotropin. In view of our results presented here, TM-Agrin could also serve as a co-receptor for the presentation of such growth factors (for review see Bernfield *et al.,* 1999). In this context it is also interesting to note that signaling of the wingless (wnt) growth factors via the Frizzled2 receptor in *Drosophila* requires the proteoglycan dally (Lin and Perrimon, 1999; Tsuda *et al.,* 1999). Moreover, one of the vertebrate homologues of wnt, Wnt-7A, has recently been implemented in presynaptic differentiation of granule cell neurons in the cerebellum (Hall *et al.,* 2000). These data suggest that TM-Agrin could also serve as a co-receptor necessary for the signaling of such small growth factor-like molecules.

Although the cytoplasmic domain of agrin is only short and does not display significant homologies to previously characterized molecules, the detection of a transmembrane form of agrin opens the possibility that agrin is a receptor capable of transmitting an extracellular signal to the cytoplasm. This could be achieved by either a direct activation of the intracellular domain of agrin or via the recruitment of adapter molecules. The low degree of conservation between the cytoplasmic parts of chick and the mammalian homologues suggests that such a signaling/adapter function of TM-Agrin is less likely. Future experiments, such as selective inactivation of the transmembrane form, may be necessary to understand the role of this form during the development of the brain.

Experimental Methods

Animals

Fertile White Leghorn (*Gallus gallus domesticus*) chicken eggs were purchased from a local hatchery and incubated at 38°C in a humid atmosphere. The stage of development was determined according to criteria de-fined by (Hamburger and Hamilton, 1951) and expressed as embryonic days of development (E). Mice were bred in-house and were derived from C57/BL6 strains.

Antibodies

The rabbit anti-agrin antiserum #46 reacts with all agrin isoforms and has been extensively characterized previously in Western blotting and immunocytochemistry (Kroger and Mann, 1996; Kroger *et al.,* 1996; Kroger, 1997; Hering and Kroger, 1999). The anti-Islet-1 monoclonal antibody (Mab 39.4D5; Ericson *et al.,* 1992) developed by T. M. Jessell was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA 52242). Other primary antibodies used in this study were: anti-chick agrin antisera (Gesemann *et al.,* 1995), anti-c-myc monoclonal antibody 9E10 (Evan *et al.,* 1985), and anti-alkaline phosphatase antibody (Medix Biotech Inc.). Appropriate secondary antibodies were all purchased from Dianova, Hamburg.

Cell Culture and Transfections

Human embryonic kidney cells (HEK293; Graham *et al.,* 1977), COS cells (Gluzman, 1981), and primary chick myotubes were grown as described previously (Gesemann *et al.,* 1995). C2C12 mouse muscle cell lines were cultured as described by Briguet and Ruegg (2000). Cells were transfected with the DEAE-dextran-based method described elsewhere (Cullen, 1987). In some cases, FuGENE (Roche Diagnostics) applied according to the supplier was used for transfecting COS cells.

Isolation of the 5'End Encoding the Transmembrane Form in Mouse and Chick

Different tissues were dissected from newborn mice. Tissues (20 mg of kidney, 60 mg brain, spinal cord, and lung) were homogenized with a Polytron (Kinematica, Lucerne) in 175 µl lysis buffer. After adding 175µl of RNA dilution buffer, the samples were incubated at -70°C for 3min and centrifuged at 13,000rpm for 5min. RNA was isolated to the manufacturer's recommendation (RNeasy Minikit, Qiagen). RNA of all tissues was reverse transcribed using the primer asFS4 (5'-TGCAGGTCACATCGGCACAG-3'), which anneals to the sequence encoding the second FS domain of agrin. Touchdown PCR using Pwo polymerase (Roche MolecularBiochemicals, Mannheim, Germany) and the primers sXhoIShATG (5'-CTCGAGATGCCNCCNYTNCCNYTNGARCAY-3'), a primer designed from the amino acid sequence of rat agrin Rupp *et al.,* 1991), and asBgIIIFS3 (5'-AGCT TAGATCTCCCACATGGCCCTTGGCGGAGT-3') resulted in PCR products in all tissues. PCR products were subcloned into the EcoRV sites of pBluescriptII KS1 (Stratagene) and sequenced, generating pKS-TM. The 5'end of chick agrin was isolated by primerextension analysis using the SMART RACE cDNA amplificationkit (Clontech). Total RNA from brain and spinal cord of 6-day-old chicken embryos was isolated according to the manufacturer's recommendation (RNeasy Minikit, Qiagen). First strand cDNA synthesis was initiated using the agrin-specific primer asccyA171 (5'-AAGAGCAGCAGAGCAAATCC-3') and the SMART II oligo (5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3'). Reverse transcription was performed on 1µg RNA using SuperscriptII reverse transcriptase (Gibco BRL). After incubation at 42°C for 60min, 1µl of RNaseH (RocheDiagnostics) was added and incubation was continued for 20min at 37°C. The resulting cDNA fragments were amplified by PCR (PCR Advantage 2 Kit, Clontech) with the nested antisense primer

asccyA131 (5'-GGATGAGGCAAATGTTGCAC-3') and the universal primer mix provided by the manufacturer. PCR fragments were analyzed on a 1% agarose gel, extracted from the gel (QIAEX, Qiagen), and cloned into the pGEM-TEasy vector (Promega). Individual colonies were grown and their inserts were sequenced.

Generation of Expression Constructs

Constructs to determine the orientation of agrin in transfected COS cells were made as follows. All constructs were cloned into the pBK-CMV vector (Stratagene). First, the human placental alkaline phosphatase (AP) gene (Berger *et al.,* 1988), originating from theAPtag-1 expression vector (Flanagan and Leder, 1990) was inserted into pBK-CMV using the *Hin*dIII and *Xho*I sites giving rise to pBK-CMV-AP. All constructs described in this section were cloned into the *Hin*dIII and *Bgl*II sites of this mammalian expression vector, giving rise to C-terminal AP-fusion proteins. To construct a transmembrane version of mouse agrin, the transmembrane segment was removed from the pKS-TM (see above) by digestion with *Hin*dIII and *Bgl*II and cloned into the corresponding sites of pBK-CMVAP. The Myc-TM-AP construct was cloned as follows. The 6 amino-terminal myc epitopes and a 5'leadersequence were obtained by digestion of the pCS2+MTvector (Roth *et al.,* 1991) with *Hin*dIII and *Xho*I. The TM-fragment was generated by digestion of pKS-TM with *Xho*I and *Bgl*II. Both fragments were then cloned into the *Hin*dIII and *Bgl*II sites of pBK-CMV-AP in a triple ligation. The SS-NTA-AP construct, containing the entire mouse SS-NtA, the first follistatin like domain (FS) and a C-terminally fused AP was generated by a series of RT-PCR on RNA isolated from PO mouse kidney and PCR on the genomic clone HB4. The resulting two PCR products were ligated into pBK-CMV-AP, yielding the final SS-NtA-AP construct. All constructs were verified by DNA sequencing. PCR conditions and DNA manipulations were done according to standard protocols, if not indicated otherwise. Full-length cDNA encoding the transmembrane form of chick agrin (TM-Agrin) was constructed by replacing the sequence encoding the 5'end of pcAgrin$_{7A4B8}$ (Denzer *et al.,* 1995) by a PCR fragment isolated from the 5'RACE experiments described above. To remove most of the 5'untranslated region and to introduce an *Eco*RI restriction site, PCR was conducted using the primer *Eco*RI-5'ccyA_27 (5'-GCGAATTCACAGCATGAC GGCTTGCCA-3') and the antisense primer agrin_as537 (5'-CGCACACCGCCCCGAACCCA-3'). The fragment was digested with *Eco*RI and *Xho*I and ligated to pcAgrin$_{7A4B8}$ΔUTR, an

expression construct whose 3'untranslated region had been removed. Because digestion of pcAgrin$_{7A4B8}$ΔUTR also removed an *Xho*I–*Xho*I fragment within the coding region of chick agrin, another ligation was necessary to reinsert this fragment. This was accomplished by *Xho*I digestion of the pcAgrin$_{7A4B8}$ΔUTR, isolation of the resulting agrin fragment and ligation to the construct from the previous ligation, cut with *Xho*I. The resulting construct pcTM-Agrin$_{7A4B8}$ΔUTR contained the entire open reading frame of the transmembrane form of neural agrin.

Quantification of AP Fusion Proteins

Two days after transfection of COS cells, conditioned medium was collected (sup), the cells were scratched off the culture dish and lysed in 1ml PBS, 1% TritonX-100. To inactivate the endogenous phosphatases, the cellular fraction and the supernatant were heated at 65°C for 5min. The fractions were subsequently centrifuged at 13,000rpm for 2min. A 50-µl aliquot of every fraction was mixed 1:1 in a microtiter plate with the Sigma FAST *p*-Nitrophenyl Phosphate (Sigma) substrate for AP. The amount of AP was determined by measuring the OD405 every 5min during 1h (Labsystems Multiscan RC) and by determining the maximal slope of the curve ΔOD/µl min over four timepoints (Genesis 1.79, Lifesciences Ltd.).

Staining of COS Cells, Metabolic Labeling and Immunoprecipitations

Transfected COS cells were grown for 2 days in DMEM containing 10% FCS before staining. For the surface staining, the cells were precooled for 15min to 4°C. The medium was replaced by precooled medium containing either anti-human placenta AP monoclonal antibody (MedixBiotech Inc.; diluted 1:500) or anti-myc monoclonal antibody 9E10 (Evan *et al.,* 1985; diluted 1:1000). The cells were incubated for 30min and washed with precooled medium. They were fixed with 4% paraformaldehyde (PFA), 11% sucrose in a 0.1M potassiumphosphate buffer, pH7.2, for 30min at 4°C, followed by another 30min at room temperature (RT). Reactive groups in PFA were subsequently blocked with PBS containing 20mM glycine. To permeabilize the cells, they were incubated with PBS containing 0.1% saponin (PBSS) and10% normal goat serum (NGS) for 20min at RT. Appropriate FITC-conjugated antibodies were incubated for 1h in the dark at RT. After washing with PBSS, the cells were mounted with Citifluor (Plano) and examined with a

microscope equipped with epifluorescence (Leica, Nussloch, Germany). For intracellular staining, cells were fixed first for 30min with 4% PFA, 11% sucrose in 0.1M potassiumphosphate buffer, pH7.2. After two washes with PBS and PBSS, the cells were incubated for 20min with PBSS containing 10% NGS. Subsequently, they were incubated for 2h with the primary antibody, then rinsed with PBSS. The incubation with the secondary antibody and the analysis was performed as described above. The procedure to metabolically label agrin-transfected COS cells, was similar to that described elsewhere (Gesemann *et al.,* 1995) except that COS cells were chased for 3h with medium supplemented with 10mM methionine before lysis.

Real-Time Quantitative PCR

For real-time quantitative PCR, the ABI PRISM 7700Sequence detector (PE Applied Biosystems) and the protocols supplied by the manufacturer were used. The following primers and probes were used for the different regions.

SS-NtA region:

sense primer: scNtA388 (5'-ACCCAGCCCCTCAGTACATGT-3')

antisense primer: ascNtA432 (5'-CATCAGGCTGGAGTTGAGCAT-3')

probe: (5'-**FAM**CCTGCCCACCGCAACGAGC**TAMRA**-3')

First FS-like region (TM-Agrin):

sense primer: scFS146 (5'-CCAACGAGTGTGAGCTGGAGAAA-3')

antisense primer: ascFS293 (5'-GTCCCTTGCTGATGACCTTGA-3')

probe: (5'-**FAM**CCCAGTGCAACCAGCAGAGGCG**TAMRA**-3')

Transmembraneregion (TM-Agrin):

sense primer: sccy-A166 (5'-TCATTGATCCCGTGCAACATT-3')

antisense primer: asccyA214 (5'-AGCAGCACAGCAAATCCCA-3')

probe: (5'-**FAM**CCTCATCCTCCTGGCCACCTCCA**TAMRA**-3')

As internal standard, we used primers and probes for glyceraldehyde3-phosphate dehydrogenase (GAPDH):

sense primer: GAPDH rev. (5'-GGGCACTGTCAAGGCTGAGA-3')

antisenseprimer: asGAPDH rev. (5'-GGTCACGCTCCTGGAAGATAGT-3')

probe: (5'-**FAM**atggcgtgcccattgatcacaagtttc**TAMRA**-3').

To determine the relative amplification efficiency of PCR reactions for specific sequences encoding the first follistatin-like (FS) domain (pan-Agrin), the transmembrane region (TM-Agrin), and the NtA domain (SS-NtA-Agrin), a construct was generated that comprised all three regions. Construct pcN25$_7$C21$_{B8}$ (Meier *et al.,* 1998a) and the subcloned PCR fragment from the primer extension analysis (see above) served as templates to generate this construct. pcN25$_7$C21$_{B8}$ was digested with *Xba*I, blunt-ended with Klenow, and digested with *Eco*RI. This generated a fragment containing the entire pcDNA1 vector and the sequences encoding SS-NtA and the first FS-like region. The sequence encoding the transmembrane region was isolated from the subcloned PCR fragment from the primer extension analysis by *Cla*I digestion followed by Klenow-treatment and digestion with *Eco*RI. After gelpurification, the two fragments were ligated. Amplification efficiencies for individual sets of primers and probes were measured using different concentrations of the construct (0.05, 0.1, 0.2, and 5 ng). Relative amplification efficiencies were SS-NtA-Agrin = 0.93 X pan-Agrin and TM-Agrin = 1.3 X pan-Agrin. All the different tissues were dissected from embryonic 11-day-old chick embryos. Total RNA was isolated using a commercial kit (RNeasy Mini Kit, Qiagen). RNA was reverse transcribed using random primers and SuperscriptII (Gibco, BRL). To determine the relative amount of agrin mRNA in each tissue, mRNA encoding the house-keeping enzyme GAPDH was used for normalization. In all tissues, the relative abundance of agrin mRNA was similar which allowed use to set the amount of mRNA encoding pan-Agrin as 100%. Relative amounts of mRNA encoding the two agrin isoforms, TM-Agrin and SS-NtA-Agrin, were calculated by accounting for the amplification efficiencies of the different sets of primers.

Characterization of the Mouse Agrin Gene

In order to determine the genomic organization of the agrin gene a Bacterial Artificial Chromosome (BAC) mouse strain 129 library (Genome Systems, Inc.), kindly provided by Dr. R. Skoda (Biozentrum), was screened with probes against the NtA (NtA-probe1) and the TM-region of mouse agrin. The probes were labeled by PCR in the presence of [α32P]dATP (Amersham PharmaciaBiotech) and subsequently purified with NucTrap pushcolumns (Stratagene) according to the manufacturer's protocol. The amount of labeled probe was determined by liquid scintillation counting (Packard Tri-Corb 2200CA). The NtA-probe1 was generated by PCR on mouse cDNA (see below) using the primer pair

sNA1 (5'-CACCACGTTCGCCTCCTCCT-3') and asNA3 (5'-GGCCACCACATCTTTGC-3') giving rise to a 128bps probe containing parts of the first two exons of the mouse NtA. The NtA-probe2 (87bps), which contains only the first exon of the NtA, was generated by PCR with sNA1 and asNA2 (5'-CCTTGCAGGAGTAGGTGTG-3'). To obtain the 132-bp TM-probe, a PCR with the primers sSh1 (5'-ATGCCCCCGTTGCCTTTTGA-3') and asSh3 (5'-CAGCACCGCAAAGCCCAACG-3') was performed. Before hybridization, the nitrocellulose filters of the BAC library were washed for 1h at 45°C in 15mMNaCl, 150mM sodium citrate, pH7 (2XSSC), containing 0.1% SDS. Subsequently, filters were prehybridized for 2h at 37°C in 300ml in 5XSSC containing 5XDenhardt's reagent, 48% deionized formamide and 100µg/ml salmon sperm DNA (hybridization solution). The labeled and denatured probe (approximately 108cpm) was incubated overnight at 37°C. Filters were washed three times at 45°C using 2XSSC containing0.1% SDS, wrapped into saran foil, and analyzed with the PhosphorImager 425 (Molecular Dynamics). One BAC contained the SS-NtA and the TM (Genome systemsclone no. 3/4/J3/130) and two other BACs contained only the TM domain (2/6/n23/96 and 4/2/b17/182). The BAC containing both sequences was subjected to further investigation. Fragments generated by restriction digestion were identified by Southern blot analysis with the described probes and subcloned into pKS (Stratagene). HB4, a 2.7kb long *Hin*dIII– *Bam*HI-fragment, was identified with NtA-probe2. It represents the most 5'clone, containing parts of the 5'UTRand the first exon. It is followed by the 4.2kb, *Bam*HI cut fragment Bam8 that was recognized by the NtA probe1. The EcoN fragment is a 6.7kb-long EcoRI fragment covering most of the first intron and the second exon. The only fragment covering the second exon oft he NtA and the TM is the *Xho*I-cut product XhoNS, which hybridizes with NtA-probe1 and the TM-probe. It overlaps in the TM domain with the 6.5kb EcoRI, clone, EcoS, which overlaps with the published sequence (Rupp *et al.,* 1992) at the 3'end. The described genomic clones were characterized by further restriction mapping and partial sequencing. Sequencing was performed using Big Dye AmpliTaq FS DNA sequencing kit (PerkinElmer) and ABI-Prism-System 377 and 310 (PerkinElmer).

Generation of Anti-Peptide Antisera

For immunization the peptide LICDNQVSTGDTRIFFVNPAP, corresponding to amino acid 96 to 116 of the chick NtA domain (Denzer *et al.,* 1995) and the peptide

TACQYPMAPGALERDRLYQHKV, corresponding to amino acid 2 to 23 of the transmembrane form of agrin, were coupled to keyhole limpet hemocyanin via an additional N-terminal cysteine and injected into two sheep each. Approximately 100ml of the bleed from day 135 of the immunization were affinity-purified using the corresponding peptide coupled to CNBr-Sepharose 4B (Pharmacia, Freiburg, Germany). The eluted IgG appeared homogeneous in SDS–page analysis after silver staining. The IgG concentration of the affinity-purified antisera was measured using the Bio-Rad protein assay as described by the manufacturer (Bio-Rad, Munich, Germany). The antisera were used at a concentration of 5µg/ml for immunocytochemistry.

Immunocytochemistry

Staining of E6 chick retinae was performed as described previously (Kroger *et al.,* 1996). Stained sections were investigated using the appropriate wedge-corrected fluorescence filter sets. In preparations stained with only the one primary or only the one secondary antibody, no signal was detected in the corresponding other channel, indicating the specificity of the fluorescence and an absence of cross-bleeding. Preincubation of the antisera with the peptide used for the immunization strongly reduced the immunofluorescence, demonstrating the specificity of the signal. The corresponding preimmune sera gave no specific staining. Images were taken with a cooled CCD camera (Spot 2, VisitronSystems, Puchheim, Germany). Brightness and contrast of the images were adjusted using Adobe Photoshop5.5.

Analysis of Agrin in Retinal Ganglion Cells

Retinal ganglion cells were purified from E6 chick retina by immunopanning with the anti-Thy-1 antibody (generously provided by Peter Jeffrey and A. Rodriguez-Tebar) as previously described (Barres *et al.* 1988) with the following modifications: Petridishes were coated with goat anti-mouse IgG (Dianova, Hamburg, Germany; 23µg/ml final concentration) overnight at 4°C and subsequently coated with the mouse anti-chick Thy-1 monoclonal antibody (Sinclair *et al.,*1987) ascites fluid diluted 1:1000 in PBS containing 1mg/ml d-glucose, 0.036 mg/ml sodium pyruvate (DPBS),supplemented with 0.2% BSA. Chick retinae were dissociated by incubating six pigment epithelium-free retinae in 10ml D-PBS, containing 80 units papain (Worthington/Technicon, Bad Vilbel Germany) and

1000 units DNase (Sigma, Deisenhofen, Germany). Proteolytic digestion was stopped by adding ovomucoid and the tissue was triturated as described by (Barres *et al.,* 1988). The single cell suspension was then added to the coated petridishes and incubated 45min at RT with gentle agitation. Afterwards, the plates were washed 10 times with D-PBS and the panned cells were released by trypsinisation in 0.125% trypsin (Sigma). Cells were collected by centrifugation at 900*g* for 15min. Only a single panning step was necessary in the avascular E6 chick retina. Analysis of the purified fraction with the anti-Islet-1 antibody, a specific marker for retinal ganglion cells (Austin *et al.,* 1995), revealed that the ganglion cells were more than 97% pure after a single panning step. RNA was isolated from the panned ganglion cells and from total retinal cells using the GlassMAX RNA isolation system (LifeTechnologies, Karlsruhe, Germany). Messenger RNA from total retina from different developmental stages was isolated using the Ultraspec reagent (Biotecx Laboratories, Houston, TX). Complementary DNA was synthesized from 1µg (panned cells) or 5µg (total retina) mRNA after DNase digestion using 5units DNaseI (Life Technologies) using oligo dT10-primers and random primers (0.5µM each, Boehringer–Mannheim, Germany), dNTPs (0.5mM each; Boehringer), DTT (10mM; Life Technologies), RNase inhibitor (100units; Boehringer), and SuperscriptII reverse transcriptase (1000 units, Life Technologies). PCR reactions were performed using the following primers:

NtA domain first PCR:

Sense primer: NTAF12 (5'-CTGGGGTTTGGCGCTGGGATTGG-3')

antisense primer: NTAB1 (5'-GGGTCGGCGGGGTCTGGGTAAA-3'); annealing temperature 63°C

Semi-nested PCR for the NtA domain:

Sense primer as in the first reaction

antisense primer: NTAB2 (5'-CATGTACTGAGGGGCTGGGTTGAC-3'); annealing temperature: 61°C

The transmembrane form of agrin was identified by a similar nested PCR approach. The first round of PCR was performed using the following primer pair:

TMF1 (5'-TCTCCTGGTGGTGCGCTACTTCA-3')

TMB1 (5'-AGGGGTCGCAGGCTCCATCAAACT-3'); annealing temperature: 62°C)

The second nested PCR was performed using the following primer pair:

TMF2 (5'-CTGGGATTTGCTGTGCTGCTCTTC-3')

TMB2 (5'-CTCGCTGCGGTAGTCCTT-3'); annealing temperature: 59°C)

The PCR reaction products were separated on 3% Tris-borate-EDTA agarose gels and stained in 2.5µg/ml ethidium bromide. Two types of controls were analyzed to exclude the presence of contaminating DNA: a nested PCR on an aliquot of the first PCR in which water was added instead of cDNA (lanes C1 in Figs. 9B and 9C) and a PCR with only the nested primers, but with water instead of cDNA (lanes C2 in Figs. 9B and 9C). In all cases the PCR products were subcloned into the TOPO cloning kit (Invitrogen) and at least three randomly chosen clones were sequenced.

Acknowledgments

We thank P. A. Marangi for his help in the initial stages of this project. We also thank Dr. Gabriela Bezakova and Dr. Thomas Meier for critical reading of the manuscript. The monoclonal anti-Thy-1antibody was generously provided by P. Jeffrey and A. Rodriguez-Tebar. We appreciate the help of F. Pfrieger with the immunopanning. This work was supported by grants to MAR from the Swiss National Science Foundation and the Schweizerische Stiftung für dieErforschung der Muskelkrankheiten and by grants from the Deutsche Forschungsgemeinschaft (KR-1039) and the Graduiertenkolleg Frankfurt to S.K. and M.A., respectively.

Note added in proof. The work cited as "R. W. Burgess, W. Skarnes, and J. R. Sanes, Abstract to Soc. Neurosci. Meeting 1999" has now been published as Burgess, R. W., Skarnes, W. C., and Sanes, J. R. (2000). Agrin isoforms with distinct amino-termini. Differential expression, localization, and function. *J. Cell Biol.* **151:** 41–52.

Chapter III

Transmembrane Agrin Reorganizes the Cytoskeleton in Neurons and Non-Neuronal Cells

Introduction

Agrin is widely expressed in the central (CNS) and in the peripheral nervous system (PNS), in neuronal as well as in non-neuronal tissue. Agrin's function is best understood in skeletal muscle where it plays a key role during formation, maintenance and regeneration of the NMJ (for review seee Sanes and Lichtman, 2001; Bezakova Ruegg, 2003). Its role in other tissues, in particular the CNS is still poorly understood and very little is known (for review see Kroger and Schroder, 2002). Although CNS neurons from mice with a targeted deletion of the agrin gene form normal synaptic specializations *in vitro* and *in vivo* (Li *et al.*, 1999; Serpinskaya *et al.*, 1999), the acute suppression of agrin expression or function by antisense probes or antibodies influences the formation and function of synapses (Bose *et al.*, 2000). The suppression of agrin expression results in the impairment of dendritic development and the formation of less synapses than in non-treated or sense-treated neurons.

Due to morphological and physiological similarities between nerve-muscle and the nerve-nerve synapse it was assumed that the role in synapse formation might be analogous. Several studies addressed the question of the involvement of agrin and its task in the CNS, in neurite differentiation and outgrowth, and there is mounting evidence that agrin is a critical determinant in neuronal differentiation (for review see Smith and Hilgenberg, 2002). Agrin isoforms are highly expressed by CNS neurons before synaptogenesis, suggesting additional functions for agrin during axonal elongation (Annies and Kroger, 2002; Halfter *et al.*, 1997; Kroger, 1997; Kroger *et al.*, 1996; Mantych and Ferreira, 2001). However, agrin's precise role during development of the CNS remains to be clairified.

Alternative first exon usage results in the synthesis of either a secreted form (SS-NtA-Agrin) or a transmembrane form of agrin (TM-Agrin). These isoforms are differentially expressed. While SS-NtA-Agrin is ubiquitously expressed in most basal lamina containing tissues, transcripts encoding TM-Agrin are preferentially enriches in the CNS, particularly on neurites during the phase of active neural growth (Burgess *et al.,* 2000; Neumann *et al.,* 2001; Annies and Kroger, 2002).

In developing central neurons agrin is localized in axons, growth cones and at synaptic sites, where it may regulate differentially axonal and dendritic elongation. Neurons grown on cells expressing different isoforms of agrin develop shorter axons but exhibit more arborization. On the other hand agrin induces longer dendrites with more braches than in agrin-depleted neurons. Those changes are associated with changes in composition of the cytoskeleton (Mantych and Ferreira, 2001). Agrin provokes and regulates neuro-specific genes that are involved in neurite elongation and branching. At nearly every phase of brain morphogenesis agrin is expressed (Cohen *et al.*, 1997; Ferreira, 1999; Mann and Kroger, 1996).

Thus agrin in the CNS seems to have alternate functions than it has in the development of the NMJ and it may be an important regulator of the maturation of dendrites and of synaptogenesis of neurons in the CNS.

The identification of the transmembrane form of agrin has led to the hypothesis that this isoform might serve as a receptor with signal-transducing activity (Neumann *et al.*, 2001). To verify this hypothesis, it has to be taken into consideration that the HSPG agrin is highly glycosylated and that it has two glycosaminoglycan (GAG)-attachment sites. A heparan sulphate proteoglycan is attached at serine/glycine (S/G)-clusters located between the 7^{th} and 8^{th} follistatin-like domain. At the centrally-located serine-threonine-rich domain there is a chondroitin sulphate-side chain (Winzen *et al.*, 2003).

These regions are involved in binding to neural-cell adhesion molecule (NCAM) and to heparin-binding growth factors, proteins involved in neuronal growth and development (Cole and Halfter, 1996; Cotman *et al.*, 1999). Agrin is able also to inhibit neurite outgrowth via glycosaminoglycan (GAG) chains (Baerwald-De La Torre *et al.*, 2004; Bixby *et al.*, 2002).

To test for the function of agrin in neurite outgrowth and to narrow down the responsible region, several non-neuronal cell lines (PC12, COS, HEK293) and primary hippocampal neurons were transfected with cDNA encoding cTM-Agrin. The protein induced the formation of actin-containing protrusions extending from the transfected cells. To address the question which part of the protein is necessary to induce these morphological changes, different chimeric constructs were generated where parts of the protein were replaced. While the intracellular and the transmembrane domains of TM-Agrin were dispensable for the formation of these processes, we show that the GAG side chains of TM-Agrin together

with close association of agrin with the plasma membrane are necessary and sufficient for the formation of these protrusions. Transfection of primary hippocampal neurons with TM-Agrin also results in changes of the morphology, as agrin, expressed in either axons and dendrites induced the formation of microprocesses. To address the question of agrin as a receptor, we took advantage of the fact that most receptors with a single transmembrane region can be activated independently of their ligands by dimerization or oligomerization using polyclonal antisera (Heldin, 1995; Weiss and Schlessinger, 1998). Anti-agrin antibodies were used to cluster agrin on primary hippocampal neurons. We demonstrate that anti-agrin antibody-mediated oligomerization of TM-Agrin induces extensive changes in the axonal cytoskeleton of neurons as well as in transfected non-neuronal cells, resulting in the extension of numerous actin-containing processes. Microspikes appeared within minutes after antibody addition, contained a complex cytoskeleton and could be induced on neurons from the PNS and CNS. Time-lapse video microscopy revealed that microspikes were highly dynamic and were formed from growth cones that transiently extended from the axon shaft and adhered to the substrate, but were subsequently retracted. Taken together, these results support the hypothesis that TM-Agrin can serve as a receptor and suggest a role of TM-Agrin during axonal elongation or axonal branching in the developing CNS.

Materials and Methods (Non-Neuronal Cells)

Generation of Expression Constructs

pcTM-Agrin

The cDNA sequence for cTM-Agrin (c=chick) have been described elsewhere (Neumann *et al.*, 2001). Chimeric constructs were generated where domains of the protein were exchanged and replaced by similar domains of other proteins. All constructs were cloned into the vector pcDNAI (Invitrogen; p = cloned into pcDNAI). All constructs were routinely sequenced to eliminate the possibility of mutations.

pmyc.cTM-Agrin

The cytoplasmic part of full-length cTM-Agrin was replaced by myc. A polymerase chain reaction (PCR) on the plasmid p_myc (Jones *et al.*, 1999) was performed with the sense primer sEcoRI_myc:

(5'-GCTGGAATTCGGATCCCAGTCGACTCTAGAGGAAGTGGTAAAGCT-3')
and the antisense primer asBspHI_myc:
(5'-CGGTCATGAACCCGAGGTCGCCCAAGCTCTCCATTTCATTCAAGT-3')
to obtain the myc-tag flanked by an *Eco*RI- and a *Bsp*HI-restriction site. The PCR product and pcTM-Agrin was cut with the restriction enzymes *Eco*RI and *Bsp*HI (both from NEB). The cytoplasmic part was cut out and replaced by myc.

pmyc.cTM.Nsk2.ECD.His

The cytoplasmic and the extracellular part were exchanged, the cytoplasmic part by myc and the extracellular part by the extracellular domain of the transmembrane protein Nsk2 (dissertation Dominik Hauser). First, the *Nhe*I- and the *Hin*dIII-restriction sites were introduced by PCR on pmyc.cTM-Agrin with the sense primer T7:
(5'-TAATACGACTCACTATAGGG-3')
and the antisense primer as_NotI_cTMdomain:
(5'-GATGCGGCCGCGAGGAAGAGCAGCACAGCAAATCCCAGCGTGGAGG-3')
The PCR product was subcloned into the TOPO-vector (Stratagene). Using the restriction sites *Nhe*I/ *Hin*dIII, the insert was cloned into the vector pCEP-Pu (Kohfeldt *et al.*, 1997). In order to replace the extracellular part of cTM-Agrin by the extracellular domain (ECD) of Nsk2, the insert was cloned into the pCEP-Pu-vector containing myc and TM-domain of agrin. The myc.cTM.Nsk2.ECD.His insert was ligated into the pcDNAI-vector using *Bst*XI/ *Xho*I.

pcTM(ASGP-R)-Agrin

This construct was generated to exchange the TM-domain of agrin by the transmembrane domain of asialoglycoprotein receptor (ASGP-R; kindly received by M. Spiess; Spiess and Lodish, 1985). Several PCRs were necessary to create this construct. The first PCR was performed on ASGP-R to isolate the transmembrane domain and to introduce a *Bsp*HI site at the 5'end with the sense primer Pr3.BspHI_ASGP-R/TM-Agrin:
(5'-ACTTCATGATCCTCCTCCTGCTCTCCCTG-3')
and the antisense primer Pr4.ASGP-R/TM-Agrin:
(5'-ATAGCTATTGGGCTTGTAGTTGATCACACAGACAACCACAAG-3')
A second PCR on pcTM-Agrin was made with the sense primer Pr1.ASGP-R/TM-Agrin:

(5'-TTGTGGTTGTCTGTGTGATCAACTACAAGCCCAATAGCTATT-3')

and the antisense primer Pr2.BssHII_TM-Agrin:

(5'-agcagcgcgccgtgcca-3')

These two PCR fragments (809bps and 89bps) were purified and used as templates for the following overlapping PCR. The short fragment was loaded on 4% MethaPhor agarose (BMA) and extracted with phenol-chloroform method. The overlapping PCR was performed with the sense primer Pr3.BspHI_ASGP-R/TM-Agrin:

(5'-ACTTCATGATCCTCCTCCTGCTCTCCCTG-3')

and the antisense primer Pr2.BssHII_TM-Agrin:

(5'-AGCAGCGCGCCGTGCCA-3')

The obtained PCR product was digested with *Bsp*HI and *Bss*HII. To overcome the dam-methylation problem of the *Bsp*HI-site in pcTM-Agrin, an additional PCR on the latter construct was carried out with the sense primer T7pcDNAI:

(5'-TAATACGACTCACTCTAGGG-3')

and the antisense primer ascyA131:

(5'-GGATGAGGCAAATGTTGCAC-3').

This PCR product was cut with *Eco*RI and *Bsp*HI. pcTM-Agrin was digested with *Eco*RI and *Bss*HII. The PCR product, and the last PCR fragment were cloned via triple-ligation into the digested pcTM-Agrin.

pcTM-PF9

This construct is a shortened version of the cTM-Agrin$\Delta^{ST1\text{-}G3}$ (kindly received from Stephan Kröger). It has a GAG attachment site between the 7th and 8th FS domain. The 5'end of cTM-Agrin$\Delta^{ST1\text{-}G3}$ was cloned into pcDNAI (Invitrogen) using *Hin*dIII and *Pst*I. The cDNA coding for the 8 FS-domains was cut out from cTM-Agrin$\Delta^{ST1\text{-}G3}$ and added to the 5'end already in the vector using *Pst*I. The correct of the insert orientation was checked by restriction digests.

pcTM-9Mc

This construct derives from 9Mc (kindly received from Willi Halfter; Winzen *et al*, 2003). It is composed of the 8 FS-domains of agrin. The three serines (S) from the SG-clusters between the 7th and 8th FS-domain are mutated to alanines (A). In order to add the DNA

sequence at the 5'end that codes for the cytoplasmic part of agrin and the TM-domain following procedure was performed: The 5'end of cTM-AgrinΔ^{ST1-G3} was cloned into pcDNAI using *Hin*dIII and *Pst*I. The mutated 3'end was cut out from 9Mc and added to the 5'end already in the vector using *Pst*I. The right orientation of the insert was confirmed by restriction digest.

Cell Culture

PC12 cells, a neuron-like cell line, were grown in DMEM high glucose (Gibco) supplemented with 5% FCS (Gibco), 10% HoS (Gibco), 0.11mg/ml sodium pyruvate (Gibco), 0.292mg/ml L-Glutamine (Gibco), 100U/ml Penicillin (Gibco), 100U/ml Streptomycin (Gibco). For human embryonic kidney cells (HEK293; Graham et al., 1977) the same medium was used supplemented with 10% FCS.

Transfection

Cells were first grown and cultured in 15cm tissue culture dishes. Before transfection they were plated with the density of 100'000 cells/ well in 4-well glass-slides that were coated overnight (oN) with poly-L-lysine (MW: 30'000-60'000 dissolved at 1mg/ml in 0.1M borate buffer; Sigma). After 24h, cells were transfected with jetPEI (Q-Biogene). The procedure followed the jetPEI protocol using 3µg plasmid DNA and 6µl transfection reagent pro well. PC12 were then treated with neural growth factor (NGF) to induce differentiation. After 24-48hs, cells were stained with appropriate antibodies (see below).

Antibodies

The following primary antibodies were used:

pNsk2	rabbit anti-mouse Nsk2 #194 (1:1000) recognizes ECD of Nsk2	unpubl., Ruegg MA
cTM-Agrin	rabbit anti-chick #3228 (1:1000) recognizes carboxy-terminal part of chick agrin	Ruegg, MA
SS-NtA-Agrin	rabbit anti-chick #3228 (1:1000) recognizes carboxy-terminal part of chick agrin	Ruegg, MA
myc.cTM-Agrin	rabbit anti-chick #3228 (1:1000) recognizes carboxy-terminal part of chick agrin	Ruegg, MA
myc.cTM.Nsk2.ECD.His	rabbit anti-mouse Nsk2 #194 (1:1000) recognizes ECD of Nsk2	unpubl., Ruegg MA
cTM(ASGP-R)-Agrin	rabbit anti-chick #3228 (1:1000) recognizes carboxy-terminal part of chick agrin	Ruegg, MA

cTM_PF9	rabbit anti-chick #706 (1:500) recognizes FS domains	unpubl., Ruegg MA
cTM_9Mc	rabbit anti-chick #706 (1:500) recognizes FS domains	unpubl., Ruegg MA

When applicable, antibodies against the intracellular part of cTM-Agrin (sheep anti-TM-Agrin; (Neumann *et al.*, 2001) were used to verify cTM-Agrin expression.

The following secondary antibody was used:

cy3-labeled goat anti-rabbit IgG (1:1000)	Jackson Laboratory

Immunocytochemistry

Transfected cells were washed twice with prewarmed PBS (phosphate-buffered saline) and then fixed with 4% PFA (paraformaldehyde) for 30min at RT (room temperature). Cells were again washed twice with PBS and twice with PBS containing 20mM glycine. Subsequenquently cells were incubated oN at 4°C with the primary AB (antibody), diluted in PBS containing 10% NGS (normal goat serum; Gibco). Next, cells were washed with PBS containing 10% NGS and permeabilized for 30min at RT with PBS containing 10% NGS and 1% saponin (Sigma). The secondary AB, diluted in PBS containing 10% NGS and 1% saponin was then added for 1h at RT in the dark. Alexa-488-labeled phalloidin (1:500; Molecular Probes) was used to visualize the cytoskeleton. Staining of the nuclei was achieved with DAPI (1µg/ml; Sigma). After washing with PBS containing 10% NGS and 1% saponin cells were mounted on glass slides and then examined under the fluorescence microscope.

Formation of processes was analyzed after fixation and staining. For quantification, only solitary cells were evaluated. Cells were scored "process-containing" in case they had more than 10 processes with a length of more than the cell's diameter. Results are given as mean +/- SEM with N=3.

Materials and Methods (Neuronal Cells)

Preparation of Mouse Primary Hippocampal Cultures

The coverslips were cleaned by incubation oN with HNO_3 and followed by two washes with NP (nanopure) H_2O and two washes with absolute EtOH for 30min each. For

sterilization, they were baked at 240°C oN. One day before plating, coverslips were coated with poly-L-lysine. After this preparatory work, pregnant mice were sacrificed by cervical dislocation. The hippocampi of E18.5 embryos were isolated and collected in 9ml precooled HBSS (Hank's Balanced Salt Solution; Gibco). Hippocampi were then treated with 1ml Trypsin/ EDTA (Gibco) and incubated in the waterbath at 37°C for 15min. The tissue was washed by fresh HBSS and replaced with 10ml MEM (Minimum Essential Medium) with Glutamax (Gibco) containing 10% FCS and 1% of P/S (Penicillin/ Streptomycin). Subsequently, cells were dissociated by pipetting smoothly (!). For staining of endogenous protein or for incubation experiments with the primary AB, the cells were plated at a density of 5-7.5 X 10^4 per coverslip. For transfection experiments cells were plated at a density of 15-20 X 10^4. The plating medium was MEM. Three hours later, when cells were attached on the coverslips, MEM was replaced with 1ml/well NBM (Neurobasalmedium; Gibco) containing 1% P/S, 0.5mMGln (Glutamine; Gibco), 25µMGlu (Glutamate; Gibco) and 2% B27 (Invitrogen). After three days in vitro (DIV) NBM was replaced with fresh NBM without Glu. Primary neurons were then stained or transfected at different time points.

Transfection of Primary Neurons

Primary Hippocampal neurons in a cell density of 15-20 X 10^4/ml were transfected the 4.DIV. The medium was replaced by 800µl of fresh NBM containing 0.5mM Gln and 2% B27. Transfection was performed with the transfection reagent Lipofectamine2000 (Invitrogen). The protocol was followed, using 3µg of cTM-Agrin plasmid DNA and 4µl Lipofectamine per well. As negative control and as control for the transfection efficiency an EGFP-construct (Invitrogen) was used.

Antibodies

The following primary antibodies were used:

mTM-Agrin	rabbit anti-mouse #204 (1:1000) recognizes	Eusebio *et al.,* 2003
cTM-Agrin	rabbit anti-chick #206 (1:1000) recognizes	Unpubl., Ruegg, MA
MAP2 in dendrites	chicken anti-MAP2 (1:200) 50µl in 10ml 3%BSA in PBS dendritic marker	abcam

synaptophysin	mouse anti-synaptophysin (1:1000) presynaptic marker	Chemicon
neurofilament	rabbit anti-mouse NF (1:500)	NovoCastra

The following secondary antibodies were used:

cy3-labeled goat anti-rabbit IgG (1:1000)	Jackson Laboratories
cy5.5-labeled goat anti-chicken (1:1000)	abcam
Alexa-488-labeled goat anti-mouse (1:1000)	NovoCastra

Immunocytochemistry

Non-transfected as well as transfected neurons were stained the 6., 10. and the 14. DIV. Cells were washed twice with PBS-1 (PBS with 120mM sucrose) and then fixed with FS (fixation solution: 4% PFA in PBS-1) for 30min at RT. After fixation, cells were washed twice with PBS-1 and twice with PBS-2 (PBS with 20mM glycine and 10nM sodium azide). For permeabilization, cells were incubated in PS (permeabilization solution: 0.25 Triton X-100 in PBS) for 10min at RT. After washing in PBS-1 cells were incubated in IN (incubation solution: 10 % BSA in PBS) for 60min in the incubator at 37°C. The incubation with the primary AB in AK (3 % BSA in PBS) was continued oN at 4°C. Next, cells were washed with PBS-1 and incubated with the secondary AB in AK for 60min at RT in the dark. For visualization of the actin cytoskeleton, Alexa-488-labeled phalloidin (1:500; Molecular Probes) was used and for the staining of the nuclei we used DAPI (1µg/ml; Invitrogen).

Induction of Microspikes with Antibodies

Before the staining procedure, microspikes were induced on hippocampal primary neurons by adding the primary antibody rabbit anti-mouse agrin #204. Neurons were incubated in early stage of development (5.DIV – before synapse formation) and at a later timepoint (10.DIV – during synapse formation). Incubation lasted 3hs in the incubator at 37°C. The AB was affinity purified using protein A and was then used at a final concentration of 10µg/ml or 50µg/ml. After treatment with AB, cells were stained against neurofilament, to visualize the dimension of the neuron. The number of microspikes was determined in double-blind experiments by counting the total number of microspikes in an axon segment with a length of 110 µm. At least 10 segments were scored in each of three independent

experiments. Microspikes on both sides of the axon were counted, but the individual branches of single microspikes were not scored.

Results

TM-Agrin Reorganizes the Cytoskeleton of non-Neuronal Cells

TM-Agrin was discovered as a highly glycosylated HSPG like the secreted isoforms but in contrast, as a protein that is predominately expressed in the CNS (Neumann *et al*, 2001). This was the reason for expression experiments in PC12 cells, a neuron-like cell line. The interest in this work was to analyse the expression in non-neuronal and neuronal cells. PC12 cells, neuron-like cell line, were transfected with full-length cTM-Agrin (C). They showed striking changes of morphology and an extension of microprocesses. Expression of SS-NtA-Agrin in parallel cultures, had no effect on the morphology of the transfected cells (B). In contrast, expression of the TM-Agrin induced a dramatic change in the morphology of the transfected cells. Transfection with Nsk2, also transmembrane protein with one TM domain, did not result in the formation of more processes compared to untransfected cells (A).

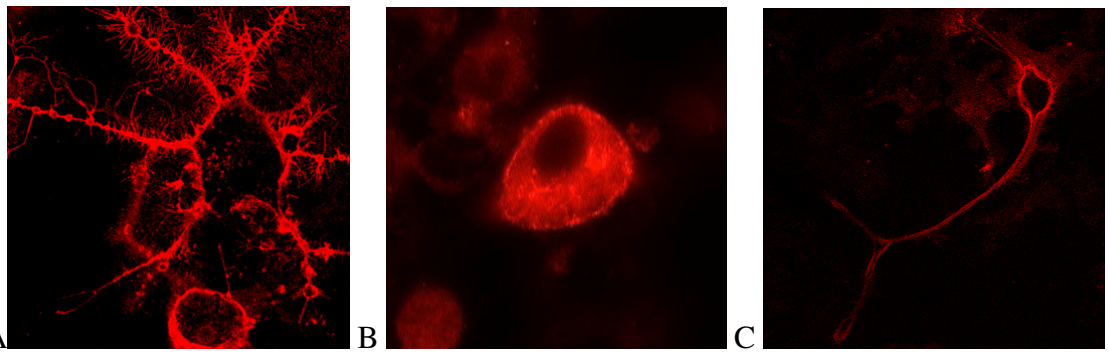


Fig. 11: PC12 cells transfected with cTM-Agrin (A), SS-NtA-Agrin (B) and Nsk2 (C)
Transfection of the neuron-like PC12 cells with cTM-Agrin results in the formation of long microprocesses in contrast to the transfection with the secreted SS-NtA-Agrin or with the transmembrane protein Nsk2.

The following transfection experiments were performed in HEK293 cells, because they showed the same alterations as PC12 cells upon transfection, and they were easier to handle. Quantification showed that more than 85% of the cells transfected with the TM-Agrin cDNA had extended processes containing actin filaments. Cells transfected with the

control cDNA of SS-NtA-Agrin and Nsk2 respektively did not exhibit the described morphological changes. Thus the formation of microprocesses is a characteristic feature of TM-Agrin.

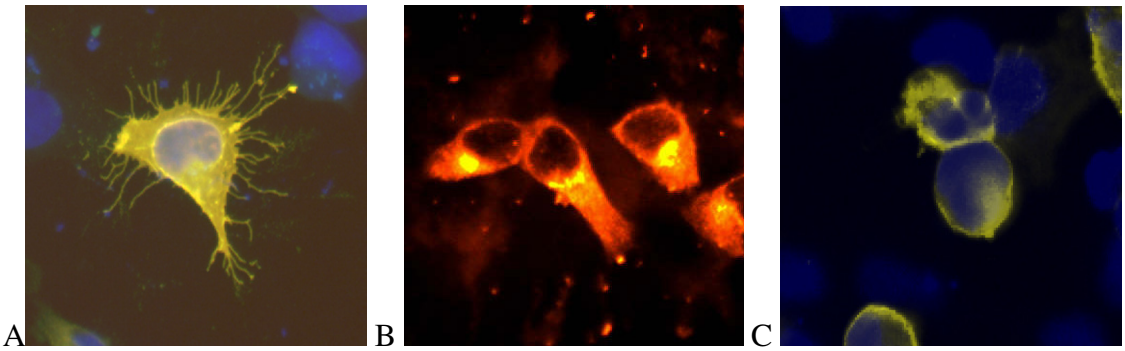


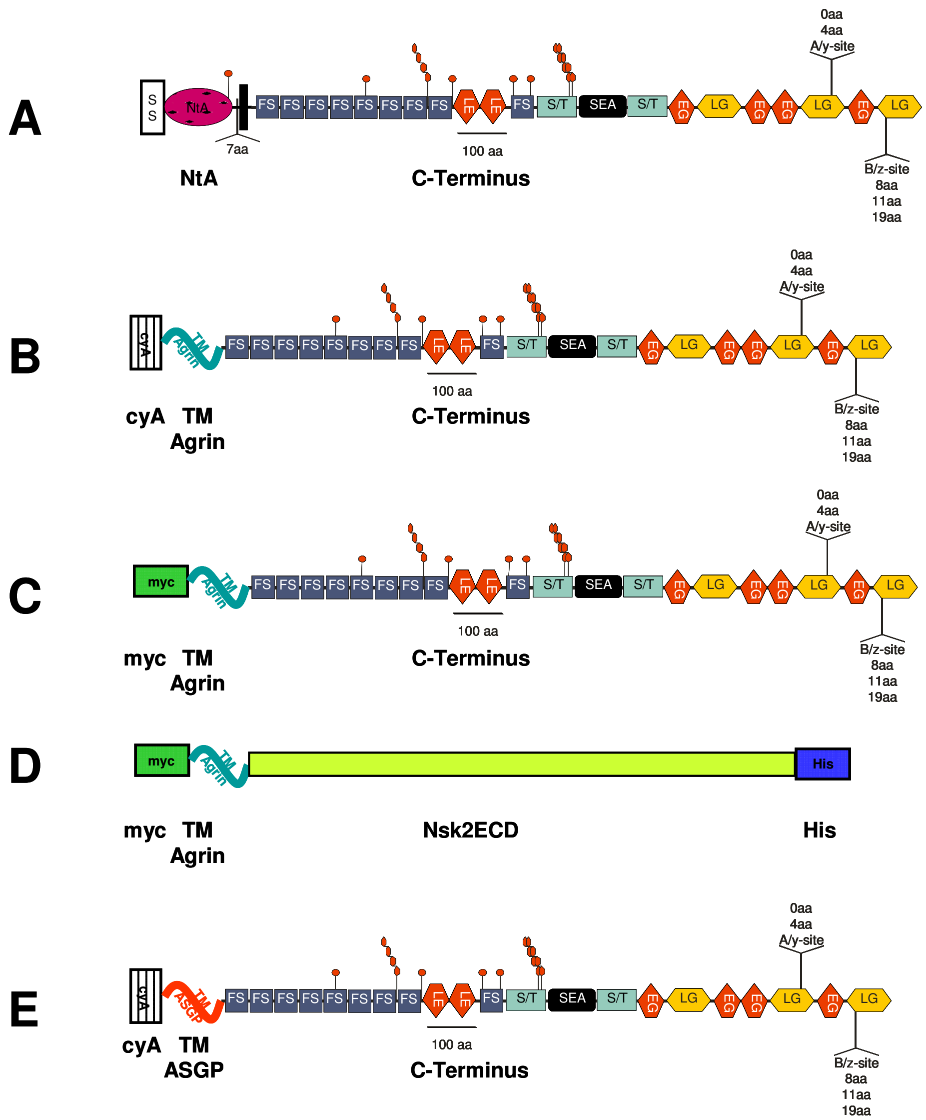
Fig 12: HEK293 cells transfected with cTM-Agrin (A), SS-NtA-Agrin (B) and Nsk2 (C)
HEK293 cells transfected with cTM-Agrin form long protrusions in contrast to the controls. Cells are stained for agrin (red), for actin with phalloidin (green) and for the nuclei (blue).

These data show that TM-Agrin is able to influence the cytoskeleton of non-neuronal cells, and that the effect on cell morphology is independent of the species of transfected cells. To determine whether protrusions contained cytoskeletal elements, they were stained with fluorescent Alexa-488-labeled phalloidin to reveal the actin filaments. The observation that transfection with TM-Agrin results in changes of phalloidin staining compared with the controls, led to the conclusion that this agrin isoform induces complex modifications of the cytoskeleton.

The Extracellular Part of TM-Agrin is Required and Sufficient for Process Formation

In order to determine the region involved in the induction of process formation, several chimera constructs were designed and expressed in HEK293 cells. Formation and extension of processes were quantified and expressed as percentage of process-containing cells per total number of transfected cells with the criteria described. Deletion and replacement of the intracellular domain of cTM-Agrin with myc-tag (C) did not abolish the process-inducing activity, indicating that the intracellular 35 amino acids are not required. 85% of the transfected cells show changes in morphology. Next followed a construct

where only the TM-domain of agrin was left, the cytoplasmic part of this chimeric TM-protein was the myc-tag and the extracellular part was substituted by the corresponding sequence of Nsk2 (D). In this case, only about 10% of the transfected cells featured microprocesses, suggesting that the TM-domain alone is not sufficient. The following construct was designed to figure out whether the TM-domain would be necessary for the modification of the cytoskeleton. Therefore this domain was replaced by the TM-domain of asialoglycoprotein-receptor, also a type II transmembrane protein (E). Transfection of HEK293 cells with this construct also resulted in the formation of microprocesses. This is conclusive evidence that the transmembrane region is not essential for these tremendous structural changes.



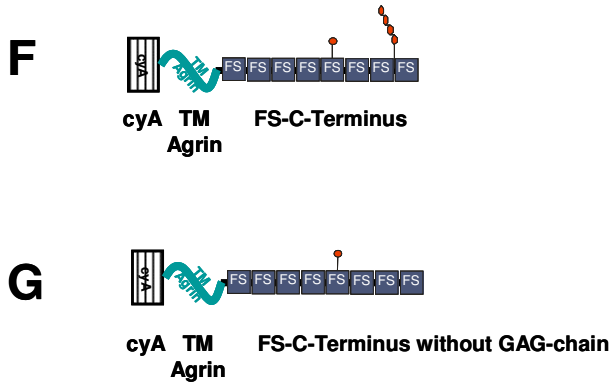


Fig. 13: Domain structures of different chimeric constructs

(A) This is the secreted isoform that binds to laminin. (B) This is the TM-Agrin that is stabilized in the membrane. (C) The intracellular part is replaced by myc.tag. (D) Only the TM domain derives from agrin, the intracellular part is the myc-tag, the extracellular region is the extracellular domain of Nsk2. (E) The TM domain is replaced by the TM domain of asialoglycoprotein-receptor. (F) Shortened version of agrin with a GAG chain attached between the 7th and 8th FS domain. (G) In this construct three serines are mutated to alanines so that GAG chain can not attach.

In summary, these data indicate that the intracellular part and the transmembrane domain of TM-Agrin are dispensable for the formation of microprocesses and suggest a crucial role for the extracellular part of TM-Agrin. Since the extracellular region of TM-Agrin is identical to the corresponding sequence in SS-NtA-Agrin, these results suggest the necessity of a close association of agrin with the membrane for the process-inducing activity. The close association of the extracellular part of TM-Agrin with the membrane is indispensable and sufficient for the process-inducing activity. Those data established the importance of the extracellular domain of TM-Agrin.

To determine the precise region within the extracellular domain, the C-terminal region of TM-Agrin was successively shortened. Deletion of the C-terminal half of agrin, a region which contained three LG domains and four EGF-like domains and which is necessary and sufficient for agrin's synaptogenic activity at the neuromuscular junction (Gesemann *et al.,* 1995), did not influence the ability to induce processes after transfection of HEK293 cells. Additional deletion of the two S/T-rich regions as well as the SEA module did not influence the process-inducing activity of TM-Agrin. In contrast, deletion of the 7th and 8th FS domain resulted in a complete loss of the process-inducing activity. This suggests that either the two FS domains or the GAG side chains, which are attached to the protein backbone between the 7th and 8th FS domain, might be responsible for the induction of processes in transfected cells. Transfection of cells with a shortened construct, containing

the intracellular part, the TM-domain and the 8 FS domains (F), results in the formation of microprocesses. Expression of a construct in which the GAG attachment site is mutated, serines in the three SG-clusters are mutated to alanines (Winzen *et al.,* 2003), did not result in the formation of processes. These results suggest that the GAG side chains of agrin localized between the 7th and 8th FS domain play a crucial role in the formation of processes.

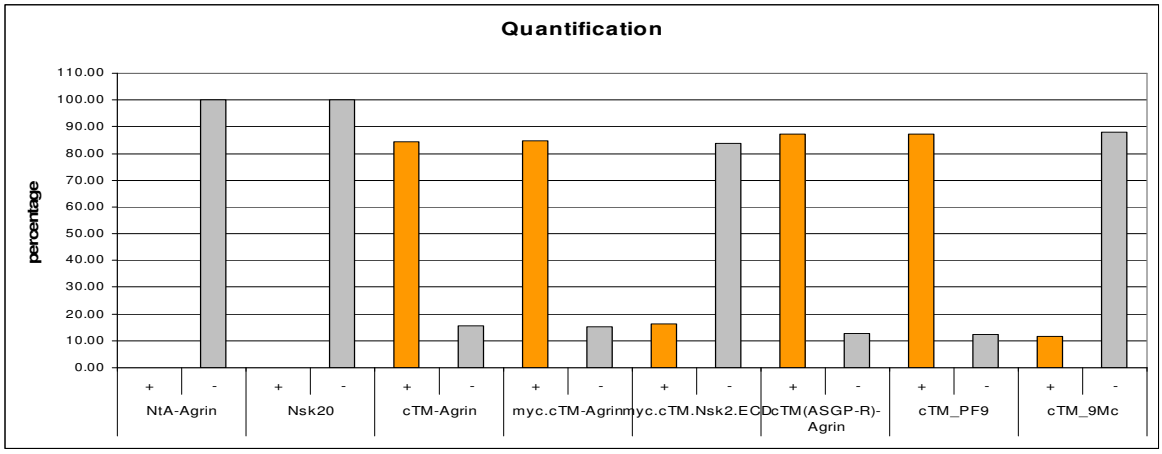


Fig. 13: Quantification of number of microprocesses

Result from this quantification is that the TM-domain of agrin is not sufficient for the induction of microprocesses. This region of the protein seems to be only necessary for the fixation of the protein in the membrane. The GAG-chain between the 7th and 8th FS domain is then closer to the membrane.

Microprocesses Form on Axons and Dendrites in the CNS

For a better understanding of the function of TM-Agrin in the brain, the full-length protein was expressed in mouse primary hippocampal neurons of E18.5. In a first step neurons were transfected and immunostained after 6, 10 and 14 DIV. The protein was expressed in axons as well as in dendrites. Dendrites could be distinguished from the axons by staining against MAP2, a dendritic marker. Agrin was stained with the rabbit anti-chick agrin antibody #228. Transfection of neurons results in changes in the cytoskeleton, after 10 and much more the 14 DIV. To confirm the assumption that the actin skeleton changed, neurites were stained with Alexa-488-labeled phalloidin to reveal the actin filaments. Non-transfected neurons, where the endogenous agrin was stained with the rabbit anti-mouse agrin #204 showed no morphological changes.

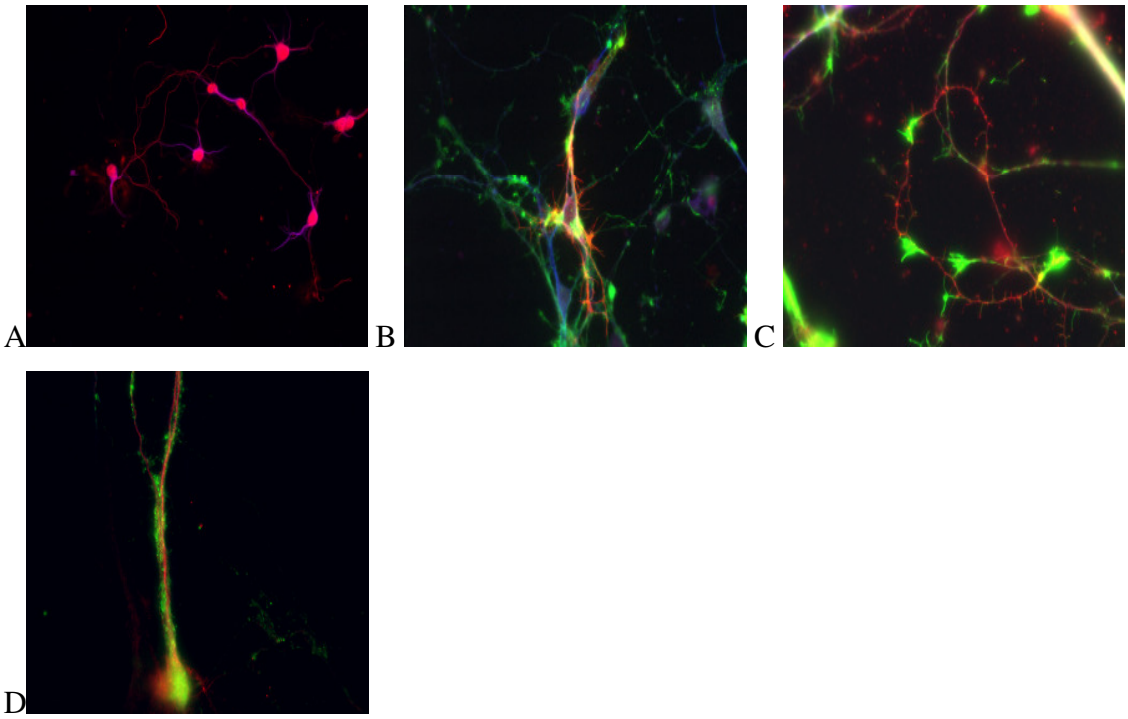


Fig. 14: Untransfected neurons (A), transfected neurons 6. (B), 10. (C) and 14. DIV (D)
In contrast to untransfected cells, neurons change their shape, when transfected with TM-Agrin.

The discovery of structure and intracellular localization of TM-Agrin led to the hypothesis that is might be a receptor or co-receptor capable of signal transduction across the membrane. It could be necessary for the signalling of small growth factor-like molecules.
We took advantage of the knowledge that most if not all receptors with a single membrane spanning region can be activated by dimerization or oligomerization via an appropriate antibody, independently of the physiological ligand (Heldin, 1995; Weiss and Schlessinger, 1998). Primary non-transfected neurons were incubated with the affinity purified rabbit anti-mouse agrin antibody #204. It has already been shown that E6 RGCs exclusively express the transmembrane form of agrin and that this form is present on the surface of growing axons *in vivo* and *in vitro* (Kroger *et al.*, 1996; Kroger, 1997; Hering and Kroger, 1999; Neumann *et al.*, 2001; Annies and Kroger, 2002). Furthermore it was found that addition of antibodies to RGC cultures dramatically changed the shape of axons. The treatment with antibodies resulted in a stronger effect than seen in transfected neurons.

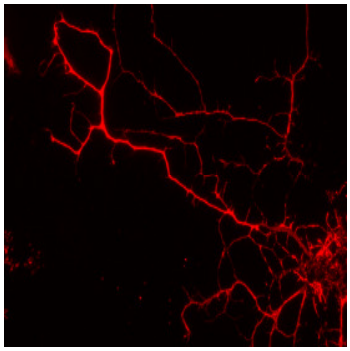


Fig. 15 Primary neurons after treatment with polyclonal antibodies
Primary neurons that are treated with antibodies grow more curvy and form many fine processes.

Anti-neurofilament antibody was used to visualize the extension of the neuronal cell. The actin cytoskeleton was labeled with Alexa-488 phalloidin. While untreated control neurons had a smooth surface and grew rather straight, neurites treated with antibodies exhibit along their entire length numerous and filopodia-like protrusions. Whereas the actin cytoskeleton in control neuronal cultures was well-defined, antibody-treated neurons show abundant protrusions spreading laterally. The phalloidin staining confirms the hypothesis that TM-Agrin influences the structuring of the actin cytoskeleton. Those structural changes could be observed on axons as well as dendrites.

Discussion

The transmembrane isoform of agrin is mainly expressed in the CNS and its discovery led to the hypothesis that TM-Agrin might be a receptor, involved in signal transduction across the membrane, and thus capable of inducing dramatic changes in cytoskeleton.

As a first step the expression of TM-Agrin was analyzed in non-neuronal cells. The striking effect on the shape of transfected cells, compared with non-transfected cells led to the question which region might be required for those changes in the cytoskeletal structure. Our results suggest that there are two requirements for the capability to induce microprocesses. The HSPG agrin is highly glycosylated and exhibits heparan sulphate proteoglycan as also chondroitin sulphate side chains which are attached at the protein backbone (Winzen *et al.,* 2003). But especially the GAG chain between the 7^{th} and 8^{th} FS domain was essential, which indicates the differences between the attached sugar chains. However, the presence alone is not sufficient, since SS-NtA-Agrin includes the same

glycosylation side and is not able to induce microprocesses (Neumann *et al*., 2001). Thus, the proximity of the GAG chain to the membrane is necessarily required. TM-Agrin has to be fixed in the plasma membrane through a transmembrane domain. Interestingly, as it was shown above, the TM-domain of agrin could be replaced by that of the asialoglycoprotein-receptor without any loss of process-inducing activity. Taken together, these results point out that TM-Agrin might play a role as co-receptor in cooperation with a signalling receptor or that TM-Agrin itself acts a signalling protein. TM-Agrin that is member of the large family of HSPGs behaves similarly as syndecans, also a HSPG (Yoneda and Couchman, 2003; McQuade et al, 2003).

In a further step, to analyse expression and function of TM-Agrin, the full-length protein was expressed in primary hippocampal neurons. With cTM-Agrin transfected neurons, show changes in the elongation and arborization of neurites. Transfected cells form more filopodia-like protrusion than controls.

Since most if not all receptors with a single TM domain can be activated by dimerization or oligomerization, independently of the physiological ligand, using polyclonal antibodies (Heldin, 1995; Weiss and Schlessinger, 1998), we analyzed potential receptor functions of TM-Agrin. Many studies have shown, that oligomerization of receptors can be imitated by using bivalent antibodies, so that a signaling cascade can be activated. This system have been used for MuSK (Hopf and Hoch, 1998) or TrkA (Clary *et al.*, 1994), adhesion molecules (Brieher *et al.*, 1996) or the transmembrane heparansulfate proteoglycan syndecan (Carey, 1997).

In order to study this potential receptor function of TM-Agrin, we incubated non-transfected primary neurons with anti-mouse agrin antibody. The effect of antibody incubation was much stronger, than that observed after transfection. Non-treated neurons have a smooth surface and grow straight whereas treated one form numerous protrusions. Antibody-treatment seems to mediate oligomerization of TM-Agrin at the surface of the cells and as a consequence neuronal cells form actin containing microspikes. These results support the hypothesis that the transmembrane isoform of agrin might exhibit signal-transducing activity that results in reorganization of the actin cytoskeleton.

Chapter IV

Upregulating Utrophin on Muscle Membrane by Overexpression of Neural Agrin: an Alternative Strategy

Introduction

Duchenne muscular dystrophy (DMD) is a widespread inherited neuromuscular disease that results from mutations/deletions in the X-linked dystrophin gene (Gramolini *et al.,* 1998). DMD is a progressive disease that affects 1 out of 3500 live males at birth (Emery, 1991). Characteristic is the degeneration of muscle fibers in repetitive cycles. Muscle fibers lose the capacity to regenerate, the muscle mass decreases and is progressively replaced by adipose and connective tissues. Children affected from DMD become wheelchair-bound in early adolescence and die in the second or third decade of life as a result of respiratory failure. Dystrophin, a large cytoskeletal protein of the spectrin superfamily, connects the extracellular matrix (ECM) with the cytoskeleton of the muscle fiber. It binds to a complex of dystrophin-associated proteins (DAP). The absence of dystrophin results in disruption of this linkage and thus mechanical damage of the sarcolemma during contraction and relaxation. Currently there is no cure, although several approaches have been investigated. One alternative consists in using a protein that is normally expressed in dystrophic muscle and that can functionally compensate the deficiency of dystrophin. Several years ago an autosomal homolog was identified which is referred to as utrophin (Love *et al*., 1989). Utrophin has also the capacity to bind to the DAP complex (Matsumura *et al*., 1992). Thus, utrophin seems to be a good candidate that could functionally compensate dystrophin. Several recent studies have indeed shown, using transgenic mouse models, that utrophin is able to fulfill this task (Rafael *et al.*, 1998; Goudemant *et al.*, 1998; Tinsley *et al.*, 1996). These two proteins have different patterns in tissue expression. Dystrophin is mostly expressed in skeletal muscle and in brain. Utrophin is widely expressed and present in most organs (Blake *et al*., 1996). Another significant difference is the subcellular localization. Dystrophin is localized extrasynaptically, at the cytoplasmic face along the entire length of the muscle fiber. Utrophin in contrast, is preferentially localized and concentrated at the NMJ.

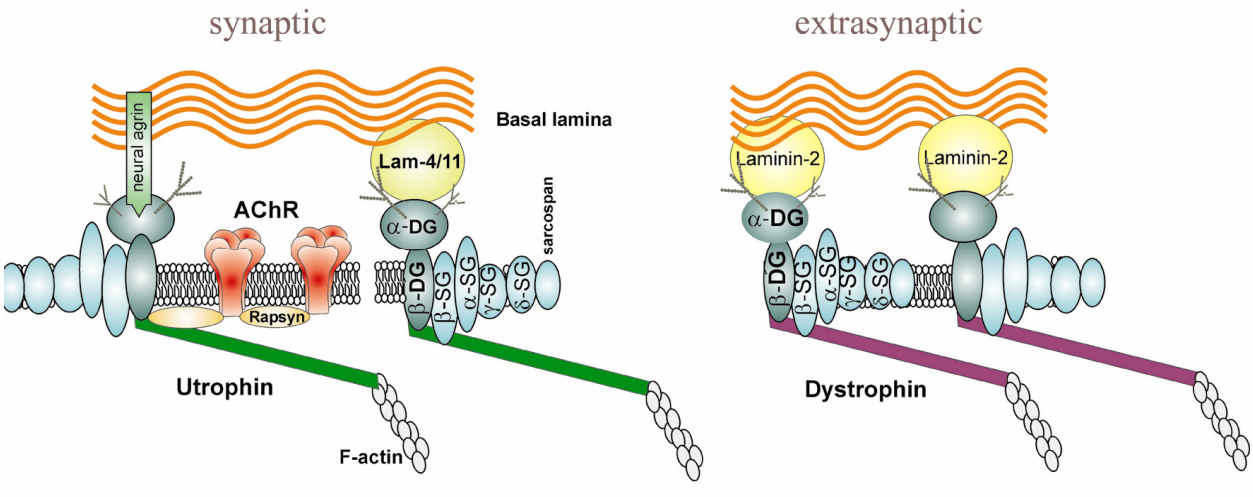


Fig. 16: Protein interaction in the synaptic cleft and extrasynaptically

The major components of the basal lamina are collagen IV, laminin, entactin, and heparansulfate proteoglycans (HSPGs). Synaptic and extrasynaptic portions of the basal lamina differ in their composition of protein isoforms.

Dystrophin and its autosomal homolog Utrophin are differ in their subcellular localization. Dystrophin is distributed extrasynaptically on the entire length of the muscle fiber wheras utrophin is preferentially expressed in the synaptic region.

It has been postulated that utrophin gene transcription has to be locally regulated within the subsynaptic nuclei via nerve-derived trophic factors (Gramolini *et al*., 1997). Nuclei in extrasynaptic regions are also capable of expressing utrophin but only after receiving specific neuronal signals. One of these factors that were focused on, was agrin (Gramolini *et al*., 1998). Cultured myotubes were treated with different agrin isoforms. The neural isoforms of agrin increases the number of AChR clusters and of postsynaptic specializations via activation of the muscle specific tyrosine kinase (MuSK). A gain of function experiment showed that microinjection of neural agrin in muscle fibers causes ectopic postsynaptic specializations und as a result clustering and concentration of synaptic proteins not only at the NMJ (Bezakova *et al*., 2001). Thus, also the presence of utrophin at the cell surface of the muscle fiber was enhanced. Furthermore it was shown that agrin treatment not only leads to the redistribution of preexisting molecules, but that it also influences and stimulates the de novo synthesis of synapse-specific proteins (Méjat *et al*., 2003).

In this work we took advantage of these mechanisms. The aim was to create a transgenic mouse where the agrin expression can be controlled and that can be a model to prevent or ameliorate the degeneration of muscle fibers in *mdx* mice, the murine model of DMD

(Tinsley *et al*., 1996; Deconinck *et al.*, 1997; Tinsley *et al*., 1998). The upregulation of utrophin seems to be a suitable method in the treatment of DMD.

Materials and Methods

Generating of the Injection Construct

The injection construct mN25C21B8_myc.pTRE was designed on the basis of mN25C95$_{00}$_myc (unpubl. Ruegg, MA). The sequence encoding mN25C95$_{00}$ (unpubl. Ruegg, MA) was inserted in the TOPO 2.1 vector (Invitrogen) using the restriction sites *Eco*RV and *Spe*I. A *Nhe*I site was inserted by performing a PCR on mN25C95$_{00}$_myc.TOPO with the sense primer sNhe_N25C21(0):

(5'-CTAGCTAGCGAGAAGGGGATAGTTGAGAAG-3')

and the antisense primer asC95_ClaI:

(5'-CCATCGATGAGAGTGGGGCAGGGTCTTAG-3').

By use of the restriction sites *Nhe*I and *Cla*I, the PCR-product was ligated into N25C95$_{00}$ The C95$_{00}$ was now replaced by the shorter C21$_0$. In the following step the B$_8$ insert was placed in by overlapping PCR. The first PCR was performed on the new obtained mN25C21$_0$_myc.TOPO by using the sense primer seq.middleNtAFS1_pos.421:

(5'-GCGGATCACTTTGCGGAACC-3')

and the antisense primer as N25C21_B8:

(5'-TGGGATC TCATTGGTCAGCTCACTCTCAGTCACAGCAT-TG-3').

The second PCR-product was made with the sense primer s N25C21_B8:

(5'-GCTGACCAATGAGATCCCAGCCGAGAAAGCGCTGCAGAGC AAC-3')

and the antisense primer asC95_ClaI:

(5'-CCATCGATGAGAGTGGGGCAGGGTCTTAG-3').

The third PCR was done by fusing those two PCR-products and using the sense primer seq.middleNtAFS1_pos.421:

(5'-GCGGATCACTTTGCGGAACC-3')

and the antisense primer asC95_ClaI:

(5'-CCATCGATGAGAGTGGGGCAGGGTCTTAG-3').

Using *Nhe*I and *Cla*I sites the new PCR-fragment was inserted into mN25C21$_0$_myc.TOPO. The new construct is referred to as mN25C21B$_8$_myc.TOPO. To be able to express agrin in a TET-off gene expression system, the sequence was cloned into

the pTRE vector (BD Biosciences) that contains the TET responsive element (TRE). The vector was digested with *Cla*I, treated with the Klenow enzyme (NEB) and then digested with *Sal*I. mN25C21B_8_myc.TOPO was cut with *Eco*RV and *Sal*I and the new agrin sequence was ligated into pTRE vector. For injection into mouse oocytes, the construct has to be linearized with *Xho*I and *Ase*I. All constructs were routinely sequenced to eliminate the possibility of mutations.

Animals

The agrin expressing transgenic mouse have been generated as described above. Mice were genotyped by PCR using the three following primer pairs:

1. primer pair:

sense primer mAgN25C21-s: (5'-CCAATGTGACCGCTAGCGAGAAG-3')

antisense primer mAgN25C21-as: (5'-CTGTAGGCCTCCAAGCCACA-3')

2. primer pair:

sense primer seq.ins.B_ms.agrin_pos.5558: (5'- CATGACAATGAGCGTGGCAACC-3')

antisense primer asC95_ClaI: (5'-CCATCGATGAGAGTGGGGCAGGGTCTTAG-3')

3. primer pair:

seq.middleNtAFS1_pos.421: (5'-GCGGATCACTTTGCGGAACC-3')

sense primer mAgN25C21-s: (5'-CCAATGTGACCGCTAGCGAGAAG-3')

PCR was performed for each pair of primer separately. Transgenic lines were identified by PCR and Southern blot, and the protein expression was confirmed by immuno-histochemistry. Positive founders were bred with C57/BL6 mice to keep the lines. As negative control we always used wild-type mice C57/BL6.

Preparation and Staining of Crossections

Soleus was taken out, embedded in tragacant and frozen in liquid nitrogen. Muscles were then ready to be cut with the cryostat in 12 µm sections and to put onto slides. After being frozen, slides were dried and sections were circled with PapPen. Sections were then permeabilized with PBS containing 0.5% Triton X-100, 1% BSA and 2% HoS for 30min at RT, and washed twice with wash buffer (0.01% Triton X-100, 1% BSA, 2% HoS in 1 X PBS facultative: NaN_3). Sections were then incubated oN at 4°C with the primary AB (1:2000; rabbit anti-NtA #206) diluted in wash buffer. Sections are next washed three

times for 10min with wash buffer, dried and incubated with the secondary AB (1:1000; cy3-labeled goat anti-rabbit IgG), diluted in wash buffer. Incubation was for 1h at RT in the dark. Slides were washed once more three times 10min with wash buffer, fixed with a few drops of EtOH for 2min, dried and then mounted with Citifluor. Before sections can be analyzed under the fluorescent microscope they have to be pressed at least 1h at 4°C:

Preparation and Staining of Bundles

Soleus was taken out and fixed with needles on a plate. It was incubated in PBS and then injected with rhodamine-α-bungarotoxin (1:200; Molecular Probes) for 30min at RT. After washing with PBS for 30min muscle was injected with 2% PFA and incubated for 10min at RT. Bundles were prepared after washing in PBS for 30min at RT. The fixed bundles were permeabilized in Falcon in 1% Triton X-100 in PBS for 30min and then blocked with 100mM glycine in PBS for 30min. Next, bundles were washed and blocked with 1% BSA in PBS for 1h at RT and then incubated with the primary AB (1:1000; rabbit Anti-NtA #206) oN at 4°C. Washing and incubation with the secondary AB (1:1000; cy3-labeled goat anti-rabbit IgG) followed oN at 4°C. Bundles were then mounted with Citifluor after a washing procedure of six times one hour.

Results

Creating a Transgenic Mouse

Aim was to design a mouse model where agrin transcription can be temporally controlled in a TET-off gene expression system. The recombinant mini-agrin construct mN25C21B_8 consists of the N25 N-terminal and the most C-terminal laminin G-like domain, containing the B_8 insert. This recombinant form of agrin is sufficient for AChR clustering (in vitro: Gesemann *et al*., 1995; in vivo: Meier *et al*., 1998). It is also sufficient to activate MuSK via phosphorylation (dissertation Patrick Scotton).

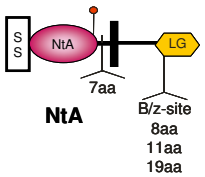


Fig. 17: Domain structure of mN25C21B_8

The injection construct consists of the NtA N-terminus and the most C-terminal localized LG-domain containing the B_8 insert.

The pTRE vector includes the TET responsive promoter element. First of all we aimed to get a transgenic mouse that expresses recombinant agrin. This was examined by immuno-histochemistry. After injection of the construct, we got 50 founders. 16 of them were potentially positive. 7 lines were examined and ectopic clusters were found in the soleus muscle of one mouse. This demonstrates that the injected recombinant agrin DNA encodes a functional protein.

From these results we conclude that the mN25C21B$_8$_myc.pTRE mouse can be an appropriate model on the way to prevent or to ameliorate muscle degeneration of *mdx* mice.

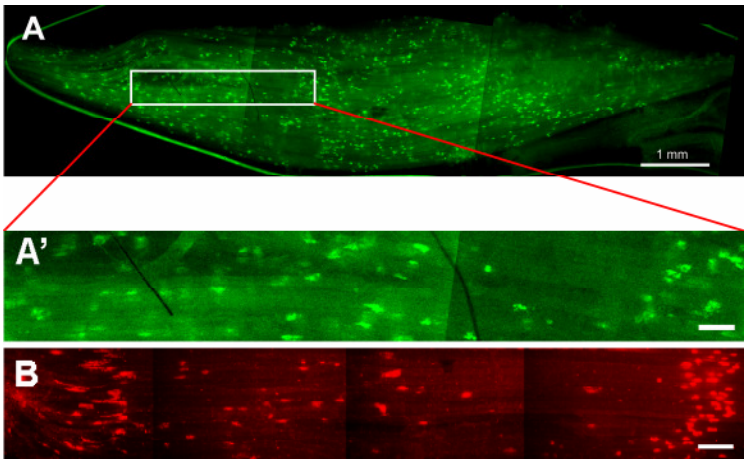


Fig. 18: Ectopic clusters

The whole soleus muscle of the transgenic mice line 1 was stained by α-bugarotoxin (green) and observed with 2.5x objective. As shown in A (scale bar: 1 mm), many ectopic clusters of AChRs are formed in the entire muscle, which have the similar size as NMJs. Compare to the AChR clusters induced by neural agrin injection (B, scale bar: 100 µm), which are mainly formed in the place close to the tendons, the ectopic clusters of AChRs in the transgenic mice are more evenly distributed in the entire muscle.

Discussion

In this work we demonstrated in vivo that overexpression of the mouse mini-agrin is sufficient to induce ectopic AChR clusters, thus provokes postsynaptic differentiation. With the increase of clusters also the utrophin level becomes higher. One still unsolved question is whether accumulation of AChR clusters is harmful for the muscle fiber. To make the system inducible, the next aim is to cross agrin-expressing mice with tTA-mice that express tetracycline-controlled transactivator protein (tTA). This protein regulates the expression of a target gene, in this case agrin, by binding to TRE. The expression of agrin

is downregulated when tetracycline or doxycycline is added. We choose the TET-off system because it was shown that it allows an efficient regulation of the transgene both in vitro and in vivo (Mizuguchi and Hayakawa, 2002). The system is inducible for the better understanding of the mechanisms during muscle fiber degeneration and regeneration.

Mice that express agrin as well as tTA will be crossbred with *mdx* mice that suffer from muscle degeneration. Agrin overexpression results in utrophin upregulation. So the muscle fibers get stabilized and stronger.

Chapter V

General Discussion

This thesis shows the meaning and importance of agrin in brain and its development and also as stabilisator of the muscle fiber. Function of TM-Agrin in the brain was elucidated by examining its expression in non-neuronal and neuronal cells. The experiments confirmed the hypothesis that TM-Agrin might be strongly involved, in all cell types, in the reorganization of the actin cytoskeleton. Transfection resulted in formation of protrusion on the cell surface and modifications lead to the hypothesis that agrin promotes neurite outgrowth. There is growing evidence that proteoglycans promote neurite outgrowth in certain neurons and inhibits it in others. These properties are often connected with the GAG chains (Bandtlow and Zimmermann, 2000; Winzen *et al*., 2003). This is consistent with our findings that the GAG chain localized between the 7^{th} and 8^{th} FS domain is involved in formation of microprocesses. Expression of different chimera constructs in non-neuronal cells elucidated the special function of those GAG chains. The precise mechanism is not yet known. In vitro studies have shown that HSPG can support neurite outgrowth by activating growth enhancing molecules such as laminin, NCAMs or integrins (Martin and Sanes, 1997; Bixby *et al*., 2002). Moreover, agrin can act as a growth factor co-receptor and can so influence axon guidance (Cotman *et al*., 1999). The role of agrin concerning axonal growth and guidance is contradictory as the mechanism of how it influences this process is not known. The hypothesis that agrin might act as co-receptor led to the analysis of agrin's function by using antibody-mediated activation by surface oligomerization. This mechanism of receptor oligomerization is universal and is found in nearly all transmembrane protein with one transmembrane domain (Heldin, 1995; Weiss and Schlessinger, 1998). TM-Agrin, a highly glycosylated HSPG has one transmembrane domain and so several lines of evidence suggest that the formation of microspikes on growing axons was the consequence of an antibody-mediated clustering of TM-agrin. Agrin, present on microspikes, induces antibody-mediated clustering of agrin on growing axons. In summary, these results support the hypothesis that activation of TM-agrin is able to elicit intracellular signaling in neurons and non-neuronal cells, resulting in a reorganization of the cytoskeleton and the formation of processes.

The multifunctional protein agrin has also an important role in the regulation of the protein level of utrophin. Utrophin that is an autosomal homolog of dystrophin, could be able to compensate for dystrophin in Duchenne muscular dystrophy where dystrophin is not-functional. We created a mouse model that expresses mouse mini-agrin and is therefore capable to induce ectopic clusters. The agrin transcription will then be negatively controlled in a TET-off system. Overexpression of agrin will not only increase cluster formation and therefore enhance the utrophin level but it can also induce de novo synthesis of utrophin by transcriptional regulation of postsynapse specific genes. This model can be a useful means for developing a therapeutic treatment for Duchenne muscular dystrophy.

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Acknowledgments

First of all, I would like to thank Dr. Markus Rüegg for the interesting years in his lab and for all the possibilities I had to learn. I also appreciated the chance to continue my project, I began during my time as diploma student.

I'm also very thankful to Dr. Stephan Kröger for his interest and his encouragement during my thesis. Our scientific discussions were interesting and were a support for my ongoing experiments.

During my work I enjoyed the nice and fruitful atmosphere in our lab and for their warmth and friendship. Therefore I'm very grateful to all the lab-members.

Especially I would like to thank Patrizia Barzaghi for her friendship and all the time we spent together. Filippo Oliveri always had time to discuss important scientific things and brought 'Sicilianità' into the lab. Special thanks to Patrick Scotton, for his friendship, his good mood and his laughing and his companionship at the weekends and when we had so much to do.

A special thank to all my friends and to my family, for their love, suggestions and their patience during my thesis.