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„The role of agrin in aquaporin 4 clustering at the
superficial and perivascular glia limitans“

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Abstract

Neuromyelitis optica (NMO) is an idiopathic, inflammatory, demyelinating disease basically affecting the spinal cord and the optic nerves. 80% of all patients carry a pathogenic autoantibody against the bidirectional water channel aquaporin 4 (AQP4). NMO is characterized by a profound loss of AQP4 expressing astrocytes. AQP4 is detected in high concentrations in astrocytes where it is polarized at the astrocytic end- feet contacting the glia limitans superficialis and perivascularis. The reason for this polarized expression remains essentially unclear.

We hypothesized that the enhanced clustering of AQP4 at astrocytic end- feet is caused by agrin. Agrin is a heparan sulphate proteoglycan known for postsynaptic clustering of nicotinic acetylcholine receptors. In the central nervous system agrin is secreted by neurons and endothelial cells and associates with the basal laminae of the blood brain barrier.

To test this hypothesis we first cocultured endothelial cells or neurons with astrocytes and made then immunocytochemical stainings to detect, whether these cells induce a clustering of AQP4 in astrocytes *in vitro*. Then, we cultured astrocytes with agrin conditioned medium derived from endothelial cells or neurons and studied the effects of these treatments on the clustering of AQP4 in astrocytes.

We found that the coculture of neurons changes the morphology of astrocytes and leads to a strong upregulation of AQP4 expression in these cells. Most likely, these effects are not caused by neuronal agrin, because addition of this molecule to astrocyte cultures did not lead to an upregulation and clustering of AQP4.

Kurzfassung

Neuromyelitis optica (NMO) ist eine idiopathische, entzündliche, demyelinisierende Erkrankung, die vor allem das Rückenmark und den *Nervus opticus* betrifft. Bei 80% aller Patienten ist ein pathogener Autoantikörper nachweisbar, der an den doppeltgerichteten Wasserkanal aquaporin 4 (AQP4) bindet. Dieser Wasserkanal ist an den distalen Fortsätzen der Astrozyten polarisiert, die mit der superfiziellen und perivaskulären *glia limitans* in Verbindung stehen. Die Ursache und der Mechanismus dafür sind unklar.

Wir haben die Hypothese aufgestellt, dass Agrin für die polarisierte Zusammenlagerung mehrerer AQP4 Moleküle verantwortlich ist. Agrin ist ein „heparan sulphate proteoglycan“, ein Protein, dass dafür bekannt ist nikotinische Acetylcholin Rezeptoren an der motorischen Endplatte zu clustern. Im Zentralnervensystem wird Agrin von Neuronen und Endothelzellen sezerniert und assoziiert mit der Basalmembran der Blut- Hirn- Schranke.

Um unsere Hypothese zu überprüfen haben wir Endothelzellen oder Neuronen zusammen mit Astrozyten kultiviert und die Auswirkungen dieser Co- Kulturen mittels immunzytochemischer Färbungen auf die Expression und Polarisierung des AQP4 in Astrozyten ermittelt. Astrozyten wurden auch mit Agrin angereichertem Medium kultiviert um die Wirkungen dieses Proteins auf Astrozyten zu analysieren.

In dieser Studie haben wir herausfinden können, dass Neuronen in Kultur mit Astrozyten Änderungen in deren Morphologie und AQP4 Expression herbeiführen. Es ist jedoch sehr wahrscheinlich, dass diese Effekte nicht durch das neuronale Agrin hervorgerufen werden.

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1. Introduction

1.1. Neuromyelitis Optica

Neuromyelitis optica (NMO) is an acute inflammatory demyelinating disease basically affecting the spinal cord and the optic nerves, causing paralysis, blindness and respiratory failure (Graber et al., 2008; Misu et al., 2007). Lesions are predominantly found in regions with high aquaporin-4 (AQP4) expression (Bradl et al., 2009; Bezakova and Ruegg, 2003).

For a long time NMO or Devic's disease was regarded as a variant of multiple sclerosis (MS). In 2004 an autoantibody against AQP4 called NMO- IgG was discovered. Since then it is recognized as a distinct disease. This AQP4 binding IgG1, that recognizes an extracellular domain of AQP4, is, among others, the marker for diagnosis of NMO even though the autoantibody is only detectable in 75% of patients. It binds to an extracellular domain of AQP4 on the surface of astrocytes and subsequently destroys them in a complement dependent way (Mandler et al., 1993).

The importance of NMO- IgG in disease mechanisms was demonstrated 2009. The group of Monika Bradl could prove that the NMO- IgG itself is pathogenic when introduced into rats with experimental autoimmune encephalomyelitis (EAE), induced by MBP- specific T cells. The transmission of the antibody in animals with an open blood brain barrier (BBB) could induce C9 and Ig deposition, macrophage and granulocyte recruitment and deplete AQP4 and GFAP from the lesions. Neurons, oligodendrocytes, myelin and NG-2 positive progenitor cells were not harmed. The study implies that there is a direct role of AQP4 specific antibodies causing astrocytopathy. This data emphasizes the importance of AQP4 and NMO- IgG in the pathology of NMO (Bradl et al., 2009).

1.1.1. Immunopathogenesis and humoral mechanisms

In NMO lesions there is an activation of the classical complement cascade by bound IgG resulting in the production of chemo- attractants for macrophages. These bind to the bound antibodies with their Fc- receptor leading to a release of cytokines and oxygen radicals, contributing to tissue injury and the destruction of myelin sheaths. Further bystander damage of grey and white matter is generated by activated macrophages, eosinophils and neutrophils that generate cytokines, proteases and free radicals, contributing to inflammation (Lucchinetti et al., 2002).

1.2. Aquaporins and Aquaporin 4

In former times scientists believed that water was moved through the lipid bilayer of the cell membrane only by diffusion, until AQP1 was discovered almost 20 years ago (Preston et al., 1992). Aquaporins (AQP) are bidirectional water channels which allow the passage of water in response to osmotic gradients. In humans 13 aquaporin molecules have been identified. They are classified into two distinct groups:

the classical aquaporins, which are only permissive for water (AQP0, 1, 2, 4, 5, 6, 8 from human and AqpZ in *E. coli*), and the

aquaglyceroporins, which are permeated by water and glycerol, (AQP3, 7, 9, 10 from human, GlpF from *E. coli*) (Graber et al., 2008; Agre, 2006). AQP1 is expressed in kidney and endothelial cells from lung microvasculature (Badaut et al., 2002) and by fibroblasts, AQP2 in the collective duct, AQP5 in submucosal glands, AQP0 in the lens, AQP7 in fat tissue, AQP9 in liver and astrocytes.

In the rodent brain, six AQP subtypes have been found: AQP1, AQP3, AQP4, AQP5, AQP8, AQP9, whereas AQP1 is restricted to the plexus choroideus and AQP9 is present in neuronal and glial subpopulations in brain stem and ependymal cells (Badaut et al., 2002).

2 years after the discovery of the aquaporin water channels in 1992, Jung et al. detected cDNA of AQP4 in the brain, amongst others primarily in astrocytes (Jung et al., 1994), confirmed by *in situ* hybridization. AQP4 is expressed only in the postnatal brain (Agre, 2006), in distal collecting tubes of the kidney and in parietal cells of the stomach. In the brain AQP4 is detected in high concentrations in the optic nerve, brainstem and gray matter of the spinal cord. AQP4 is also expressed in ependymal cells and endothelial cells (Nagelhus et al., 1998), but not in neurons, oligodendrocytes or choroidal epithelial cells. In astrocytes AQP4 is particularly enriched in astrocytic endfeet at the perivascular or superficial glia limitans directly contacting the basal lamina of the blood- brain- barrier (BBB), endothelium and pia mater, and in astrocytes ensheathing glutamatergic synapses, see figure 2 (Nielsen et al., 1997). This

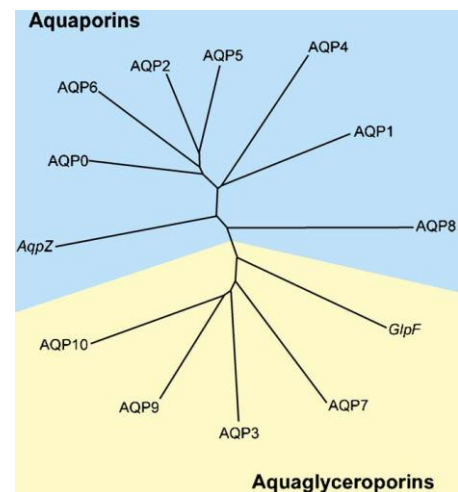


Figure 1: Human AQP repertoire; Figure taken and legend adapted from (Agre, 2006)

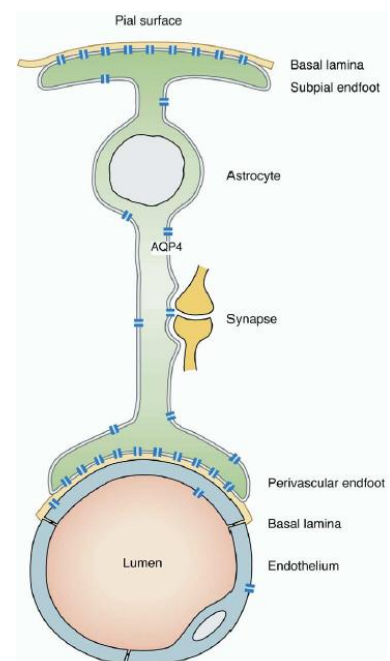


Figure 2: Schematic view of AQP4 clustered in astrocytic endfeet ; Figure taken and legend adapted from (Nagelhus et al., 2004)

polarized distribution of AQP4 in astrocytes indicates that there might be specialized functions for water flux in these areas discussed below. The mechanisms of clustering AQP4 at astrocytic end- feet are not known.

1.2.1. AQP4 isoforms and orthogonal arrays of particles

In the rat six different isoforms of AQP4 are known (Moe et al., 2008) Two different AQP4 isoforms are generated by alternative transcripts and different initiation sites for translation. AQP4 M1 has 323 amino acids and AQP4 M23 has 301 amino acids. Variant M23 assembles in large OAPs, M1 in small arrays. If both isoforms are transfected in cells *in vitro* they form arrays that resemble those found *in vivo* (Nicchia et al., 2009; Noell et al., 2007). These two 2 AQP4 isoforms assemble in the membrane as heterotetramers (Neely et al., 1999). Recently a third AQP4 isoform has been discovered, called Mz. It is larger than M1 but its expression level is lower than M1 and M23.

Several studies suggest that in brain and kidney AQP4 is clustered in geometric structures called orthogonal array of particles (OAP) that are detectable by freeze fracture imaging (Wolburg et al., 1994; Badaut et al., 2002; Rash et al., 1998) and by a new biochemical method that separates different isoforms by size, called Blue Native- Sodium Dodecyl Sulphate- Polyacrylamids Gel Electrophoresis (BN- SDS/PAGE) (Nicchia et al., 2009). Moreover, OAPs were absent when the AQP4 gene was disrupted (Wolburg et al., 1994).

Today we know that NMO- IgG autoantibodies target AQP4 water channels only when they are clustered in orthogonal arrays of particles (OAPs). Nicchia et al. (2009) showed that NMO IgGs from patients serum immunoprecipitate both AQP4 isoforms that can be detected by anti- AQP4 antibodies in western blot. NMO IgG themselves do not recognize AQP4 after SDS- PAGE, indicating that their epitope is conformational and not a linear sequence. It could be demonstrated that NMO IgG preferentially recognized the M23 isoform in a ratio of 2,22 compared to M1 in AQP4 transfected HeLa cells. Further on, M1 transfected cells could not be immunohistochemically stained with NMO- IgG (Nicchia et al., 2009).

1.2.2. AQP4 function and pathophysiology in the brain

In vitro studies of astrocytes show that AQP4 mediates volume changes. This might contribute to maintaining right K⁺ concentrations of extracellular spaces by clearing excess or avoiding too little water resulting from alternations in ion concentrations by neuronal activity. AQP4 may contribute to

potassium take up by inwardly rectifying K^+ channels (Kir4.1) that colocalize in astrocyte membranes (Nicchia et al., 2000).

Active neurons release K^+ and glutamate leading to a local shrinkage of the extracellular volume (Sykova and Chvatal, 2000). The decrease of the extracellular space arises when astrocytes take up K^+ by Kir4.1 and bicarbonate by NBC leading to a water influx through AQP4.

This shrinkage is locally confined and includes a widening of the extracellular space by efflux of water and K^+ distally from the site of neuronal activity, see figure 3.

Another evidence supporting this model is that Kir4.1 is colocalized with AQP4, determined by double immunogold stainings (Nagelhus et al., 2004) and the fact that K^+ clearance is delayed in *syn^{-/-}* mice, lacking properly clustered AQP4 at glial endfeet (Amiry-Moghaddam et al., 2003).

AQP4 might also contribute to the worsening of pathological situations in the brain. In tumors that are related to BBB breakdown (e.g. glioblastoma), the upregulation of AQP4 is believed to contribute to edema formation. Studies of traumatic brain edema showed that after 24 hours there was a decrease in AQP4, believed to be a protective effect against vasogenic edema. After 3 days there was an increase of AQP4 at the border of the injury, suggested to be essential for the clearance of excess water during cytotoxic edema (Ke et al., 2001).

In AQP4 knockout mice brain edema were delayed and reduced (measured by hemispheric enlargement), neurologic recovery was improved and showed a better survival after water intoxication or ischemic stroke by artery occlusion (Manley et al., 2000).

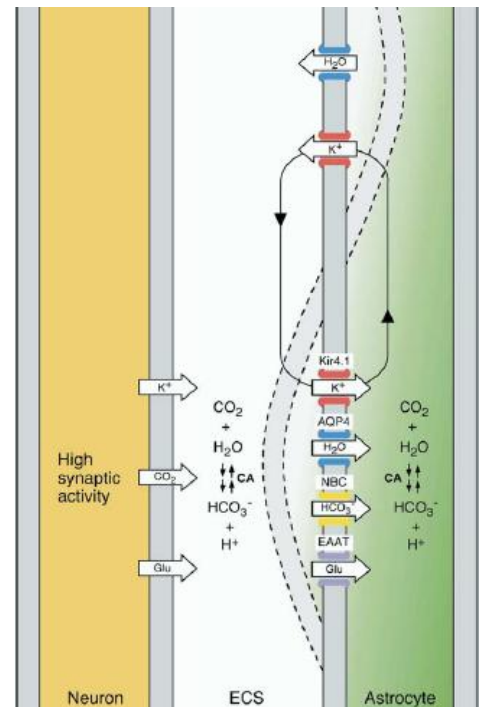


Figure 3: AQP4: the role of K^+ siphoning in the brain ; Figure taken and legend adapted from (Nagelhus et al., 2004)

1.2.3. AQP4 structure and organisation

AQP4 is a type III transmembrane protein with intracellular N- and C- termini. AQP4 monomers form homotetramers with four pores per tetramer. Each monomer has six transmembrane α - helices from which two are responsible for selection of water molecules. They have a highly conserved Asn- Pro- Ala motif (Jarius et al., 2008). The C- terminus with its conserved Ser- Ser- Val- COOH motif binds to

PDZ domains that are protein- protein association domains, named after PSD- 95, Discs Large and Z- Occludins, shown in figure 4.

These domains are associated with α - syntrophin. Immunoprecipitations revealed that α - syntrophin is associated with AQP4. α - syntrophin knockout mice have strongly reduced AQP4 expression in muscle, hence the proper localization is dependent on α - syntrophin (Neely et al., 2001; Warth et al., 2004).

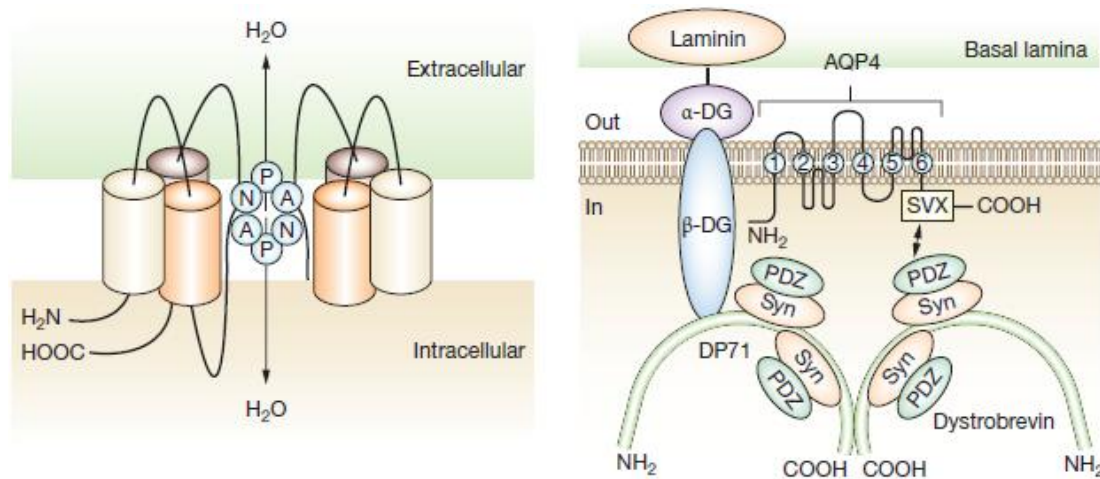


Figure 4: Left pannel: AQP4 homomers in the cell membrane. Right pannel: AQP4 anchoring in the cell membrane by the DDC. Abbreviations: α/β -DG, α/β -dystroglycan; DP71, dystrophin variant DP71; PDZ, PDZ- domain; syn, α - syntrophin; SVX, Ser-X-Val domain of AQP4; SYN, α - syntrophin. APN, Asn- Pro- Ala; Legend adapted and figure taken from (Jarius et al., 2008)

α - syntrophin, dystrophin, dystroglycan and dystrobrevin are part of the dystrophin- dystroglycan- complex (DDC). α - syntrophin is intracellularly bound to the scaffold proteins dystrobrevin and dystrophin. Dystrophin connects the cytoskeleton with the extracellular matrix and forms a bridge between filamentous actin and β - dystroglycan, a transmembrane protein, that is associated with α - dystroglycan, a laminin and agrin- binding protein (Neely et al., 2001; Jarius et al., 2008). In *mdx* mice, which have a mutation in the dystrophin gene and therefore lack dystrophin, AQP4 expression was reduced in muscle (Neely et al., 2001; Agre et al., 2002) and astrocytic end- feet were swollen due to impaired water uptake by the disturbed organization of AQP4 water channels (Badaut et al., 2002).

These data show that the proteins of the DDC have an important role in AQP4 distribution and expression level.

1.3. Agrin

Agrin is a heparan sulphate proteoglycan with 1937 aminoacids and a molecular mass of 225kDa. Extensive N- and O- linked glycosylations at the amino- terminal is likely to increase the molecular weight to up to 600kD (Rupp et al., 1991).

Agrin is important for the postsynaptic clustering of nicotinic acetylcholine receptors. It was isolated from basal lamina extracts from the electric organ of *Torpedo californica* for the first time (Nitkin et al., 1987). In a trial with agrin deficient mice that died of breathing failure shortly after birth, it was proven that agrin was essential for the formation of a functional postsynaptic structure at the neuromuscular junction (NMJ) (Gautam et al., 1996). Another evidence for the function of agrin was that the injection of plasmids or purified recombinant agrin into non- synaptic regions of innervated rodent muscle could induce nAChR clustering and consequently resume proceedings *in vivo* (Rupp et al., 1991).

1.3.1. Agrin isoforms and expression patterns

There are two main agrin isoforms resulting from alternative splicing. One isoform encodes a cleaved single sequence (SS) followed by the amino terminal agrin (NtA) domain (SS- NtA- agrin). Only this isoform can bind to laminins in the basal lamina, is present at the NMJ and can be associated with the extracellular matrix. The other isoform has a shorter amino terminus, which makes it a type II transmembrane (TM) protein (TM- agrin) and is inserted into the cell membrane. It might play a crucial role in neuron- neuron interactions. These two isoforms share about 1900 amino acids. The TM isoform has a unique 49 amino acid sequence and SS-NtA- agrin a 150 amino acid sequence. Mice lacking secreted LN- agrin, lack agrin in their basal laminae. The proper formation of the NMJ was impaired like in agrin knockout mice, indicating the necessity of this isoform for living. Both amino terminal domains are encoded by two different exons, TM- agrin lying downstream a 8kb intron, suggesting that there are two different promoters regulating the expression. Hence, there are agrin isoforms with different functions in different tissues such as muscle or brain (Burgess et al., 2000; Bezakova and Ruegg, 2003).

These splicing variants, SS-NtA- agrin (long amino termini (LN)) and TM- agrin (short amino termini (SN)) (Burgess et al., 2000), can further be spliced at two other positions in the mRNA, referred to as A/Y and B/Z sites. A and B is designated for chick and frog, Y and Z for mammals (Smith and

Hilgenberg, 2002). These sites are present at the carboxy- terminal laminin- globular (LG) domains LG2 (A/Y) and LG3 (B/Z). Splicing at these sites means that the resulting proteins have different numbers of amino acids resulting in several functions (Ruegg et al., 1992).

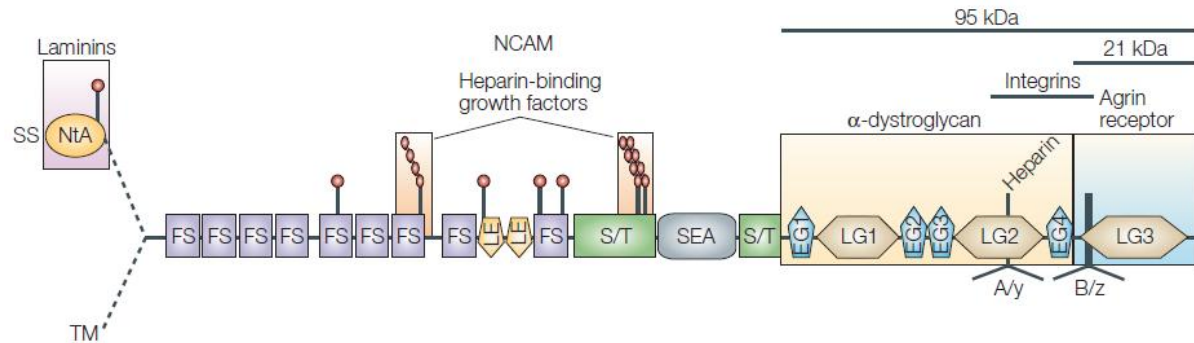


Figure 5: Schematic drawing of agrin. FS, follistatin- like domain; LE, laminin - like domain; LG, laminin globular domain; EG, EGF- like domain; SEA, sperm protein; S/T, serine/ threonine rich domain; α - dystroglycan, laminin, NCAM, Integrin binding sites; A/y B/z- splice sites; Figure taken and legend adapted from (Bezakova and Ruegg, 2003)

The A/Y site has 0 or 4 amino acid inserts and the B/Z site has 0, 8, 11 or 19 amino acid inserts, written for instance A/Y4B/Z8. The four amino acids insert at A/Y (Lys- Ser- Arg- Lys), encoded by a separate exon, is required for heparin binding and modulates the binding of agrin to α - dystroglycan, that is present on astrocytes. α - dystroglycan also binds the B/Z with high affinity (Hopf and Hoch, 1996; Gesemann et al., 1996).

The splicing variant is dependent on the tissue (Ruegg and Bixby, 1998). The main sources of agrin Y4Z8 are neurons, motoneurons and Schwann cells in the peripheral nervous system (PNS).

In the brain and *in vitro*, agrin is expressed by neurons, to lesser extend in astrocytes, but not in oligodendrocytes and microglia (Sole et al., 2004). Agrin Y0Z0 is mainly expressed by vascular endothelial cells in the CNS and periphery, in lung epithelial cells and kidney tubules (Stone and Nikolics, 1995). The table below gives an overview about tissues that express agrin and the different isoforms they express.

It is also known that agrin is expressed outside the CNS micro vessels, namely in testis, thymus and kidney glomeruli (Smith and Hilgenberg 2002).

Tissue	Form of agrin	A/y site*	B/z site†
Periphery			
Lung	SS-NtA	A/y ⁻	B/z ⁻
Kidney	SS-NtA	A/y ⁻	B/z ⁻
Central nervous system			
Overall	SS-NtA ≈ TM	A/y ⁻ << A/y ⁺	B/z ⁻ and B/z ⁺
Glia	ND	A/y ⁻ << A/y ⁺	B/z ⁻
Neurons	TM >> SS-NtA	A/y ⁺	B/z ⁻ and B/z ⁺
Retinal ganglia cells	TM	A/y ⁺	B/z ⁻ and B/z ⁺
Neuromuscular junction			
Motor neurons	SS-NtA >> TM	A/y ⁺	B/z ⁻ and B/z ⁺
Skeletal muscle	SS-NtA	A/y ⁻ >> A/y ⁺	B/z ⁻
Embryonic Schwann cells	ND	A/y ⁻	B/z ⁻
Intact adult Schwann cells	ND	ND	B/z ⁻ >> B/z ⁺
Regenerating adult Schwann cells	ND	ND	B/z ⁻ < B/z ⁺
Immunological synapse			
Thymocytes	TM?	ND	B/z ⁻
Splenocytes	TM?	ND	B/z ⁻

Figure 6: The table features the tissues that express agrin and also the isoforms expressed by each tissue.
A/y⁻ = 0, A/y⁺ = 4; B/z⁻ = 0, B/z⁺ = 8, 11, 19; Figure taken and legend adapted from (Bezakova and Ruegg, 2003).

1.3.2. Roles of agrin in the periphery

1990 McMahan formulated his agrin hypothesis, postulating that agrin was necessary for the assembly of the postsynaptic apparatus (AChR) *in vivo*. Gautam et al. (1996) generated agrin deficient mice and confirmed McMahan's hypothesis, because mice died of breathing failure due to impaired aggregation of nicotinic acetylcholine receptors (nAChR). In the same year DeChiara et al. generated MuSK- deficient (muscle specific receptor tyrosine kinase) mice with a highly similar phenotype indicating that MuSK is part of the receptor complex at the NMJ. This observation was reconfirmed when Glass et al. could crosslink agrin and MuSK chemically (Glass et al., 1996). Given that knockout mice for agrin and MuSK die perinatally by breathing failure, these proteins are crucial for the aggregation of AChRs.

At the NMJ, SS- NtA- agrin binds to laminin and induces the AChR aggregation (Burgess et al., 2000), whereas the 21kD fragment of agrin, representing the LG3 domain, has the same activity like full length agrin in inducing this effect (Gesemann et al., 1996), demonstrating its crucial role of amino acid inserts for varying tissues.

1.3.3. Agrin in the blood- brain- barrier

Hugh Davson was one of the founders of the modern concept of the blood- brain- barrier (BBB) (Davson, 1976). Little was known about its function, induction and maintenance. Today, after more than 30 years of investigation, we know that the BBB protects the brain from excess and toxic substances from the blood and allows the controlled entry of nutrients (Wang and Bordey 2008).

As shown in figure 7, the BBB is built up by different cell types: endothelial cells, pericytes and astrocytes. The inner layer is formed by endothelial cells that line cerebral microvessel. They are connected by tight junctions and thus inhibit the transcellular passage of molecules. There is extremely low pinocytotic activity and only restricted diffusion of hydrophilic, charged molecules in comparison to endothelium in the periphery (Abbott 2002).

Pericytes are embedded into the basement membrane. They are thought to support and regulate the function of endothelial cell during wounding and non- pathological situations (Lindahl et al., 1997).

Astrocytes surround CNS microvessels with their end- feet, regulate BBB permeability and deposit a second basal lamina referred to as glia limitans. Astrocytes buffer excess potassium and neurotransmitters, provide nutrients and contribute to the structural support around synapses, together with endothelial cells and the basement membrane (Bushong et al., 2004).

The basement membrane is a specialized extracellular matrix composed of collagens, fibronectin, perlecan, agrin and laminin (Ekblom et al., 2003) and functions as a charge and molecular size filter (Smith and Hilgenberg, 2002). Several immunohistological studies have shown, that agrin is associated with brain microvasculature and represents one out of many components of the basal lamina of BBB. It accumulates during proper development of the BBB (Stone and Nikolics, 1995).

The endothelial and astrocytic basement membranes differ in their laminin composition. Laminins 411 and 511 are found in the endothelial basal lamina and laminins 111 and 211 in the glia limitans

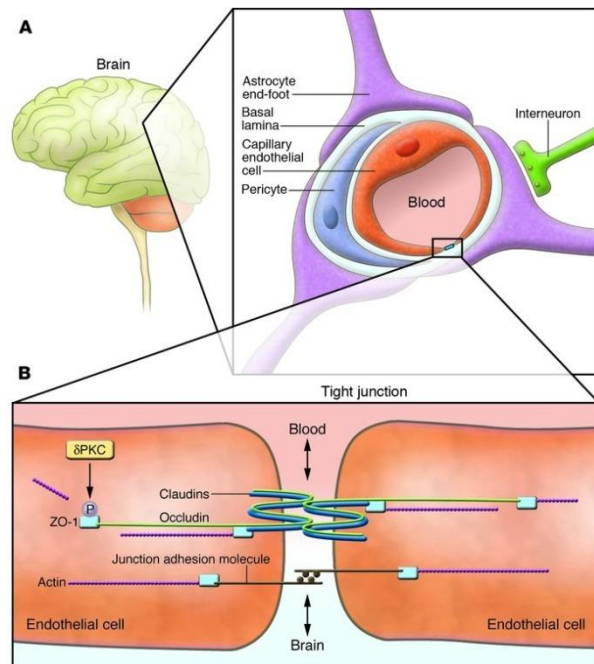


Figure 7: The blood brain barrier and the participating cells: astrocytes, endothelial cells , neurons, pericytes and basal lamina. Endothelial cell tight junction is depicted in higher magnification; Figure taken and legend adapted from (Chou and Messing, 2008)

(Wolburg-Buchholz et al., 2009). Astrocytes secrete amongst others basic fibroblast growth factor (bFGF) and hydrocortisone to regulate the BBBs properties. On the other hand endothelial cells also have influences on astrocytes by regulating their growth, glutamate synthetase activity and laminin production (Wang and Bordey, 2008).

1.4. Aims of the study

AQP4 is detected in high concentrations in astrocytes where it is polarized at the astrocytic endfeet contacting the glia limitans superficialis and perivascularis. The reason for this polarized expression remains essentially unclear.

We hypothesize that the enhanced clustering of AQP4 at astrocytic end- feet is caused by agrin that is associated with the brain microvasculature as one component of the basal lamina and one part of the dystrophin- dystroglycan- complex (DDC) that anchors AQP4 in the membrane.

To test this hypothesis we first cocultured astrocytes with endothelial cells or neurons to observe, whether different isoforms of agrin are able to induce a clustering of AQP4 in astrocytes. Secondly, we cultured astrocytes with medium conditioned with agrin derived from endothelial cells (Y0Z0) or neurons (Y4Z8) to study the effects of these treatments on the clustering and expression level of AQP4 in astrocytes.

2. Materials and Methods

2.1. Isolation of rat brain endothelial (RBE) cells and rat heart endothelial cells

2.1.2. Materials

Buffer A, pH 7,4 in PBS, sterile filtered

153mM NaCl (Merck)

5,6mM KCl (Merck)

2,6mM MgCl₂ 6H₂O (Riedel de Häen)

2,3mM CaCl₂ 2H₂O (Merck)

15mM Hepes (Sigma)

Collagenase type CLS II, in PBS, sterile filtered

1,5% Collagenase (CLSII, 238U/mg, Biochrom AG)

0,1% BSA (PAA)

25% BSA in PBS, sterile filtered (PAA)

DMEM/ 10% FCS (Lonza BioWhittaker)

Percoll 50, sterile filtered

22,5ml Percoll, density 1,132g/ml (GE Healthcare)

2,5ml DMEM (Lonza BioWhittaker)

25ml DMEM/ 10% FCS (Lonza BioWhittaker)

Endothelial Cell Medium (PAA)

L- Glutamine, 200mM (Lonza BioWhittaker)

Penicillin- Streptomycin (Lonza BioWhittaker)

1000U Potassium Penicillin/ml, Streptomycin Sulfate/ml

Endothelial Cell Medium (PAA)

Puromycin dihydrochloride, streptomyces alboniger 0,1mg/ml (Sigma)

Collagen type 1, from calf skin (Sigma)

2.1.1. Methods

Rats were sacrificed with CO₂ and decapitated. The abdomens were washed with 70% ethanol, the skin covering the head and abdomen was removed. The brains and hearts were washed, dissected and placed into a dish filled with buffer A in the lamina. From brains, the cerebelli, meninges and white matter were removed. The remaining cortices and the hearts were cut with scalpels to small pieces and homogenized with a Pasteur pipette. The homogenates were transferred into 50ml tubes and centrifuged at 1100rpm for 6 minutes at 20°C.

The supernatant was discarded and the tissues were resuspended in 5ml buffer A and 1ml collagenase per animal for an incubation of 60' at 37°C in a water bath. For proper degradation of extracellular collagen the homogenate was shaken every 10 minutes.

The digestion was stopped by adding buffer A to a final volume of 45ml and centrifugation at 1100rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 15ml 25% BSA. The next centrifugation step was at 2600rpm for 20 minutes at 4°C. After centrifugation gradients became visible. The upper gradient consisted of myelin and BSA. In the pellet capillaries and red blood cells appeared. The upper layer was transferred into another tube by decanting. Myelin and BSA were centrifuged for a second time at 2600rpm for 20 at 4°C.

The pelleted cells were pooled, resuspended in 5ml 1,5% collagenase, and incubated for further 45 minutes at 37°C. In the meanwhile two Percoll 50 gradients were prepared for the separation of the cells by means of a density gradient. 14ml Percoll 50 were centrifuged at 14500rpm for 60 minutes at 20°C. After the incubation the cells were centrifuged at 1200rpm for 5 minutes at 20°C and the pellet was resuspended in 1ml DMEM with 10% FCS. The established Percoll 50 gradient was gently overlaid with the cell suspension and was centrifuged at 2600rpm for 10 minutes at 20°C.

The layers above and beyond the grey layer were transferred into a new tube and double volume DMEM with 10% FCS was added. After centrifugation with 1800rpm for 5 minutes at 20°C the supernatant was discarded and 10ml DMEM with 10% FCS were added for washing. After another centrifugation step with 1200rpm for 5 minutes at 20°C the pellet was resuspended with Endothelial Cell Medium with additional 4µg/ml or 3µg/ml puromycin for endothelial cell selection for a period of two or three days. The cells were plated on collagen coated 35mm cell culture dishes.

2.2. Endothelial cell culture

2.2.1. Materials

Endothelial Cell Medium (PAA)

L- Glutamine, 200mM (Lonza BioWhittaker), 1%

Penicillin- Streptomycin (Lonza BioWhittaker), 1%

1000U Potassium Penicillin/ml, Streptomycin Sulfate/ml

Puromycin dihydrochloride, streptomyces alboniger 0,1mg/ml (Sigma)

Hydrocortisone 5µg/ml in Endothelial Cell Medium (Sigma)

Recombinant human FGF basic 146aa, E. coli derived 5µg/ml (R&D Systems)

2.2.2. Methods

After the isolation of endothelial cells from rat brains the cells were cultured in endothelial cell medium. For the selection of endothelial cells they were treated for 2 to 3 days with 4 or 3µg/ ml puromycin. After the treatment the medium was supplemented with basic fibroblast growth factor (bFGF) and hydrocortisone for recovery from puromycin treatment. These factors were kept in the medium for two days until cells were used for further investigations. The medium was changed every 48 to 72 hours.

2.3. Astrocyte culture

2.3.1. Materials

Dulbecco's Modified Eagle Medium (DMEM, Lonza BioWhittaker)

Microglia Medium supplements were added to RPMI 1640 without L-Glutamine (Lonza BioWhittaker)

10% Fetal Bovine Serum (PAA)

1% Penicillin/Streptomycin 10.000 U/ml (Lonza BioWhittaker)

1% L-Glutamine 200 mM in 0,85% NaCl (Lonza BioWhittaker)

Poly- L- lysine solution (PLL solution) (Sigma)
PLL hydrobromide powder in PBS 5µg/ml (plastic dishes)

2.3.2. Methods

Primary mixed glia cell cultures were established from newborn to 2 days old Lewis rat brains. The animals were decapitated and their heads cleaned in 70% ethanol, the brains were removed from the head, placed in DMEM and meninges were removed. The brains were then transferred into 10 ml microglia medium, where they were mechanically dissociated by pipetting until a homogenous cell suspension was reached. The suspension was then placed in Poly-L-Lysine coated tissue culture flasks (approximately 1 brain per flask) at 37°C and 5% CO₂ for 24h to allow cell attachment. The culture medium was exchanged every 24 to 72 hours with 10 ml of microglia medium until the cultures were mature and confluent. After 7 to 10 days, the flasks were placed in a rotary shaker at 170rpm at 37°C for at least 4h and up to 18h. The loosely attached microglia and progenitor cells were thus released into the supernatant and discarded.

Coating of plastic cell culture dishes

PLL powder was dissolved to a final concentration of 5µg/ml in sterile PBS. Dishes were coated overnight or until usage in the incubator. Before seeding the cells dishes were washed 3 times with PBS.

2.4. Coculture of endothelial cells and astrocytes

2.4.1. Materials

Trypsin EDTA (Lonza Biowhittaker)

FCS (PAA)

RPMI (Lonza Biowhittaker)

Endothelial Cell Medium (PAA)

Accutase (PAA)

PBS (Lonza Biowhittaker)

Collagen coated glass coverslips (BD Biosciences)
Rat tail collagen type I, 22mm

L-Glutamine (Lonza Biowhittaker)

2.4.2. Methods

Endothelial cells were cultured on collagen coated cell culture dishes until confluency. Then cells were detached with 1ml accutase/ 35mm culture dish for 10 to 20 minutes and centrifuged with 300g for one minute. Cells were plated as a droplet in a volume of 300µl per coverslip on collagen coated coverslips. After 30 minutes to one hour cells were adherent and the remaining medium could be added. Endothelial cells could not be counted, because it was not possible to bring them to a single cells suspension without harming them. Depending on how confluent endothelial cells were, one dish contained enough cells for 4-6 coverslips. Cells were grown over night on the collagen coated coverslips. After 24 hours cells regained their spindle shaped morphology and were ready for cocultures.

Astrocyte cultures were grown until confluency for 7 to 10 days and were shaken off over night. The supernatant of mixed glia cultures was discarded and the monolayer was washed twice with PBS. 2ml of Trypsin EDTA were added for 5 minutes. The reaction was stopped with 8ml microglia medium and resuspended well. This suspension was centrifuged at 300g on room temperature. Astrocytes were resuspended with endothelial cell medium and plated on top of the endothelial cells on collagen coated coverslips.

Staining procedure: The primary antibodies ZO-1 and AQP4 are both derived from rabbit. This means, that the secondary antibodies are both anti- rabbit but labeled with either CY2 (for ZO-1) or CY3 (for AQP4). Therefore incubations of secondary antibodies had to be one at a time. Anti- rabbit CY3 (red) for AQP4 was incubated last also staining ZO-1. GFAP was labeled with CY5 (blue) and ZO-1 with CY2 (green/red or yellow).

2.5. Embryonic hippocampal neuron culture

2.5.1. Materials

Neuronal Medium, sterile filtered

- 5ml 10x MEM (Invitrogen)

- 26mM NaHCO₃ (Merck)

- 1mM sodium pyruvate (Invitrogen)

- 2mM L- Glutamine (Lonza BioWhittaker)

- 33mM D- Glucose (Merck)

- 2% B27 supplement (Invitrogen), stored on -20°C and thawed over night at 4°C

- Filled up to 50ml with ddH₂O

HBSS (Hank's Buffered Salt Solution) pH 7,3 sterile filtered

- 20mM HEPES

- 2mM CaCl₂

- 5,4mM KCl

- 1mM MgCl₂

- 136mM NaCl

- 1mM Na₂HPO₄

- 5,6mM glucose

Trypsin EDTA solution

- 0,05% 1x trypsin/EDTA (PAA)

- 0,01% HEPES

- 0,1% Pen/Strep, 10000U/ml (Lonza Biowhittaker)

- Stored on -20°C, thawed over night at 4

PLL coated glass coverslips

- 1M HCl

- 50%, 70%, 96% ethanol

- Borate buffer, pH 8,5

 - 400ml distilled water

 - 1,24 boric acid

 - 1,9g borax

- Poly- L- lysine solution (PLL solution) (Sigma)

 - PLL hydrobromide powder in borate buffer 5µg/ml (glass coverslips)

 - PLL hydrobromide powder in PBS 5µg/ml (plastic dishes)

2.5.2. Methods

Before starting the isolation the neuronal medium was prewarmed and adjusted to pH 7,4 in the incubator at standard conditions. We used Lewis rat embryos from E17 to E18,5.

The pregnant rats were anesthetized with CO₂ and killed by cervical dislocation. The abdomen was sterilized with Barrycidal and incisions were made along the midline. The uterus was taken out together with the embryos and placed into a sterile plastic culture dish. Embryos were removed from the placenta and embryonic sack and placed into a second dish. Embryos were decapitated, brains were removed carefully and incubated in prewarmed HBSS. The hippocampi were dissected as good as possible to avoid glial and cortical neuron contaminations. They were transferred into a 15ml falcon containing HBSS in a water bath at 37°C. After collecting all hippocampi the HBSS was decanted and replaced by trypsin- EDTA for an incubation of 10 minutes at 37°C. The solution was decanted and cells were washed two times with HBSS. After the second washing step 1-2ml HBSS were left inside and cells were triturated by gently pipetting 20 to 30 times with a Pasteur pipette that was previously polished by heat. For the second trituration a Pasteur pipette was polished until its opening was reduced to half of its size. 10µl of the cell suspension were pipetted into a counting chamber. 200.000 to 600.000 cells were plated on coverslips covering the bottom of a 6cm tissue culture dish filled with 5ml neuronal culture medium. Medium was changed weekly by replacing only two thirds of the old medium with fresh medium.

Coating of glass coverslips

Coverslips were treated with 1M HCl at 60°C over night. After cooling down to room temperature they were rinsed with ddH₂O. Afterwards they were sonicated three times for 30 minutes in ddH₂O, followed by an ascending ethanol series to 96%, 30 minutes each. After the coverslips were dried at 70°C, three paraffin dots were placed on the borders. 100µl of the PLL- solution in a concentration of 5µg/ml in borate buffer were pipetted over each coverslip and incubated over night in the incubator. Coverslips were rinsed with sterile ddH₂O once for 5 minutes and once for two hours. Coated coverslips were stored dry in sterile culture dishes in the incubator.

The protocol for coating of plastic culture dished see 2.3.2.

2.6. Coculture of neurons with astrocytes

2.6.1. Materials

Neuronal Medium, sterile filtered
5ml 10x MEM (Invitrogen)
26mM NaHCO₃ (Merck)

1mM sodium pyruvate (Invitrogen)
2mM L- Glutamine (Lonza BioWhittaker)
33mM D- Glucose (Merck)
2% B27 supplement (Invitrogen), stored on -20°C and thawed over night at 4
Filled up to 50ml with ddH₂O

Trypsin EDTA (Lonza Biowhittaker)

2.6.2. Methods

The same procedure was applied for detaching the astrocytes and seeding them on neurons as for coculturing endothelial cells with astrocytes, sees 2.4.2.

2.7. Laminin and agrin treatment of astrocytes

2.7.1. Materials

Poly- L- lysine solution (PLL solution) (Sigma)
PLL hydrobromide powder in borate buffer 5µg/ml (glass coverslips)

Microglia Medium, supplements were added to RPMI 1640 (Lonza BioWhittaker)
10% Fetal Bovine Serum (PAA)
1% Penicillin/Streptomycin 10.000 U/ml (Lonza BioWhittaker)
1% L-Glutamine 200 mM in 0,85% NaCl (Lonza BioWhittaker)

Human recombinant laminin 211, 0,1 mg/ml (BioLamina)
Human recombinant laminin 411, 0,1 mg/ml (BioLamina)
Human recombinant laminin 511, 0,1 mg/ml (BioLamina)

We received agrin condition medium from Dr. Ruth Herbst. It was produced by HEK 293 cells that were transfected with either the endothelial isoform 0.0., or the neuronal isoform 4.8.

2.7.2. Methods

To estimate the right concentration of agrin conditioned medium where the astrocytes responded best, the first treatment of astrocytes on poly-L- lysine coated glass coverslips was for 72 hours in concentrations ranging from 10µl/ml to 150ml/ml agrin conditioned medium in microglia medium.

The concentrations were adopted from Dr. Herbst laboratory. 20µl/ml for 4.8., 30µl/ml for 0.0. were incubated with cultured astrocytes for the same amount of time like astrocytes were in coculture with endothelial cells and neurons.

Laminin 211 and 411 derived from endothelial cells and laminin 511 derived from astrocytes were air-dried on poly-L-lysine coated glass coverslips in a concentration of 10µg per coverslip.

2.8. Immunocytochemistry

2.8.1. Materials

Collagen Coated Coverslips (BD Biosciences)
Collagen Type I, rat tail, 22mm round coverslips

Poly-L-lysine solution (PLL solution) (Sigma)
PLL hydrobromide powder in borate buffer 5µg/ml (glass coverslips)
Borate buffer, pH 8,5
400ml distilled water
1,24 boric acid
1,9g borax

PBS (Lonza BioWhittaker)

FCS (Lonza BioWhittaker)

4% PFA
40g PFA in heated 500ml 0,2M Sörensenbuffer, filled up to 1l with ddH₂O
0,2M Sörensenbuffer (pH 7,4)
40mM NaH₂PO₄

0,1% Triton X-100 (Sigma) in PBS

Dako diluent (DAKO)

PBS/ 10% FCS

PBS/ 10% Dako diluent

Antibodies

Primary antibodies (AB):

Mouse monoclonal anti GFAP (Invitrogen)
Rabbit polyclonal anti ZO-1 (Zymed)
Rabbit anti AQP4 (Sigma)

Rabbit polyclonal anti agrin (abcam)
NMO- IgG (kindly provided from Dr. Fujihara)
SMI 311(abcam)
SMI 313(abcam)

Secondary antibodies (AB):

Donkey anti- rabbit CY2 (molecular probes)
Donkey anti- rabbit CY3 (molecular probes)
Donkey anti- mouse CY2/ dyelight 488 (molecular probes)
Streptavidin CY5 (molecular probes)
donkey- bi- α - rabbit (Jackson Immuno Search)
donkey- bi- α - mouse (Jackson Immuno Search)

Geltol

6g glycerine
2,4g mowiol
6ml dH₂O
12ml Tris 0,2M pH 8,5
Stirred for 10 minutes at 50°C, centrifuge at 5000g for 15 minutes

0,1M Gallate in Geltol

2.8.2. Methods

The cocultures of endothelial cells or neurons with astrocytes were treated as followed.

For fixation of the cells the medium was removed and cells were washed carefully twice with PBS. Coverslips with cells were incubated upside down for 5 minutes with 1ml 4% PFA at room temperature and washed three times with PBS for 5 minutes. Cells were permeabilized with permeabilization reagent 0,1% Triton X- 100 for 5 minutes at room temperature and washed three times with PBS for 5 minutes. For blocking, coverslips were incubated for 30 minutes with Dako diluent. For the protection of the cells all treatments were performed with wax dots and cells facing the bottom of the dish.

Primary antibodies were diluted in PBS/ 10% Dako diluent as followed:

Mouse anti GFAP	1:100
polyclonal rabbit anti ZO-1	1:50
Rabbit anti AQP4	1:100
Rabbit anti agrin	1:100
SMI311	1:100

The cells were incubated over night at 4°C in wet chambers, washed 3 times for 5 minutes with PBS. Coverslips were incubated for one hour at room temperature on a provided droplet of secondary antibodies in dilution (10%FCS/PBS):

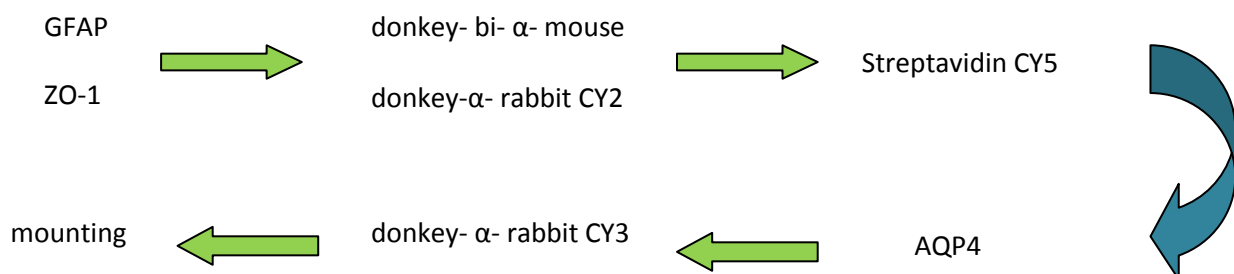
Donkey anti- rabbit CY2	1:100
Donkey anti- rabbit CY3	1:100
Donkey anti- mouse CY2	1:100
Streptavidin CY5	1:100
donkey- bi- α- rabbit	1:100
donkey- bi- α- mouse	1:100

After washing 3 times for 5 minutes the wax dots were removed and coverslips were mounted cells facing the microscope slide on a provided gallate/ geltol droplet.

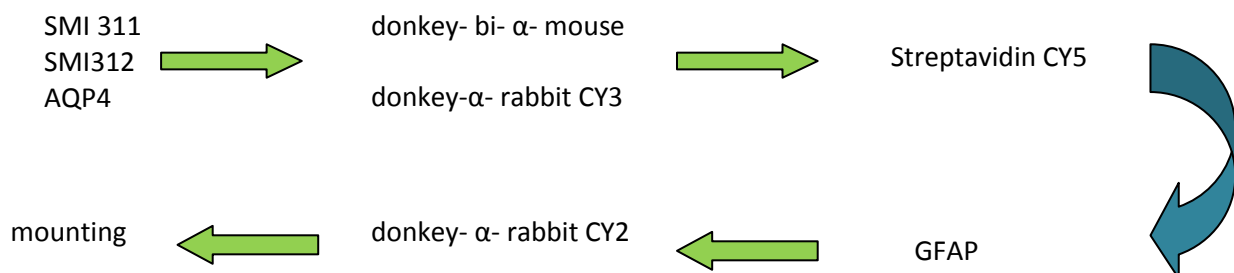
For proper polymerisation they were kept overnight at 4°C and on room temperature before examination under the confocal fluorescent microscope.

Following protocols were used for triple stainings of cocultures:

Endothelial cells with astrocytes:



Neurons with astrocytes:



2.9. Immunohistochemistry

2.9.1. Materials

FCS (Lonza Biowhittaker)

DAKO buffer (Dako)

Xylene (Sigma)

Methanol (Riedel-de Haën)

H₂O₂ (Merck)

TBS pH 7,6

50mM Tris (AppliChem)

150mM NaCl (Merck)

Deionized water to 1l

PBS pH 7.4

137 mM NaCl (Merck)

2.7 mM KCl (Merck)

10 mM sodium phosphate dibasic (Merck)

2 mM potassium phosphate monobasic (Merck)

Primary Antibodies

Rabbit polyclonal anti agrin(abcam)

Rabbit anti α 1- syntrophin RA-16 (Sigma)

Mouse anti α - dystroglycan (Hycult Biotechnology)

Rabbit anti AQP4 (Sigma)

pre- treatment

EDTA pH 9

EDTA pH 9

EDTA pH 8,5

none

Dilution

1:250

1:5000

1:1000

1:250

Secondary antibodies

Donkey anti rabbit 1:2000

Sheep anti mouse 1:500

EDTA 1mM, 10mM Tris in PBS

Avidin- Peroxidase (Sigma), 1:100 in Dako buffer/ 10% FCS

Copper sulfate 2%/ 0,9% NaCl

DAB 25mg/ml in PBS (Sigma)

Haemalum (Sigma)

0,5% HClOH (Merck)

Eukitt (Kindler)

2.9.2. Methods

Tissues were fixed with 4% paraformaldehyde (PFA) for 24 hours or up to 5 days depending on their size and stored in PBS at 4°C. Afterwards, they were dissected, dehydrated over night, embedded in paraffin and cut in 3µm thin sections with a microtome. Sections were mounted on superfrost slides and baked for 3- 5 hours at 57°C. For deparaffination the slides were placed two times 15 minutes in xylene and washed twice in 96% ethanol. The endogenic peroxidase was blocked with 0,2% H₂O₂ /methanol for 30 minutes. After a decreasing ethanol series to aqua dest. a pre-treatment was made for one hour in the steamer, depending on the primary antibody, in either citrate buffer, EDTA pH 8,5 or EDTA pH 9. After washing with TBS the unspecific background was blocked for 20 minutes with DAKO buffer/10% FCS on room temperature. Afterwards the primary antibody was incubated in 10% FCS/DAKO buffer over night at 4°C.

The slides were rinsed with TBS and the secondary antibody in 10% FCS/DAKO buffer was incubated for one hour at room temperature. After washing 5 times with TBS, avidin- peroxidase 1:100 in 10% FCS/DAKO buffer was applied for one hour.

The slides were washed with TBS. Staining was finished by 1ml DAB in 50ml PBS/ H₂O₂. The slices were counterstained with haemalum eosin for 10 seconds, washed in water, immersed in 0,5% hydrochloric acid ethanol, washed with water, blued in scott's blueing solution for 5 minutes, washed with water and dehydrated to 96% ethanol. The slices were kept in ester until they were embedded in eukitt.

2.10. Fluorescence activated cell sorting (FACS)

2.10.1. Materials

Stain- buffer (BD Pharminogen)

Subcuvia (Baxter) 160g/l

Sheep serum (Sigma)

Blocking solution: stain buffer/10% sheep serum

Antibodies:

Human NMO IgG (kindly provided from ...)	1:100
Subcuvia(Baxter) 160g/l	1:100
Sheep- bi- α - human (Amersham)	1:100
APC- α - biotin (MACS Miltenyi BiotechAmersham)	1:10

2.10.2. Methods

1,5 confluent 75cm² tissue culture flasks were trypsinized for 5 minutes, resuspended and plated on 3 PLL- coated 10cm culture dishes (protocols see 2.5.2.). Dishes were grown for 72 hours for proliferation.

Cells were washed twice with PBS and detached again for 15 minutes with 5ml trypsin- EDTA. Before transferring them to a 50ml falcon tube dishes were washed and remaining cells were obtained by pipetting up and down several times. After a centrifugation step of one minute per ml at 300g the supernatant was discarded. Cells were resuspended and portioned on 5 V- shaped 96-well plate slots. Cells were centrifuged at 300g for 5 minutes at 4°C and the supernatant was discarded by decanting. Cells were blocked for 30 minutes on 4°C in the blocking solution (containing the serum of the tertiary antibody) and centrifuged with 300g for 5 minutes at 4°C.

Then, cells were incubated for 30 minutes at 4°C with primary antibodies. Additionally, controls without the primary antibody (unstained control) and with subcuvia 1:100 (IgG control) were made.

After blocking the samples were centrifuged 5 minutes at 4°C with 300g and washed twice, each time with 100µl and a centrifugation under the same conditions. Now cells were resuspended in 100µl stain buffer 1:100 with the secondary AB for 30 minutes at 4°C. After secondary AB binding the samples were centrifuged under the same conditions and washed twice with 100µl staining buffer. For tertiary antibody incubation cells were resuspended in 100µl APC- α - biotin containing buffer, incubated 30 minutes at 4°C and centrifuged under standard conditions. For FACS analysis cells were resuspended in 300µl staining buffer and transferred to FACS tubes on ice.

2.11. RNA isolation and cDNA transcription

2.11.1. Materials

RNAeasy Mini Kit (250) (Qiagen)

QIAGENshredder (250) (Qiagen)

10x Reaction Buffer with MgCl (Fermentas)

RiboLock™ RNase Inhibitor (Fermentas)

DNase I (Fermentas)

25mM EDTA (Fermentas)

RNase free H₂O (Ampuwa)

M-MLV RT, RNase H(-), point mutant, 200U/μl (Promega)

M-MLV reverse transcriptase 5x reaction buffer(Promega)

dNTPs (Roche PCR box) (Promega)

T7-N7 primer:

5'- CCA AGC TTC TAA TAC GAC TCA CTA TAG GGA GA (AGCT) (AGCT) (AGCT) (AGCT) (AGCT)- 3'

2.11.2. Methods

Before starting the isolation the RLT buffer had to be supplemented with β- mercaptoethanol 10μg per 1ml. Cells were washed with PBS and 600μl RLT buffer mix were added to harvest cells from a 35mm dish. The lysate was transferred into a QIAshredder spin column. Total RNA was also isolated from frozen tissue. 30μg per shredder tube were cut off from total tissue and homogenized in RLT buffer with β- mercaptoethanol and centrifuged 2 minutes at 15000g. For RNA isolation from tissue, the flow through was centrifuged again for 2 minutes at 15000g. The lysate was transferred into a new tube, supplemented with 400μl 70% ethanol and mixed properly. It was added to an RNeasy spin column and centrifuged for 15 seconds at 8000g. The flow through was discarded and 700μl RW1 buffer were added. The sample was centrifuged for 15 seconds at 8000g and the flow through was discarded. Additional 500μl RPE buffer were added. Centrifugation for 15 seconds at 8000g and flow through was discarded. This step was repeated but centrifuged for two minutes at 8000g. The column was placed into another collection tube and centrifuged for 1 minute at 15000rpm to dry.

The RNA was diluted from the mini column into a 1,5ml eppendorf tube by the centrifugation of 50µl RNase- free water at 7500rpm for 1 minute.

DNA digestion (Fermentas enzymes)

RNA sample	50µl
DNase Buffer (10x)	7µl
RiboLock Rnase Inhibitor	2µl
DNase 1	7µl
RNase- free water	4µl
Total volume	70µl

The DNA digestion mastermix was added to total RNA, mixed, spun down and incubated for 30 minutes at 37°C. Afterwards 7µl stop solution EDTA were added, mixed and incubated for 10 minutes at 65°C.

RNA Cleanup, Qiagen:

30µl RNase- free water and 350µl RLT buffer with β- mercaptoethanol were added to total RNA and mixed. After the addition of 250µl 100% ethanol, the mixture was transferred into an RNeasy Mini spin column and centrifuged for 15 seconds at 8000g. The flow- through was discarded and 500µl RPE buffer were added. The last two steps were repeated. After centrifugation at 8000g for 2 minutes the column was placed into a new collection tube. The column was again centrifuged for 1 minute at 8000g and then placed into a new 1,5ml eppendorf tube. 30µl RNase free water were pipetted into the column and centrifuged for 1 minute at 8000g.

After RNA isolation and clean up it was transcribed into cDNA for polymerase chain reaction (PCR). All 30µl RNA were used for cDNA transcription and incubated with 2µl of T7-N7 primer for 5 minutes at 70°C and 5 minutes on ice.

Mastermix for each sample:

M-MLV RT buffer	10µl
dNTPs	3µl
RNase Inhibitor	3µl
ddH ₂ O	4µl
Total volume	20µl

The mastermix was added to the probe and incubated for 2 minutes at 40°C. Afterwards 2µl of M-MLV reverse transcriptase were added per sample and incubated for 10 minutes at room temperature, 50 minutes at 40°C and 15 minutes at 70°C. The cDNA was frozen at -20°C.

2.12. PCR

2.12.1. Materials

Ethidium bromide solution 10mg/ml (Sigma)

peqGold 100bp DNA ladder (Invitrogen)

PCR- set (Roche)

Agarose (BD Pharmingen)

2.12.2. Methods

Primers were designed online with Primer3Plus and BLASTed.

Agrin Forward:

Sequence: TGG TTT CCC ACT TTC TTT ACG

Start: 3633bp; Length: 21bp; Tm: 60 °C

Agrin Reverse:

Sequence: GAT TCT GGC AAG GGT TTC AC

Start: 3915bp; Length: 20bp; Tm: 60 °C

Product size: 282 bp

GAPDH Forward:

Sequence: GGC ATT GCT CTC AAT GAC AAC

Start: Length: 21bp Tm: 64

GAPDH Reverse:

Sequence: TGA GGG TGC AGC GAA CTT TAT

Start: Length: 21bp Tm: 60

Product size: 310bp

MAP2 Forward:

Sequence: ATG AAG GAA CCG ATG ACA AA

Start: 2789bp Length: 20bp Tm: 56°C

MAP2 Reverse:

Sequence: AGG TCT CAG TTC AGT GCC AG

Start: 3149bp Length: 20bp Tm: 62°C

Product size: 381bp

GFAP Forward:

Sequence: TTG TTT GCT AGG CCC AAT TC

Start: 1359bp Length: 20bp Tm: 58°C

GFAP Reverse:

Sequence: CCT CGG GAT CTT TTC CTT TC

Start: 1695bp Length: 20bp Tm: 60°C

Product size: 336bp

Mastermix per sample was:

1µl template cDNA	
Buffer with MgCl	5µl
Primer for	1µl
Primer rev	1µl
dH ₂ O	40,6µl
Polymerase	0,4µl

PCR- programm:

10'	95°C	Denaturation	} 40 cycles
30''	95°C	Denaturation	
45''	Tm-5°C	Primer annealing	
30''	72°C	Synthesis	
∞	4°C		

3. Results

3.1. Endothelial cell cultures from rat brain

In order to test cellular interactions needed for the clustering of AQP4 and for its focused expression at the glia limitans, it was first necessary to culture and characterize all possible different cellular interaction partners including endothelial cells, astrocytes and neurons.

Endothelial cells were grown in endothelial cell medium in collagen coated dishes for 5 to 7 days until cells were confluent. Afterwards they were detached, split and cultured on collagen coated coverslips. To guarantee highly pure endothelial cell cultures, they were treated for 2 to 3 days with 4 or 3 µg/ml puromycin. Puromycin treatment is an effective way of selecting cells. Endothelial and epithelial cells with physiological barrier functions in lung, kidney, gut epithelium, testis, placenta and brain over- express a multidrug resistance- associated protein, called P- glycoprotein (P-gp) that works as an efflux- pump. Rat brain endothelial cells express highly active P- gp and when cultured *in vitro* can survive high concentrations of puromycin (Perriere et al., 2005; Perriere et al., 2005).

In the beginning the cells grew in small star- shaped colonies, before they arranged in whirls as they became confluent (figure 9A, B). The characterization of endothelial cells was performed by immunocytochemical studies.

They were based on the selective expression of zona occludens (ZO-1) proteins in tight junctions of two neighbouring endothelial cells, which are good markers for immunocytochemistry stainings of endothelial cells (Wolburg, Neuhaus et al. 1994). The antibody binds along membrane borders and encircles endothelial cells, as shown in figure 9C and D. They do not establish tight junctions at the site that they are not in contact with another endothelial cell.

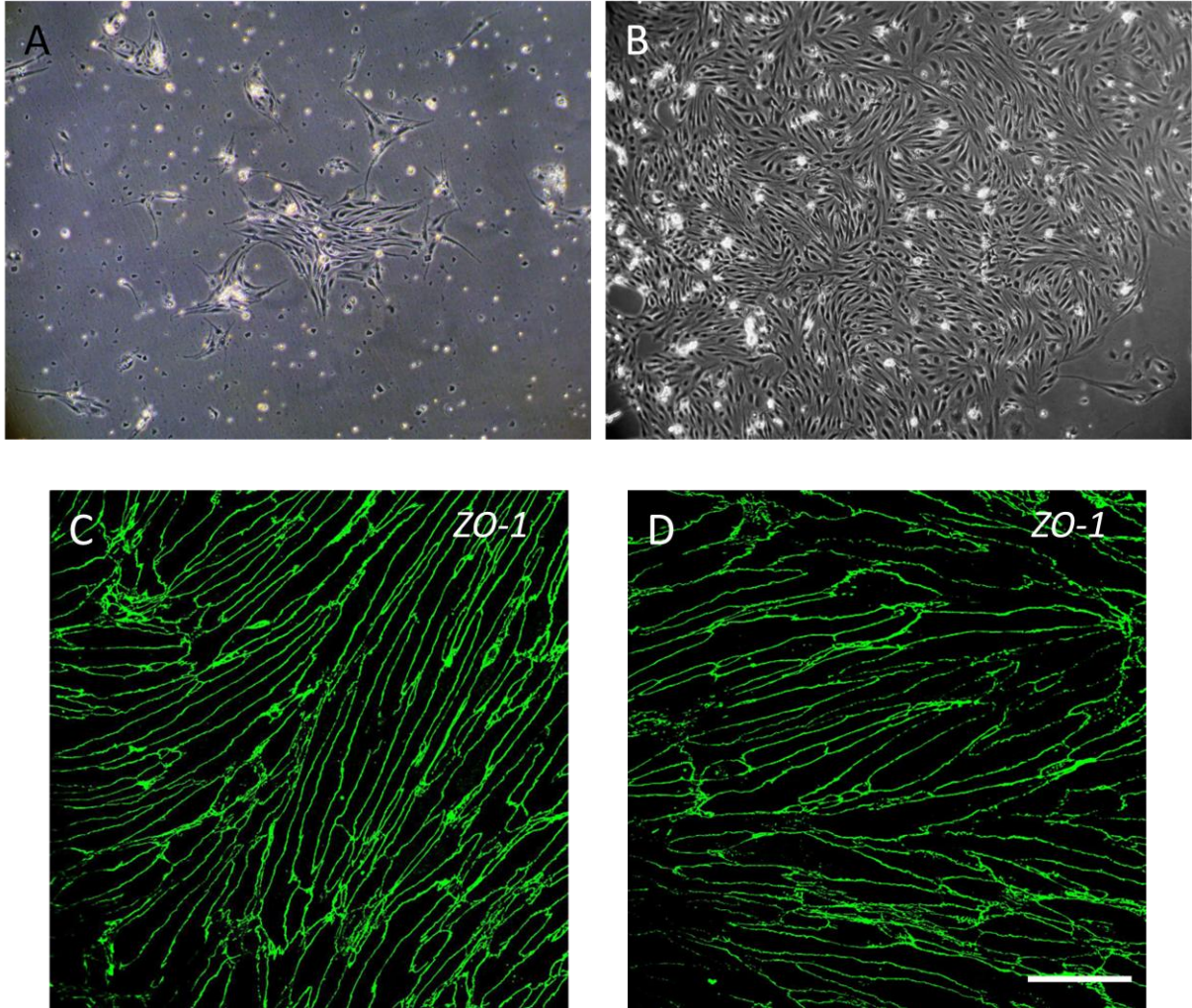


Figure 8: Rat brain endothelial cells in culture. (A) Rat brain endothelial cells after puromycin treatment. (B) Partially confluent rat brain endothelial cells in their characteristic spindle shaped morphology growing in whirls. Magnification 10x. (C) and (D) show the staining pattern of ZO-1 antibody in cultured rat brain endothelial cells. Pictures were taken from confluent parts of a coverslip. Scale bar 50µm.

3.2. Rat heart endothelial cell culture

It was also possible to isolate endothelial cells from heart with the same protocol that was used for the isolation of brain endothelial cells. The morphology of *in vitro* cultured endothelial cells from heart in comparison to brain differs essentially. Cells are rather angular- or polygonal-shaped than spindle- shaped (Frye and Patrick, Jr., 2002; Frye and Patrick, Jr., 2002). They often grow separately in single cells and not in small colonies like brain endothelial cells. Only if treated with bFGF and hydrocortisone for recovery from puromycin treatment they form brain endothelial cell- like colonies.

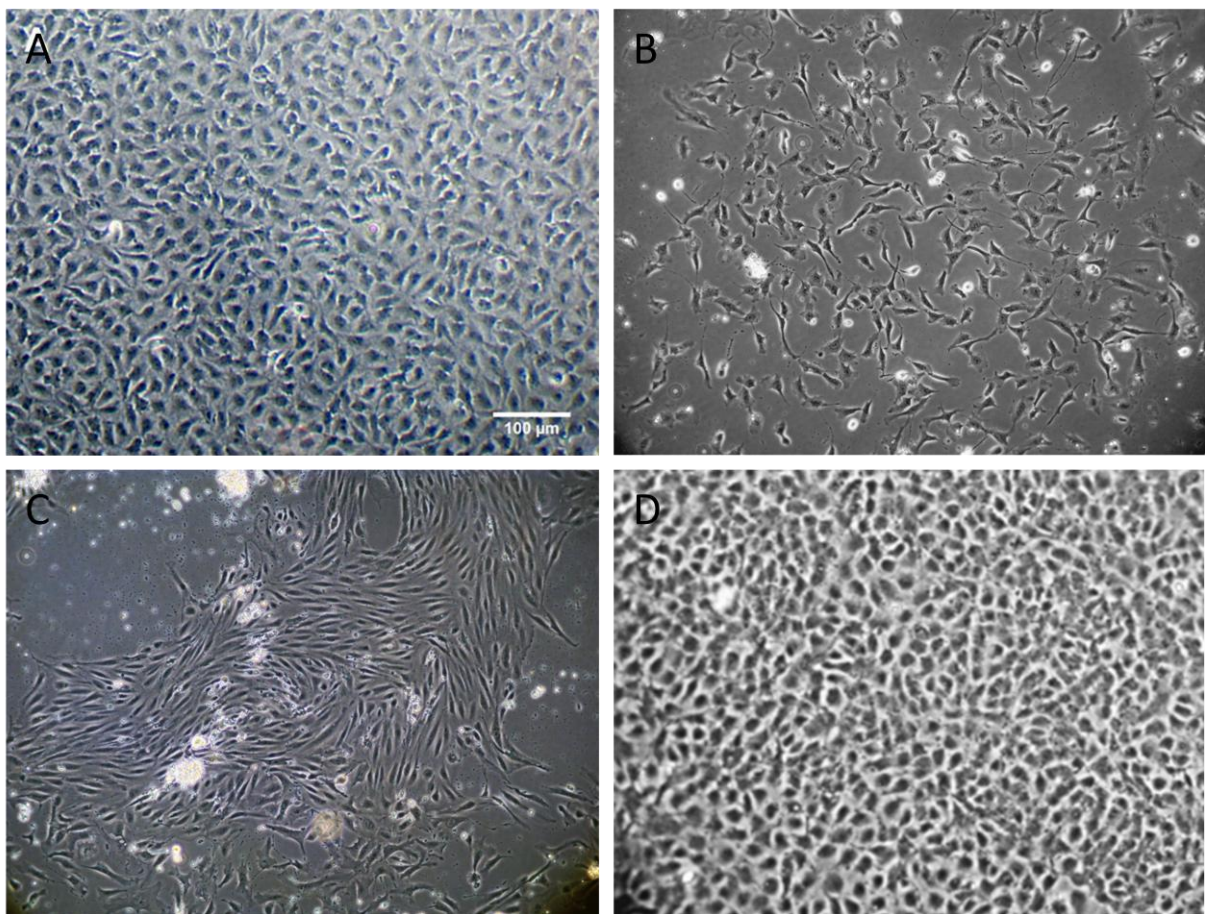


Figure 9: Rat heart endothelial cells. (A) Confluent endothelial cells *in vitro*. The picture is from the homepage of The University of Toronto (Cheung et al. 2008). (B) Non- confluent rat heart endothelial cells, treatment without factors (untreated). (C) A colony of endothelial cells treated with hydrocortisone and bFGF. (D) Confluent endothelial cells after one to two weeks in culture. They have the same cobblestone morphology as in picture (A). The scale bar is only valid for the first picture. Magnification of B, C and D 10x.

To confirm that the cells in culture were endothelial cells they were stained with antibodies which target a cell type specific antigen. Relevant antigens are: CD31 (PECAM1), CD34 or von Willebrand Factor (VWF) (Pusztaszeri et al. 2005). We stained the cells with anti- vWF antibodies. VWF is a protein that circulates in plasma as multimers and induces the adhesion of platelets to the endothelium at sites of vascular damage. VWF is also a carrier protein for the blood clotting factor VII. It is synthesized by endothelial cells and megakaryocytes (McCarroll et al., 1985). 95% of VWF in endothelial cells is secreted and the remaining molecules are stored in cytoplasmic granules called Weibel- Pallade- bodies, which are cell type specific. They are rod- shaped organelles (Sadler, 1998). All rat endothelial cells could be nicely stained, hence containing the endothelial cell specific von Willebrand factor (figure 11).

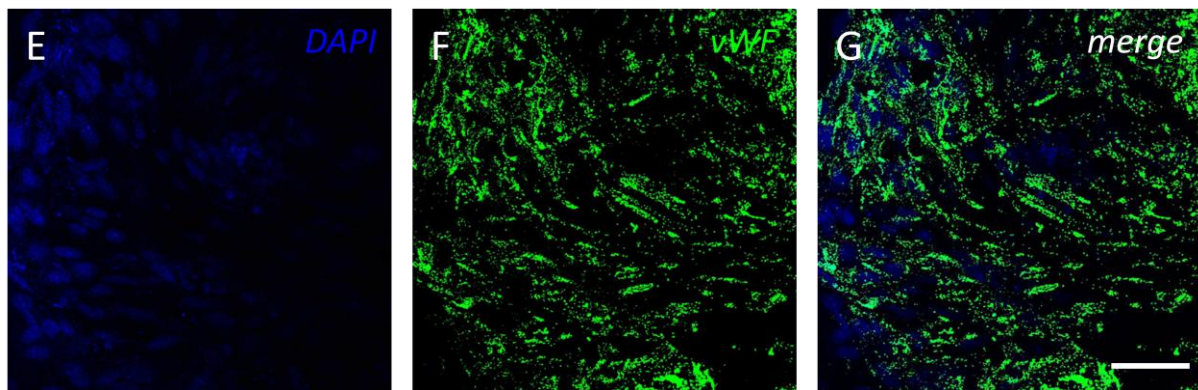


Figure 10: Rat heart endothelial cells. (A) Nuclei stained in blue with DAPI. (B) vWF staining in green as an endothelial cell marker. (G) Merged picture of (A) and (B). Scale bar 50µm.

3.3. Hippocampal neuron cultures

Hippocampal neuron cultures were established from E17 to E18,5 old Lewis rat embryos. After 24 hours in culture all cells were adherent. Neurons that arranged in colonies already formed some processes. Single cells often had no process in comparison to the cells that grew in a colony, indicated by an arrow in figure 12D. 48 hours after the isolation all cells established processes and cells in advanced differentiation stage had arborizations of their processes. After 72 hours cells were ready to be cocultured. Neurons build up very long processes.

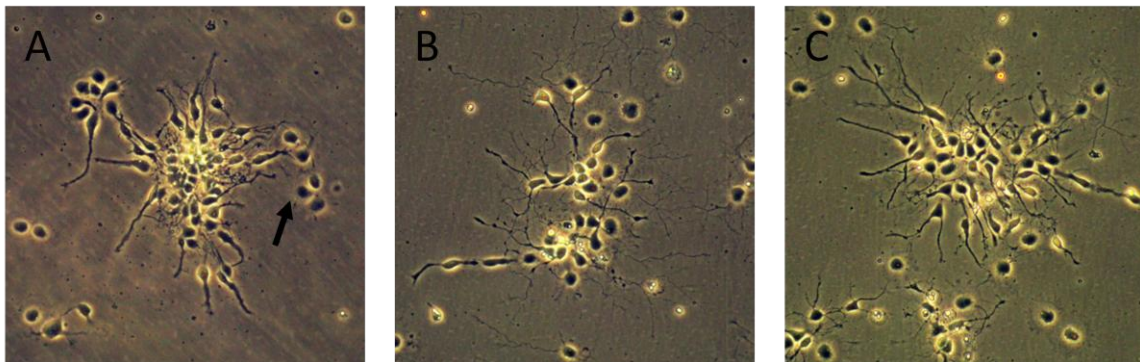


Figure 11: Hippocampal neurons in culture. (A) 24 hours after isolation from embryonic rats. Single cells do not have any processes yet. (B) 48 hours after isolation. (C) 72 hours after isolation. Magnification 40x;

We next characterized these cells in more detail. Specific antigens of neurons in culture are neurofilaments which are the earliest feature of neurons that differentiate. Therefore we used anti-SMI 311 and anti- 312 antibodies binding to neurofilaments. Neurofilaments are heteropolymers that belong to the family of intermediate filaments and can be phosphorylated in different degrees. Dendrites and cell somata have non- phosphorylated or poorly phosphorylated neurofilaments and are detected with anti- SMI 311 antibodies, whereas highly phosphorylated neurofilaments in axons are detectable by anti- SMI 312 (Ulfing et al., 1998). These were used to characterize neurons *in vitro*.

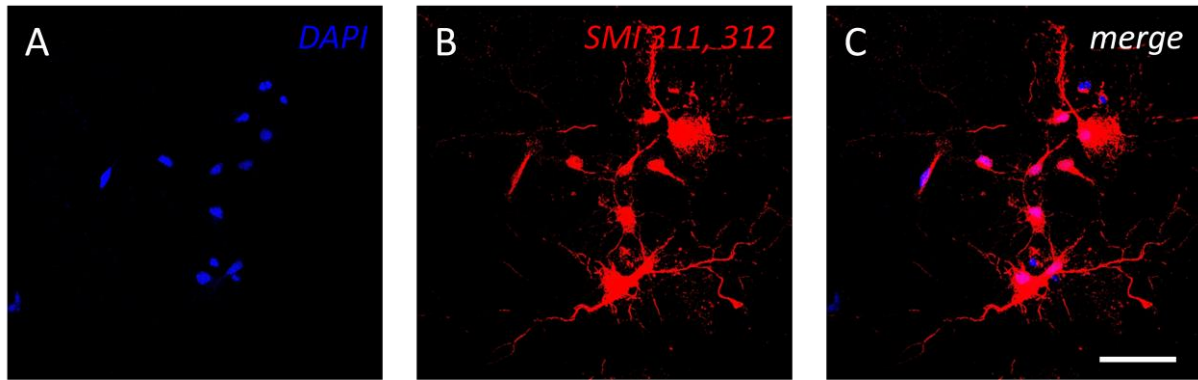


Figure 12: Hippocampal neurons in culture. (A) DAPI staining of the nucleus. (B) Neuronal cell bodies and processes were stained with SMI 211 and SMI 311 in red. (C) Merged picture of (A) and (B). Scale bar 50µm.

3.4. Astrocyte cultures

Astrocyte cultures were established from mixed glia cultures, which contained: ependymal cells, microglia, progenitor cells, fibroblasts, oligodendrocytes and astrocytes. The white appearing cells on top of the cell layer (figure 13 A and B) are microglia and progenitor cells that were shaken off. Except of ependymal cells, that can be recognized easily by their cilium (Bruni, 1998, refman), all other cells have similar morphology. It is, therefore, not possible to distinguish astrocytes from fibroblasts unless the cells were specifically stained, see figure 13.

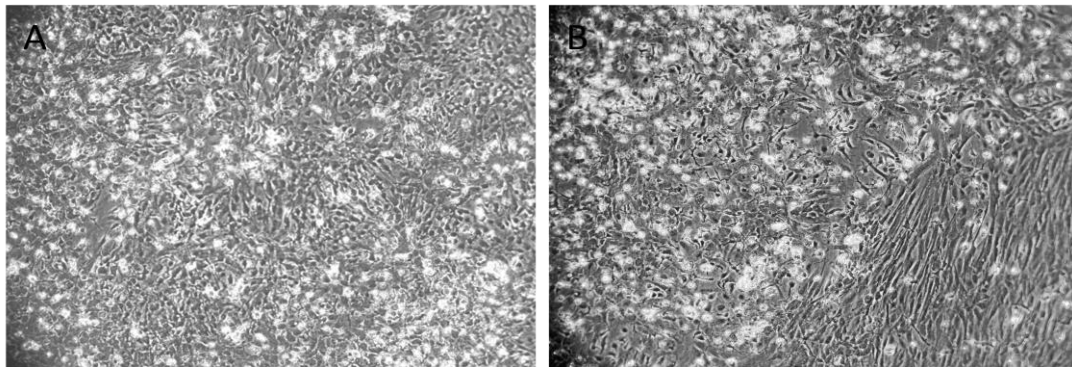


Figure 13: A and B show total mixed glia cultures established from newborn Lewis rats. (A) and (B) are pictures taken from 7 days old cultures.

There are two main astrocyte markers available. Glial fibrillary acidic protein (GFAP) is a major component of glial fibrils. It is expressed in higher levels by protoplasmic astrocytes and a distinct marker, even though it is also expressed by other cell types and tissues, namely: ependymal cells, kidney glomeruli and peritubular fibroblasts, osteocytes, pancreas and liver (Bushong et al., 2004). Another marker for astrocytes is S100 β , a glutamine synthetase. With this marker it is possible to detect even the finest ramifications, but the stainings look diffuse, there is no clear boundary of neighboring cells (Wang and Bordey, 2008). Astrocytes also express the water channel AQP4. We used anti- GFAP and anti- AQP4 antibodies to specifically stain astrocytes in culture.

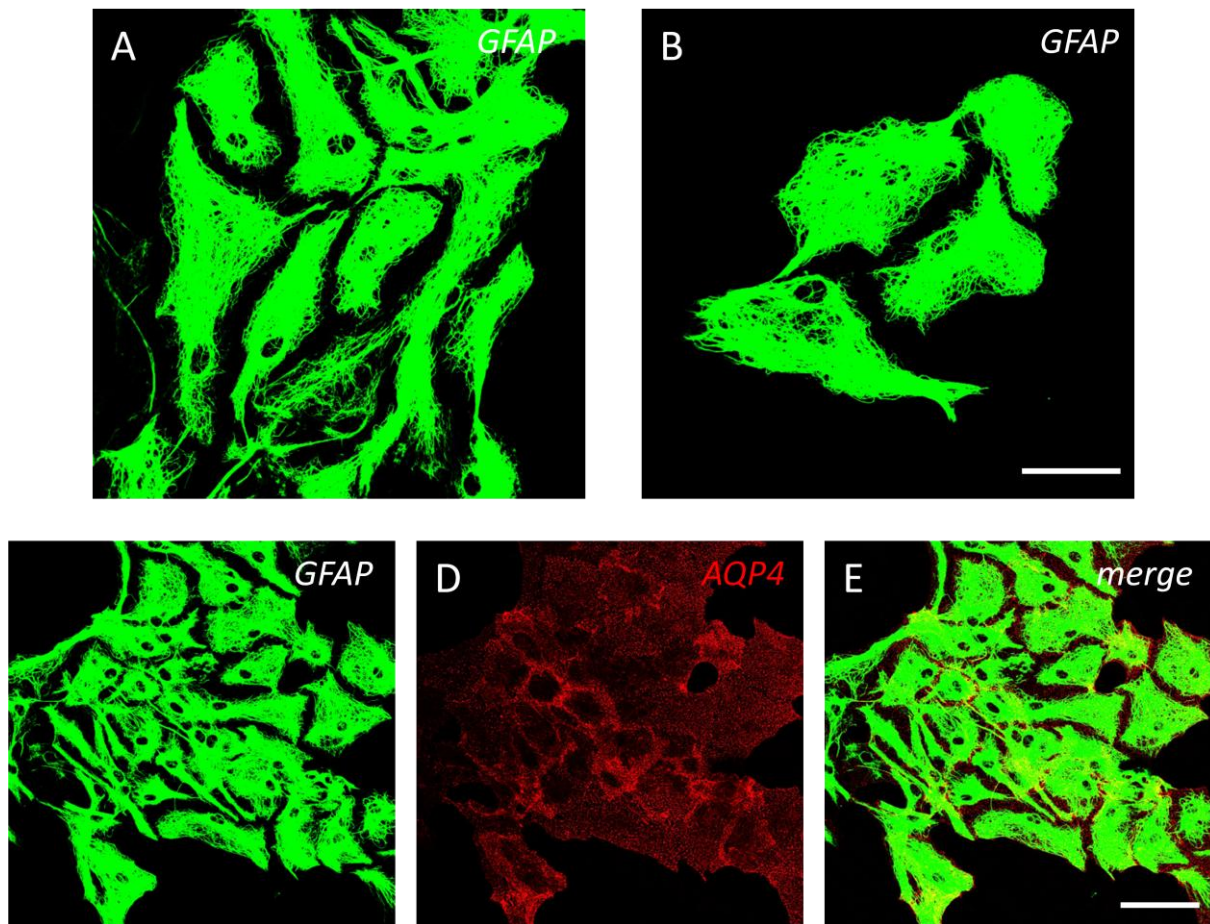


Figure 14: (A) A colony of protoplasmic astrocytes stained with anti- GFAP antibody. (B) A smaller astrocyte colony. (C) and (D): Double staining of astrocytes that were cultured for 72 hours on PLL- coated glass coverslips after detaching from mixed microglia tissue flasks. (E) Merged picture of E and F. Scale bars: 50µm.

3.5. Coculture of brain endothelial cells and astrocytes

Once all different cell cultures were established, we could coculture the different cell types to study AQP4 expression in more detail. The cocultures of endothelial cells with astrocytes were incubated for at least 72 hours in endothelial cell medium. Astrocytes grew well under these conditions. After 24 hours of coculture, astrocytes were already in contact with endothelial cells as indicated by arrows in the picture below. They were diffusely scattered between endothelial cell colonies. After 48 hours of coculture, glial cells had proliferated and almost completed the remaining space between endothelial cell colonies. After 72 hours of incubation astrocytes and endothelial cells were often confluent.

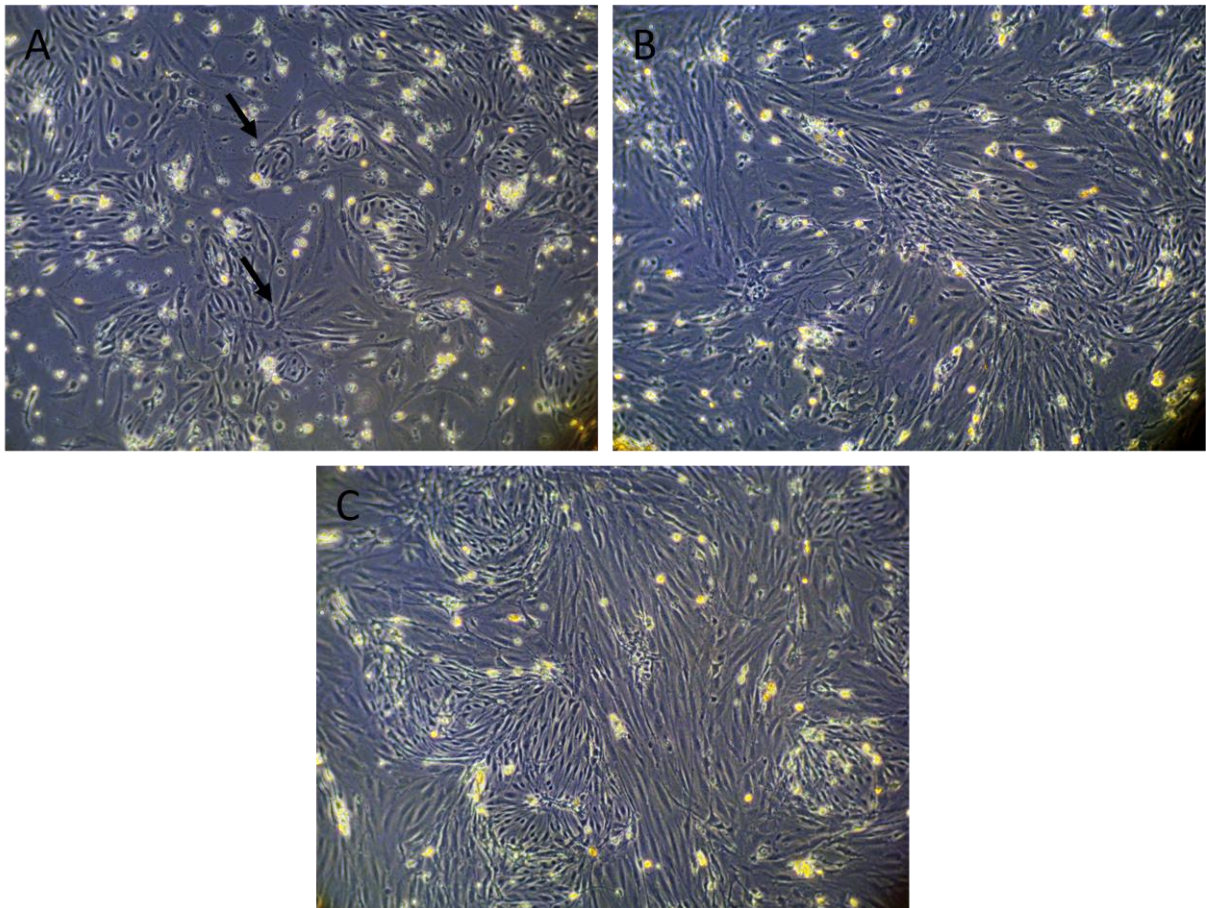


Figure 15: (A) Rat brain endothelial cells in coculture with astrocytes cocultured for 24 hours. Arrows indicate astrocytes contacting endothelial cells. **(B)** Coculture after 48 hours. Astrocytes have already proliferated. **(C)** After 72 hours there was a confluent monolayer.

Cells in coculture were further characterized by immunocytochemical stainings. Endothelial cell cells looked very healthy. They kept their arrangement of characteristic whirls and spirals throughout. Astrocytes seemed not to be harmed in endothelial cell medium and did not change their morphology. Figure xA and C shows that the end- feet of the astrocytes are in close proximity to or even contacting endothelial cells.

Double stainings were made with a primary antibody against ZO-1, a marker protein for endothelial cells tight junctions and GFAP, to identify astrocytes.

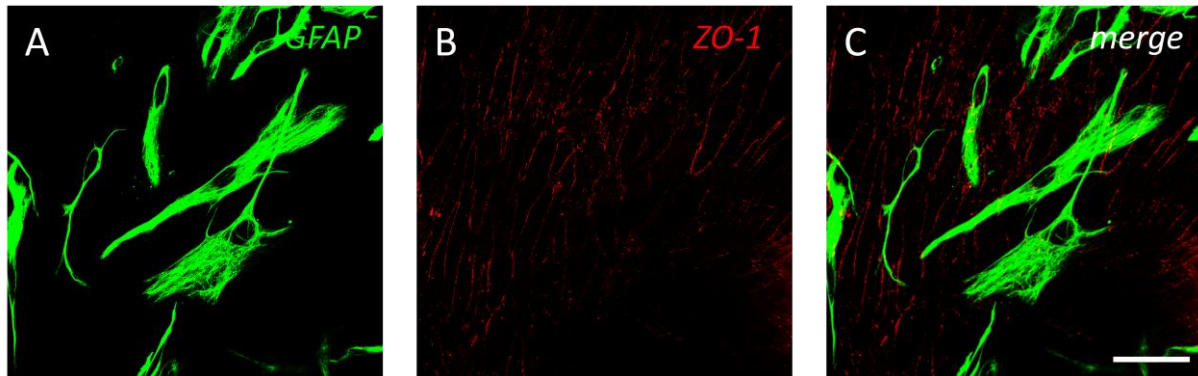


Figure 16: Immunocytochemical staining of endothelial cells and astrocytes in coculture. (A) GFAP as a marker for astrocytes, in green, (B) ZO-1 in red for endothelial cells.(C) Astrocytes grown in coculture in endothelial cell medium for 24 hours. Astrocytes seem to adhere and proliferate between, above and underneath endothelial cells. Scale bar 50µm.

We then examined how this coculture affected the AQP4 expression. For this purpose, I made triple stainings with ZO-1, AQP4 and GFAP. Rat brain endothelial cells in coculture with astrocytes did not alter AQP4 expression nor the degree of AQP4 clustering in astrocytes. In the picture above it even seems, that AQP4 is downregulated in astrocytes that are in coculture with endothelial cells. To prevent a miss- interpretation by false negative AQP4 stainings, some cocultures where only stained for GFAP and AQP4 (figures 14E- G).

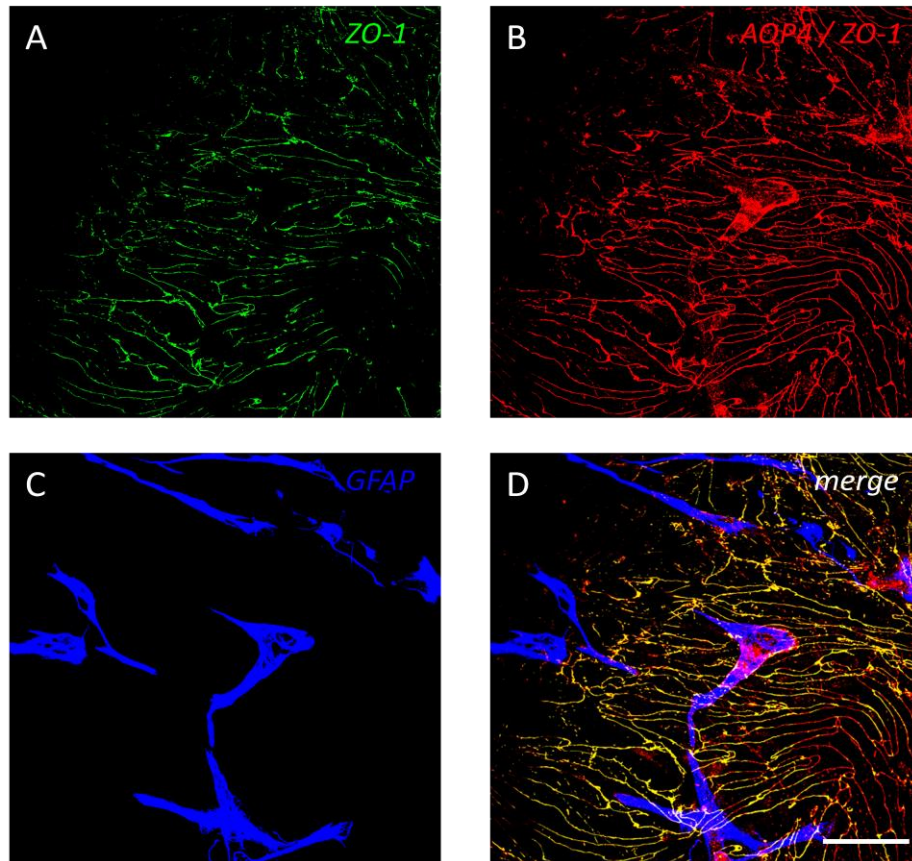


Figure 17: Rat brain endothelial cells in coculture with astrocytes for 72 hours. (A) Endothelial cells stained for ZO-1 in green. (B) AQP4 and ZO-1 in red (because both primary antibodies are derived from the same animal). (C) Staining pattern of GFAP as the marker protein of astrocytes, in blue. (D) Merged picture.

This control staining revealed that in comparison to pure astrocytes cultures the AQP4 immunoreactivity was not altered in cocultures with brain endothelial cells that secrete the agrin isoform 0.0 (compare to figure 12 E- G).

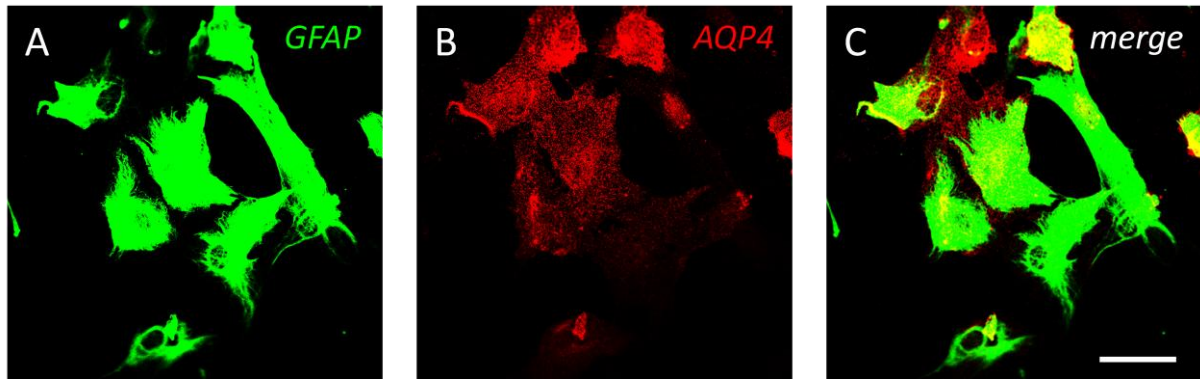


Figure 18: Rat brain endothelial cells in coculture with astrocytes for 72 hours. Endothelial cells were not stained. (A) Astrocytes stained with anti- GFAP antibody, (F) anti- AQP4 staining, (G) merged picture. Scale bars: 50µm.

3.6. Coculture of heart endothelial cells with astrocytes

Similar findings were made upon coculture of astrocytes with rat heart endothelial cells. Astrocytes processes are often contacting endothelial cells with their endfeet and cellbodies.

A triple staining with VWF, GFAP and AQP4 did not work. Therefore cocultures were only stained with GFAP and AQP4. Figure 16 shows astrocytes that have an AQP4 immunoreactivity identical to control cells in culture.

This confirms the finding from brain endothelial cells that agrin 0.0. cannot be responsible for AQP4 clustering and expression level increase in astrocytes.

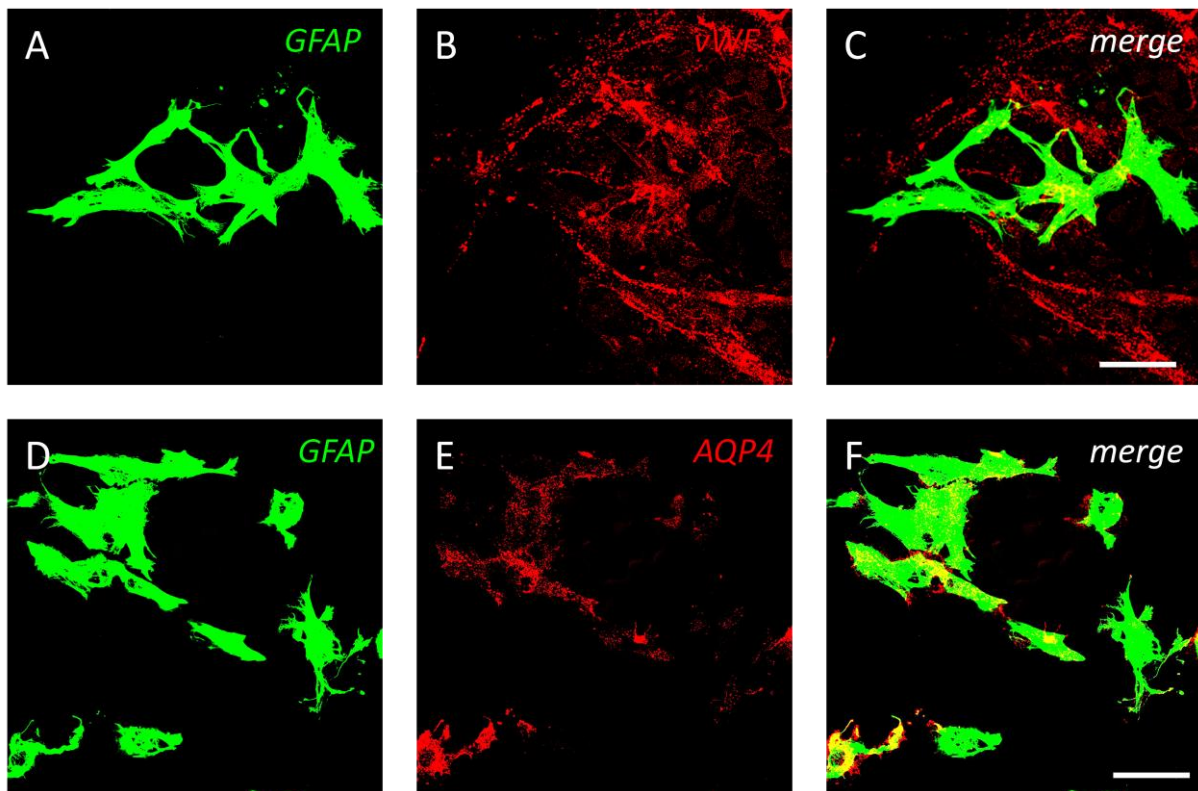


Figure 19: Rat heart endothelial cells in coculture with astrocytes. (A) astrocytes in green labeled for GFAP, (B) heart endothelial cells in red labeled for vWF, (C) merged picture of A and B. D to F: detection of AQP4 immureactivity of astrocytes in coculture without VWF staining. Scale bar 50 μ m.

To further test whether the neuronal isoform of agrin is able to induce AQP4- clustering and increase in AQP4 expression, we cocultured astrocytes with neurons.

3.7 Coculture of neurons with astrocytes

In the following experiments astrocytes were cocultured with neurons, to see whether neurons have any effect on the AQP4 expression level and on its subcellular distribution. In this experiment neurons and astrocytes were cocultured for 72 hours in neuronal medium. Astrocytes in coculture with neurons change their morphology dramatically. There are only few protoplasmic astrocytes in comparison to astrocytes in coculture with endothelial cells from brain and heart. It is also possible that they simply change their morphology. Further on neurons seem to provide a trigger that alters astrocytes processes in regard of a membrane ruffling. These ruffles or arborizations cannot be visualized by GFAP staining, but by AQP4 staining. They are highly AQP4 positive.

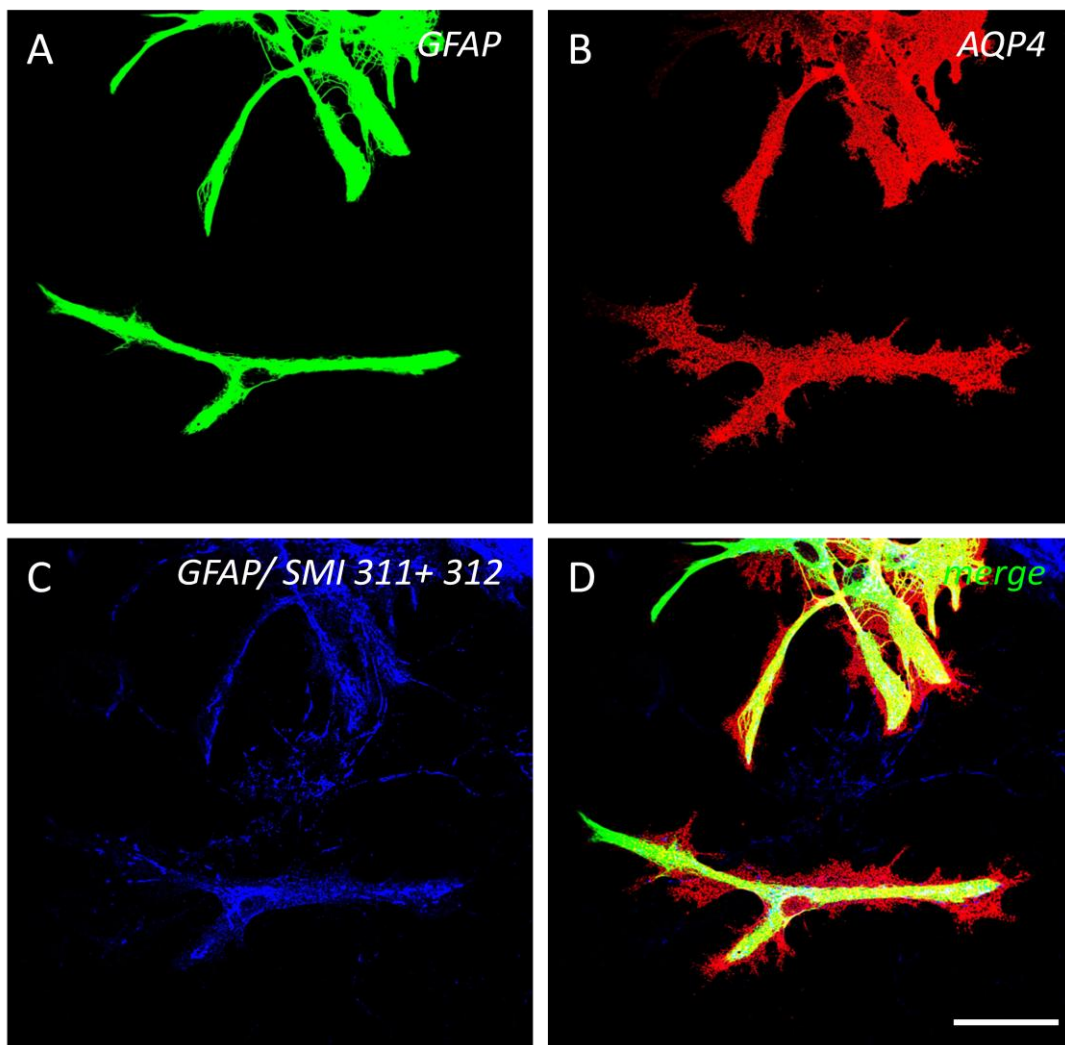


Figure 20: Astrocytes in coculture with hippocampal neurons for 72 hours. (A) GFAP staining illustrates astrocytes, (B) staining of AQP4, (C) double staining of GFAP and SMI as their primary antibodies are derived from the same animal, (D) merged picture of A- C.

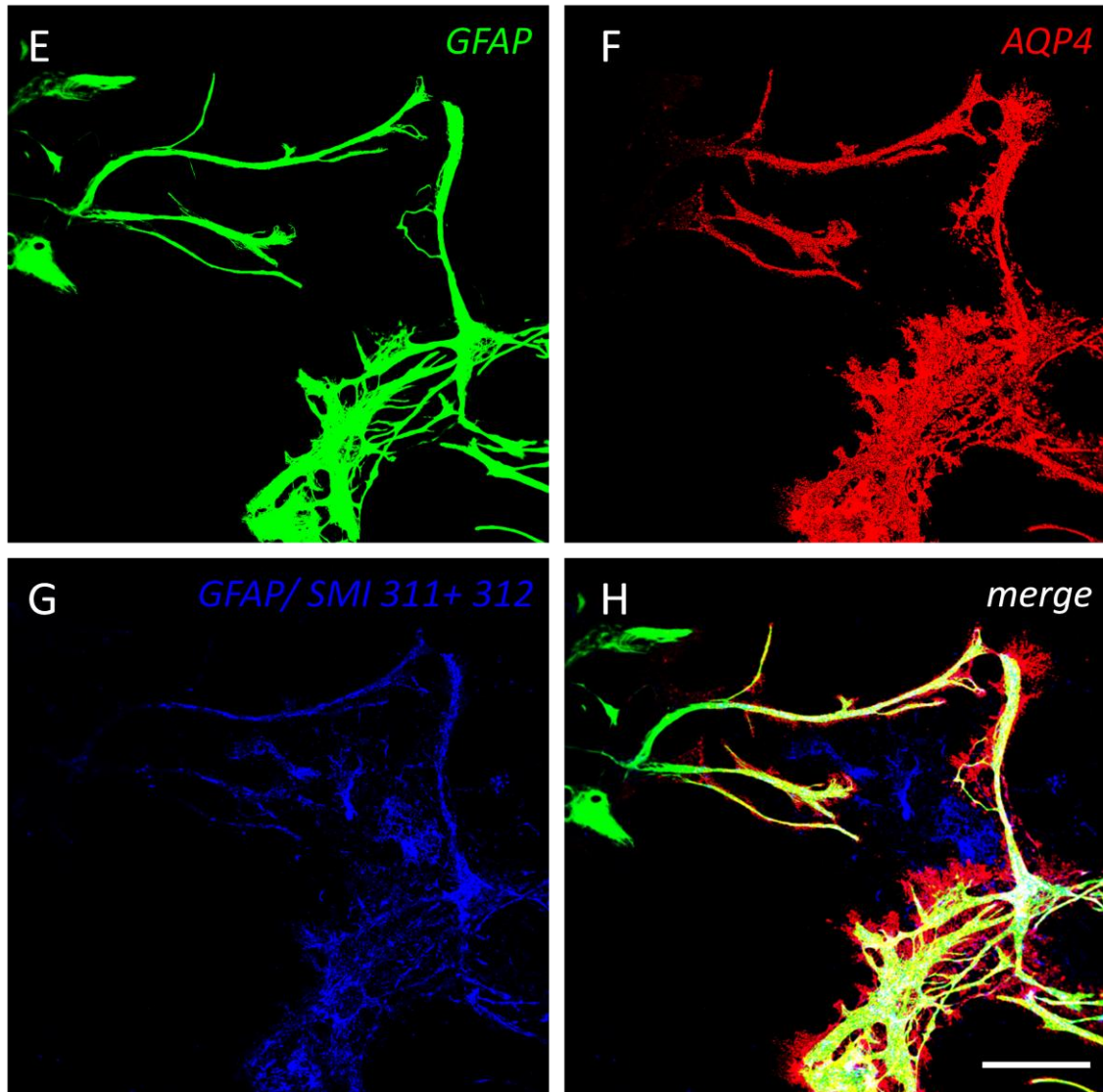


Figure 21: Enhanced AQP4 immunoreactivity in comparison to the coculture with endothelial cells. (E) GFAP visualizes astrocytes, (F) staining with anti- AQP4 antibody, (G) double staining of GFAP and SMI as their primary antibodies are derived from the same animal, (H) Merged picture of E- G. Strong arborizations and high AQP4 expression of astrocytes processes. Scale bars 50 μ m.

As a control, astrocytes were cultured in neuronal medium to rule out, that the medium itself would have any effect on astrocytes. The pictures below show that astrocytes are less in number, the cells are much smaller and colonies consist of very few cells in comparison to astrocytes in endothelial cell medium or microglia medium. There were many “rolled up” cells, visible in the left corner. Furthermore, GFAP positive cells that could probably be progenitor cells of glia and neurons, did not express AQP4 as they did in coculture with neurons.

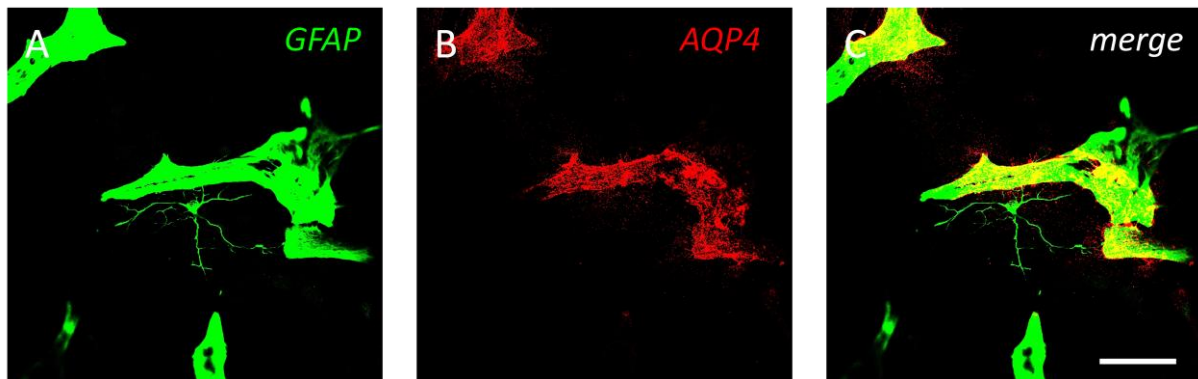


Figure 22: Astrocytes grown in neuronal medium as control. (A) GFAP staining, (B) AQP4 staining, (C) merged picture of (A) and (B). 3 AQP4 positive protoplasmic astrocytes. The other two astrocytes at the party of the pictures seem to be out of focus from the AQP4 level. Scale bar: 50µm.

This indicates that, independently from the medium, astrocytes change their morphology or cell fate dependent on the environment established by neurons. As neurons secrete the agrin isoform 4.8., it could be possible that this protein isoform may be responsible for an enhancement of AQP4 expression, as indicated by literature (Noell et al., 2007).

Next, we wanted to investigate whether the effect of neurons on astrocytes is a phenomenon that is limited for short period of time or whether the effect persists. For this purpose we cocultured neurons we astrocytes for one week. Figure 18 shows that neuronal processes extended and that astrocytes kept their fine arborizations of membrane ruffles that are strongly AQP4 positive, 18.

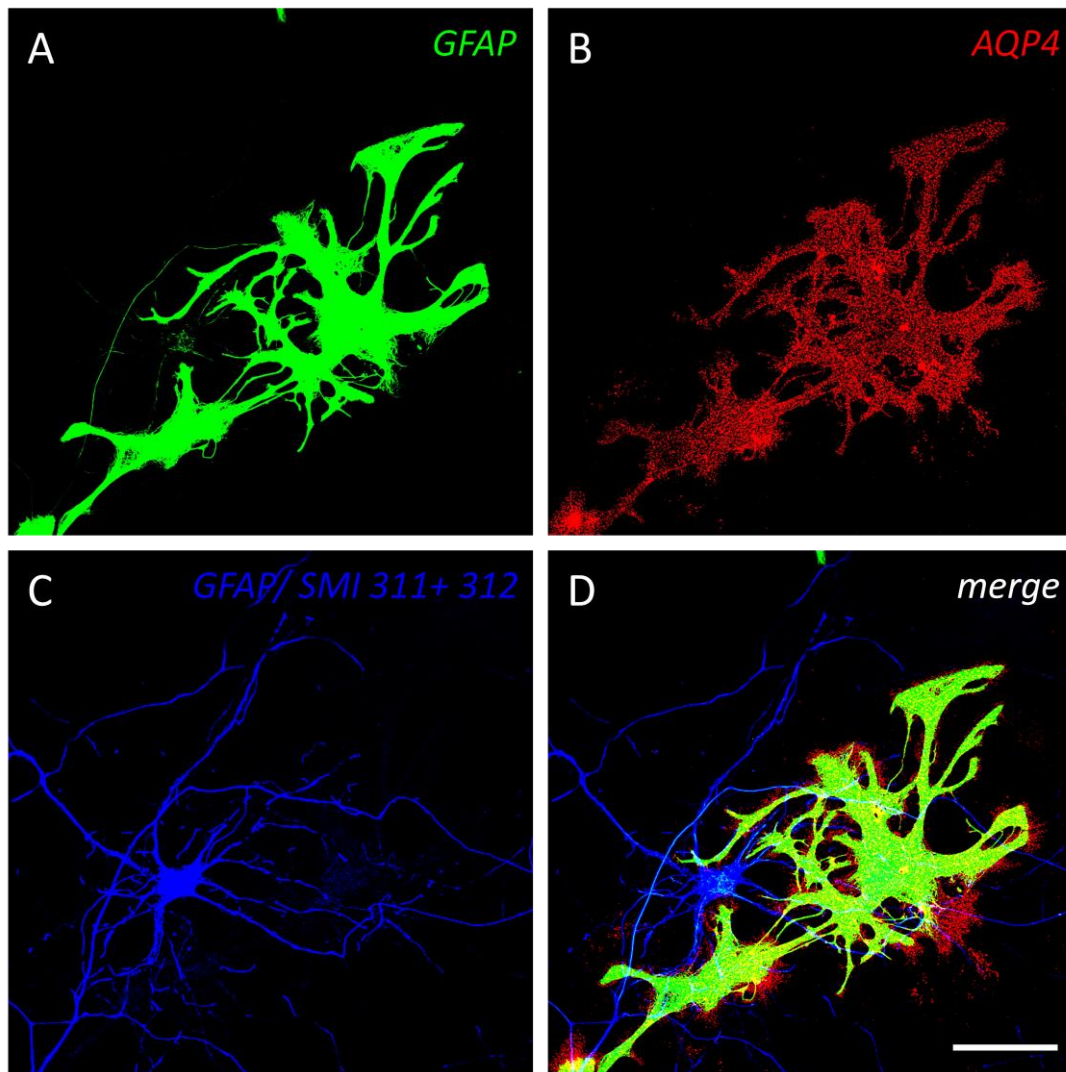


Figure 23: Astrocytes in coculture with hippocampal neurons for one week. (A) GFAP staining illustrates astrocytes, (B) staining of AQP4, (C) double staining of GFAP and SMI as their primary antibodies are derived from the same animal, (D) merged picture of A- C. Enhanced AQP4 immunoreactivity in comparison to the coculture with endothelial cells. Scale bar 50µm.

Further on, we confirmed the specificity for the anti- AQP4 antibody, clustering and correct surface expression by the staining with a NMO- IgG antibody, isolated from a human NMO patient. The pictures below show that the staining pattern of NMO- IgG is equal to the staining of the anti- AQP4 antibody. The fine arborizations of AQP4 positive astrocytes also become visible with the NMO-IgG. This indicates that the anti- AQP4 and the NMO-IgG recognize the same protein in astrocytes. More detailed experiments with the aim to characterize the epitopes of NMO- IgG and the AQP4 antibody (Nicchia et al., 2009) will be discussed in the discussion.

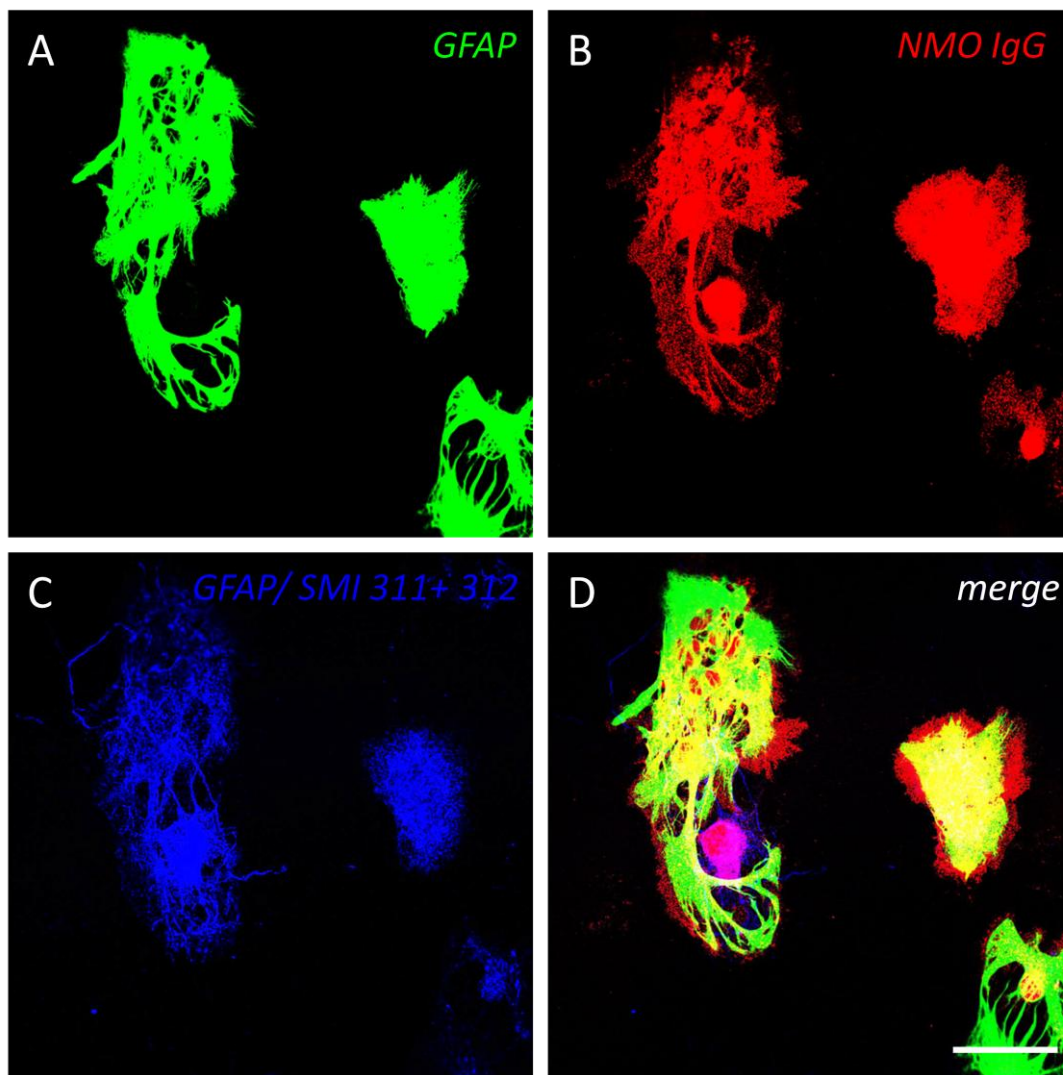


Figure 24: Astrocytes in coculture with hippocampal neurons for one week. (A) GFAP staining illustrates astrocytes, (B) staining of AQP4 by NMO-IgG, (C) double staining of GFAP and SMI as their primary antibodies are derived from the same animal, (D) merged picture of A- C. Enhanced AQP4 immunoreactivity in comparison to the coculture with endothelial cells is also detectable with specifically binding NMO- IgG. Scale bar 50µm.

3.8. Agrin expression in different cell types

In order to verify whether agrin is expressed by endothelial cells, neurons, astrocytes and heart endothelial cells I performed PCRs for each cell type. While endothelial and neuronal cultures were quite clear, the astrocyte cultures had a purity of only 30%, with fibroblasts as the main contamination population, determined by FACS cell sorting (data not shown). Amplified PCS products were sequenced.

Agrin expression was high in the brain at postnatal day one (P1) and much lower in the adult brain. In comparison to P1 total brain, agrin expression in adult total brain decreased almost 2 thirds. It is also reported in literature that the expression decreases postnatal (Stone and Nikolics, 1995). Agrin expression was high in neurons, astrocytes and heart endothelial cells.

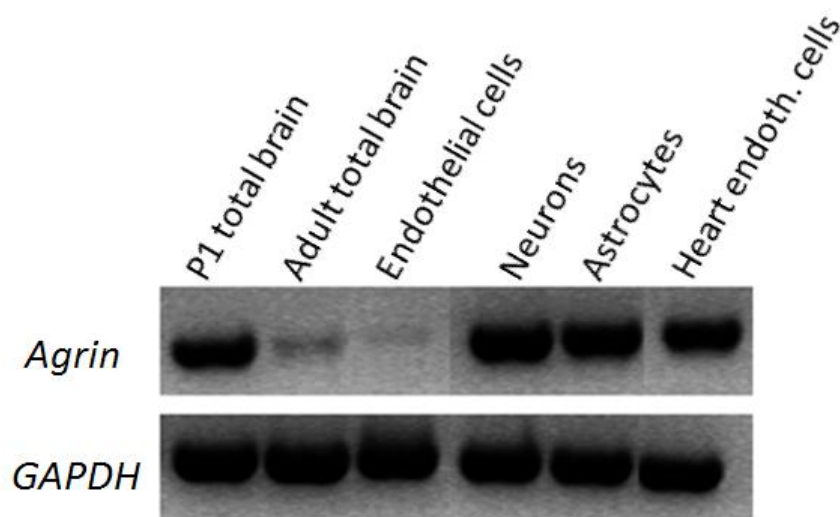


Figure 25: Detection of agrin expression in postnatal day one (P1) total rat brain, adult total brain, rat brain endothelial cells, embryonic day 18 neurons, newborn astrocytes, rat heart endothelial cells. GAPDH was used as a reference gene.

3.8. Agrin treatment of astrocytes

Because of the fact that neurons had a great effect on astrocytes morphology and AQP4 expression we assumed and hypothesized that agrin might be the protein responsible for triggering these mechanisms. For this reason we cultured astrocytes for 72 hours to 2 weeks in medium conditioned with agrin: agrin 0.0., the endothelial cell isoform and agrin 4.8., the neuronal isoform, to investigate the action of this protein on astrocytes.

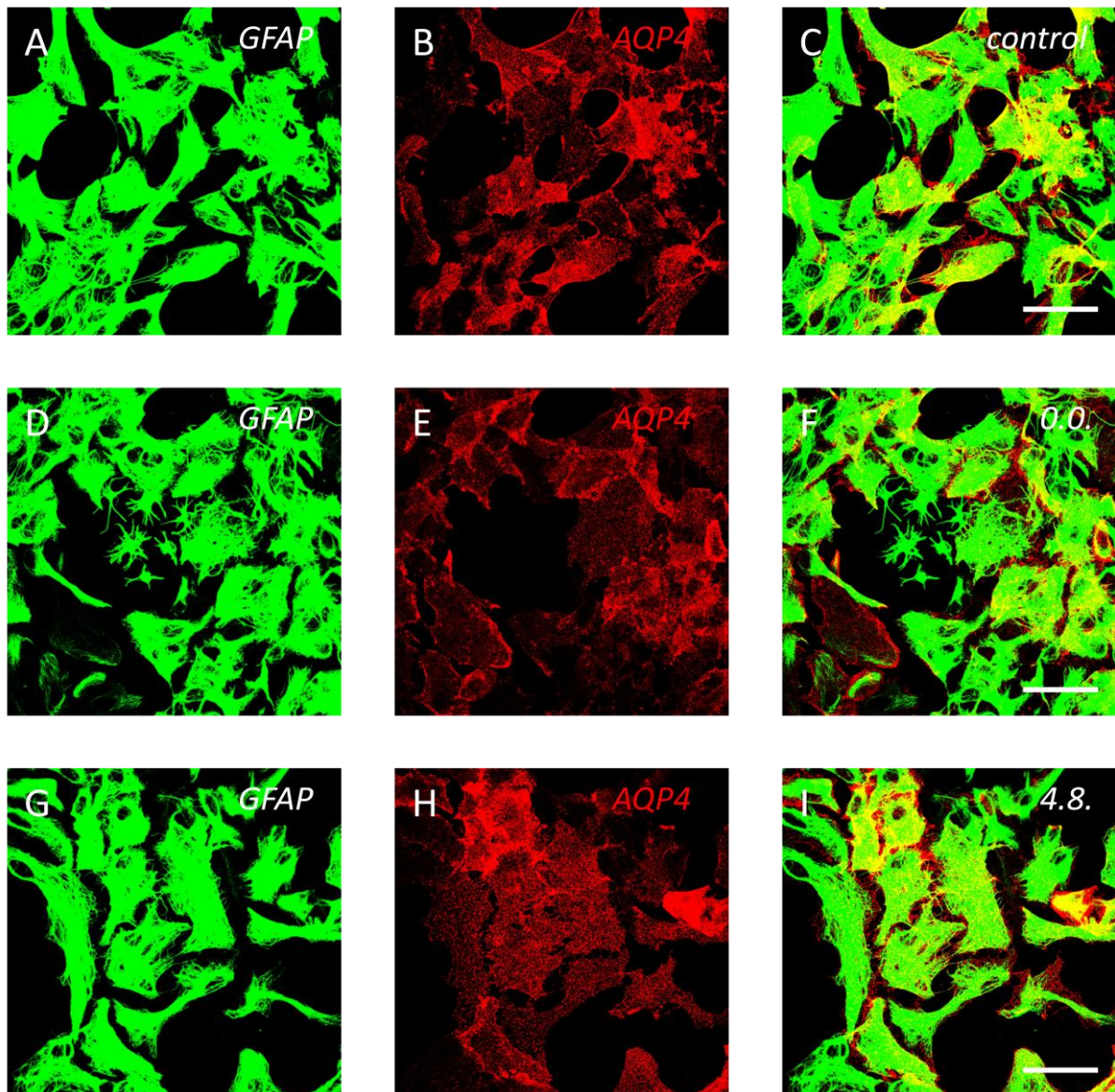


Figure 26: Astrocytes treated with agrin conditioned medium for 7 days. A, D and G are stained for GFAP as a marker for astrocytes. B, E and H are stained for AQP4. C, F and I are merged pictures. The first row are control stainings of non-treated cells. D-F depict astrocytes treated with agrin isoform 0.0. and pictures G to I depict astrocytes treated with agrin isoform 4.8. (agrin 0.0.:30 μ l/ml, 4.8.: 20 μ l/ml). Scale bar: 50 μ m

3.9. Agrin and laminin treatment of astrocytes

After 72 hours to 7 days of agrin treatment no considerable differences could be detected in 4 independently performed experiments. These findings raised the question whether laminin, another protein contributing to the basal lamina in the BBB, is needed to induce effects in astrocytes that are treated with agrin conditioned medium.

In comparison to the control astrocytes that were not treated with any agrin and laminin the immunoreactivity of the agrin/laminin treated cells did not change. Neither the AQP4 distribution, nor the AQP4 levels, nor astrocytes morphology was altered when treated with laminin and agrin, see figure x.

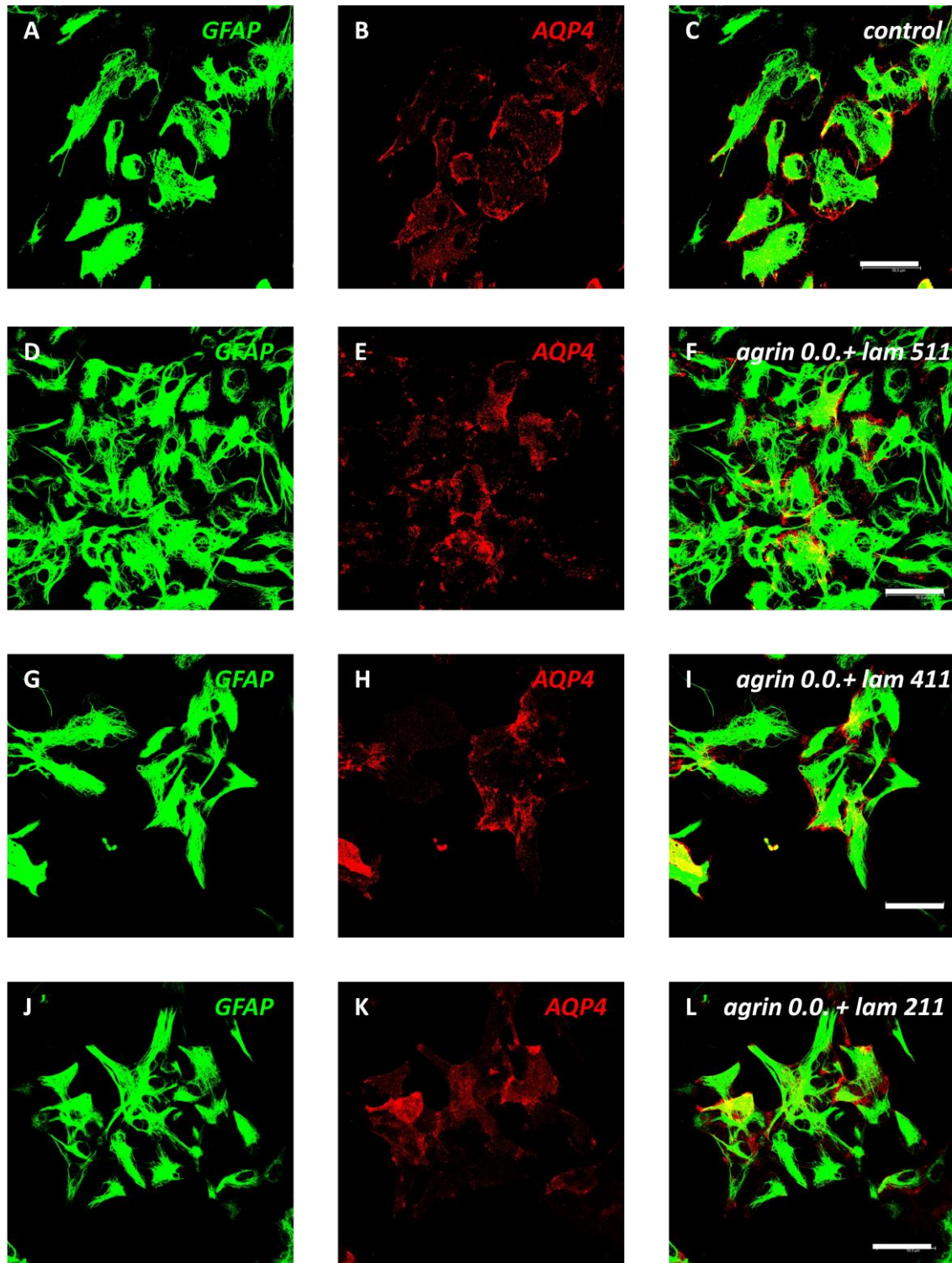


Figure 27: Astrocytes treated with agrin 0.0. conditioned medium for 7 days. A, D, G and J are stained for GFAP as a marker for astrocytes. B, E,H and K are stained for AQP4. C, F, I and L are merged pictures. The first row are control stainings of non- treated cells. D-F depict astrocytes treated with agrin isoform 0.0. with laminin 511, pictures G to I depict astrocytes treated with agrin isoform 0.0 with laminin 411 and pictures J to L shows astrocytes treated with agrin 0.0. and laminin 211. Scale bar: 50µm

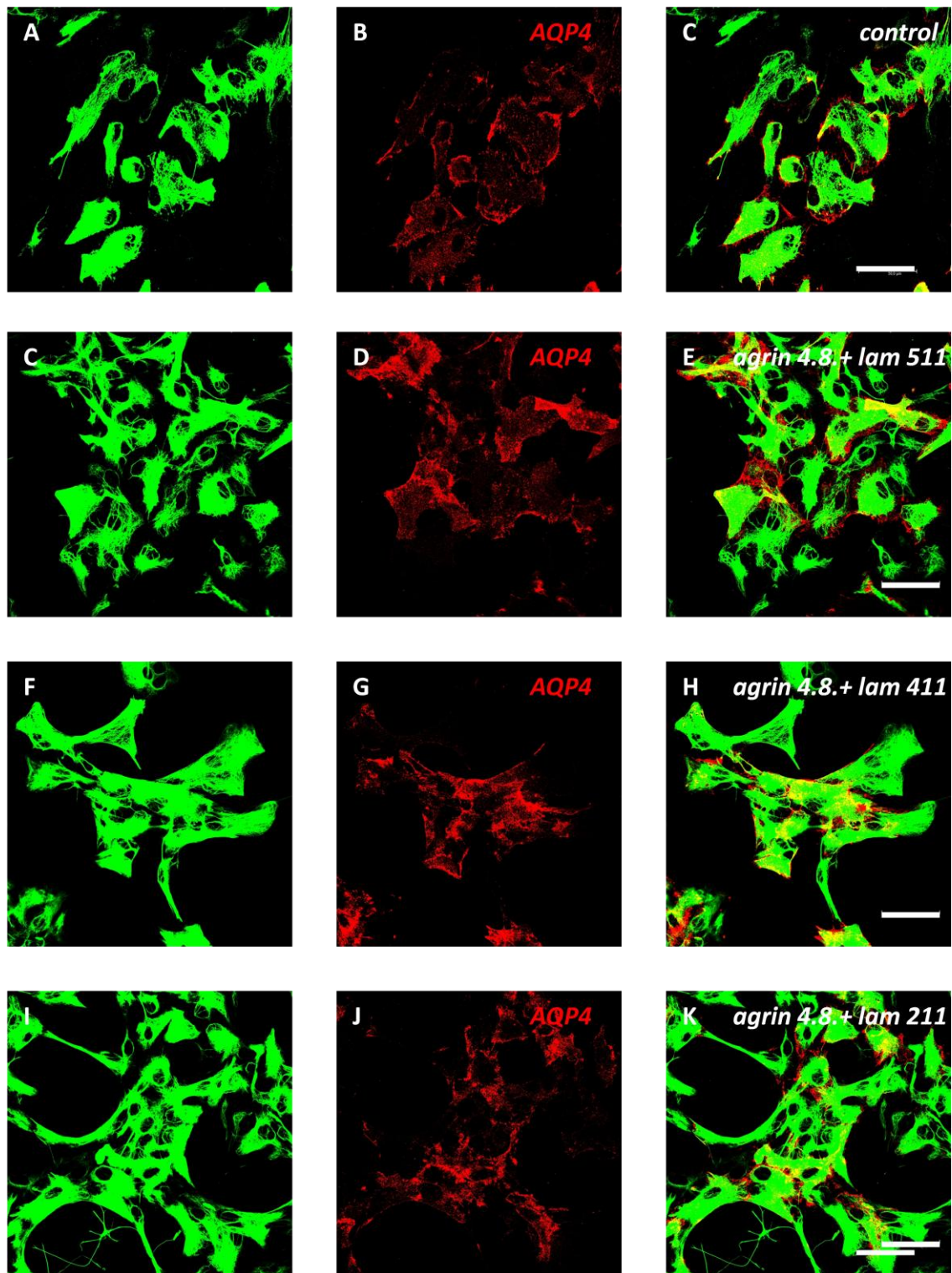


Figure 28: Astrocytes treated with agrin 4.8. conditioned medium for 7 days. A, D, G and J are stained for GFAP as a marker for astrocytes. B, E, H and K are stained for AQP4. C, F, I and L are merged pictures. The first row are control stainings of non- treated cells. D-F depict astrocytes treated with agrin isoform 4.8. with laminin 511, pictures G to I depict astrocytes treated with agrin isoform 4.8. with laminin 411 and pictures J to L shows astrocytes treated with agrin 4.8. and laminin 211. Scale bar: 50μm

4. Discussion

Until now the mechanism is not known why AQP4 is focally clustered at the perivascular and superficial glia limitans in the central nervous system. We hypothesized that the enhanced clustering of AQP4 at astrocytic end- feet is caused by agrin that is secreted from endothelial cells and neurons at the BBB and is associated with basal laminae. Several studies support this hypothesis (Smith and Hilgenberg, 2002; Bezakova and Ruegg, 2003; Noell et al., 2007; Noell et al., 2009; Warth et al., 2004; Jarius et al., 2008).

In the recent years there was more and more evidence that agrin has an effect on the expression and distribution of AQP4. It was shown by Noell et al. (2007) by culturing astrocytes in agrin- conditioned medium, produced by HEK 293 cells, transfected with two agrin isoforms: Y0Z0 that corresponds to the non- neuronal and endothelial cell derived agrin and Y4Z8, the neuronal agrin variant. They reported about an increase of AQP4 immunoreactivity in agrin treated cells.

In order to test our hypothesis, we cocultured postnatal day 1 astrocytes with endothelial cells, secreting the agrin isoform Y0Z0, and neurons, secreting the agrin isoform Y4Z8. The effect of these cells on astrocytes was judged by two different criteria: the AQP4 immunoreactivity and the astrocytes morphology. In culture astrocytes have an equally distributed immunoreactivity of AQP4 over the whole cell membrane. Regarding their morphology there are 2 types of astrocytes in the brain that are also visible *in vitro*: protoplasmic and stellate astroglial cells. Protoplasmic, also described as spongiform, astrocytes have many delicate branched leaflet processes and are known for their intimate association with excitatory synapses from neurons and cover the blood vessels in the brain (Bushong et al., 2004; Wang and Bordey, 2008). Stellate or fibrous astrocytes have long, thin and unbranched processes, that envelope nodes of Ranvier *in vivo* (Wang and Bordey 2008).

We could detect and distinguish these two isoforms by their morphology and GFAP positive stainings in our cultures. Stellate and protoplasmic astrocytes are distinguishable by their morphology, fig. 29 E and F. Stellate astrocytes are often stained weaker for GFAP and weakly AQP4 positive or AQP4 negative. In addition to stellate and protoplasmic astrocytes we could also observe another GFAP positive and AQP4 negative cell type with many delicate processes which probably represent neuronal precursors (Ihrie and Alvarez-Buylla, 2008), fig. 29 E.

When astrocytes were cultured with rat brain endothelial cells we could not detect any change in the morphology and in AQP4 immunoreactivity level or AQP4 distribution in astrocytes. To further

verify these findings we cocultured rat heart endothelial cells that also secrete agrin Y0Z0, which we showed by means of PCR and was reported in literature (Stone and Nikolics, 1995). Again, we could not detect any effect of heart endothelial cells in regard of their ability to alter AQP4 expression or its organization in the cell membrane.

The staining rather resembled astrocytes that were not cocultured and therefore had no agrin treatment. In consequence, agrin isoform Y0Z0 (and other factors that derive from endothelial cells) might not be responsible for inducing changes in AQP4 expression level, clustering or morphology.

When we cultured astrocytes with neurons we could detect a great impact of neurons that synthesize the agrin isoform Y4Z8 on astrocytes.

First, there was a striking difference of the morphology of astrocytes. Fig. 29 A- D show astrocytes that were two weeks in coculture with neurons and are strongly AQP4 positive. Astrocytes encircled neurons, stained in blue, and changed their morphology dramatically from a rather amoeboid- like morphology as shown in figure 29 E- G (non- treated pure astrocyte culture), to cells with small cell bodies and many processes that are partially arborized and strongly AQP4 positive.

Next, astrocytes started to built up fine arborizations of membrane ruffles that were strongly AQP4 positive and GFAP negative. This was not the case in untreated cells where the immunoreactivity of AQP4 and GFAP overlaps completely. An important observation was that these AQP4 positive membrane specializations were present not only when astrocytes were in contact or close proximity with neurons, but also in areas where astrocytes were not facing any neuron. This indicates that a neuron- derived, soluble factor might be responsible for these morphological changes, e. g. agrin.

Another effect of neurons on astrocytes is that there is an induction of AQP4 expression in cells that are normally AQP4 negative. Figure E- G show astrocytes that were not treated but kept in pure astrocyte cultures. There are several protoplasmic cells and two GFAP positive cells on the lower left corner that could possibly be stellate astrocytes, radial glia or precursor cells. They are completely AQP4 negative.

In comparison, when astrocytes are cocultured with neurons, formerly AQP4 negative cells, that share the morphology of the untreated AQP4 negative and GFAP positive cells, start to express AQP4. Hence, there must be a neuronal factor supplying astrocytes with a trigger to become AQP4 positive and that enhances the AQP4 expression level.

Neurons are not contacting all astrocytes that show these altered features in coculture. This indicates that the factor responsible for a higher AQP4 expression of astrocytes is soluble and derived from neurons.

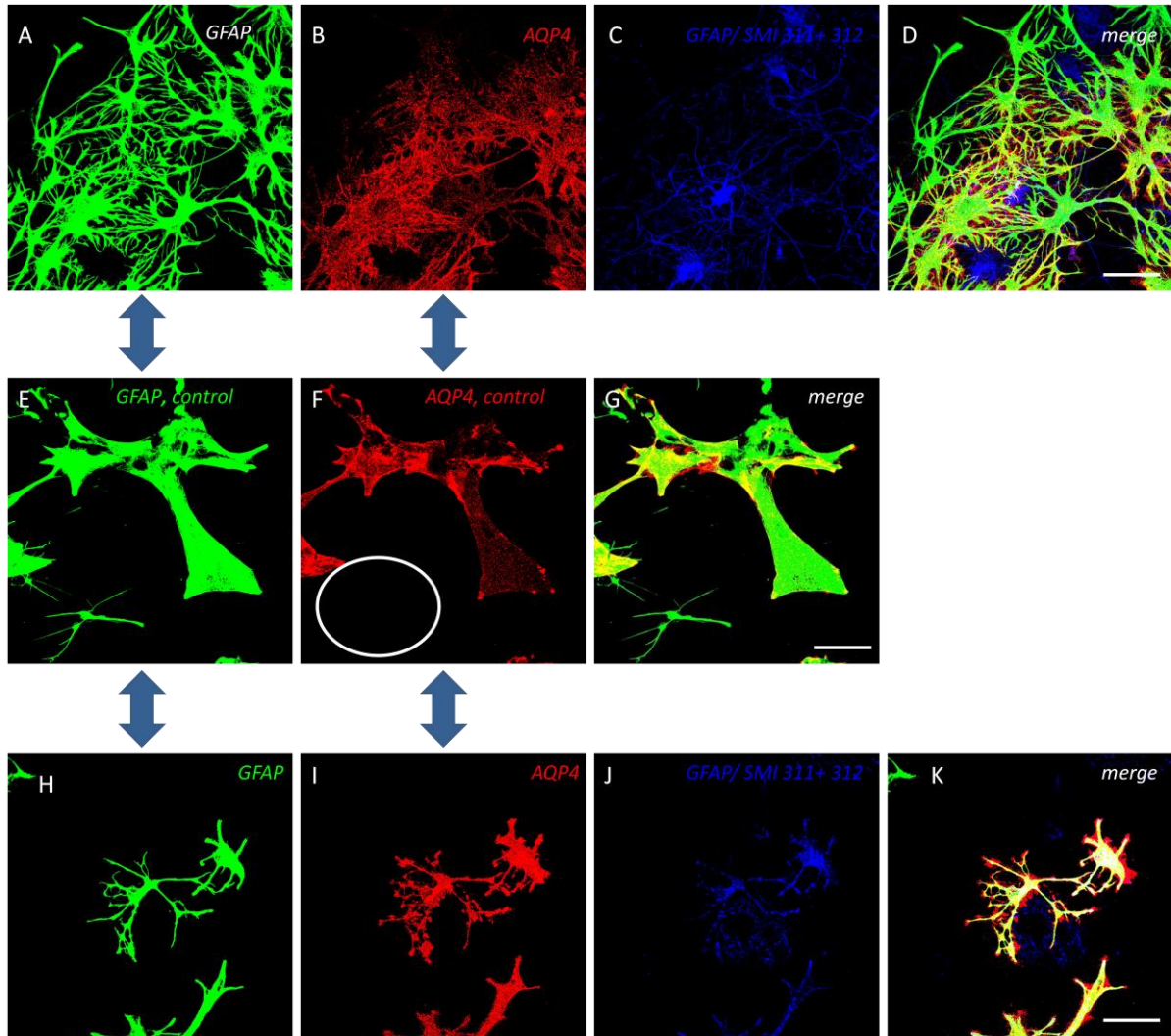


Figure 29: A- D: astrocytes in coculture with neurons for 2 weeks; H- K: astrocytes in coculture with neurons for 72 hours. E- G: astrocytes cultured without neurons. (A), (E), (h) GFAP staining of astrocytes; (B), (F) and (i) anti- AQP4 staining; (C) and (j) are stained for GFAP and SMI312/311 as their primary antibody derives from the same species; (D) and (K): merged pictures. Scale bar: 50µm.

This finding is supported by immunohistochemical stainings. Rats reveal a more intense AQP4 immunoreactivity in the grey matter, where neuronal cell bodies are located, than in the white matter, see fig. 30. Is the driving force for enhanced AQP4 expression and the alternation of cell morphology and AQP4 level *in vitro* also deriving from neurons *in vivo*?

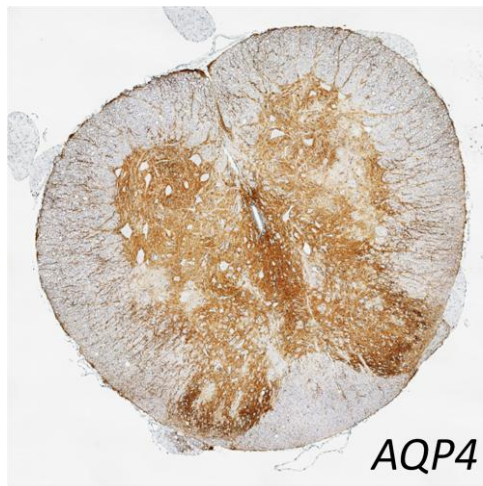


Figure 30: AQP4 staining pattern of a rat spinal cord.

Is the driving force for these effects agrin? Neurons do synthesize agrin isoforms that are inserted into the membrane and some, that are secreted into the extracellular matrix (Burgess et al., 2000), in our case into the cell medium. We speculate that the neuronal isoform of agrin Y4Z8 might be responsible for the alternation of AQP4 expression and astrocyte morphology *in vitro*.

To test this, the next step was to culture astrocytes with medium that was conditioned with agrin. For this reason seven to ten days old astrocytes from newborn Lewis rats were cultured for up to two weeks with medium conditioned with agrin isoform Y0Z0 or Y4Z8 and control medium without supplemental agrin. Four independent tests were performed from different cultures for several incubation times: 3 days, 7 days, 10 days and 14 days.

We could detect several cells with a "focal clustering" of AQP4 immunoreactivity on astrocytes when treated with both agrin isoforms. But, this was also the case in many non- treated cells. No difference in AQP4 polarization could be seen in agrin Y0Z0 or Y4Z8 treated astrocytes in comparison to the control, independently from the time of treatment. Therefore neuronal and endothelial cell derived agrin might not be the protein responsible for the upregulation of AQP4 and for the morphology changes seen in astrocytes cocultured with neurons. Therefore we did not ascribe these changes to the action of agrin in the medium.

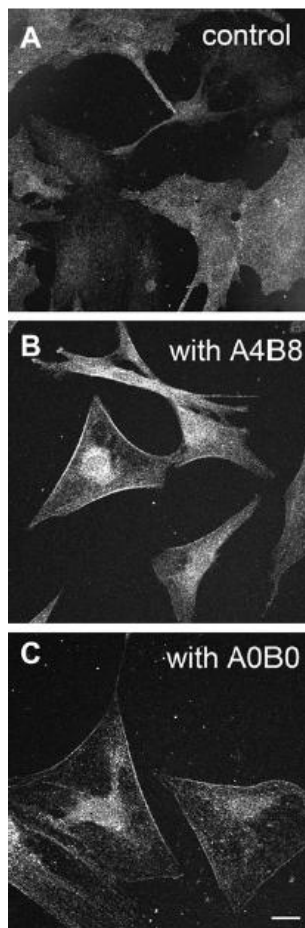


Figure 31: Astrocytes in culture: (A) control, (B) treated with agrin A4Z8 and (C) treated with agrin A0B0 ; Figure taken and legend adapted from (Noell et al., 2007)

A comparable test was performed by Susan Noell et al. (2007). They cultured murine wild type astrocytes for 14 days with agrin conditioned medium of both isoforms, Y0Z0 and Y4Z8. They reported an increased immunoreactivity for AQP4 in the presence of agrin Y4Z8 and an increase of the M23 AQP4 mRNA isoform. Figure 32B shows a more intensely stained astrocyte when treated with agrin Y4Z8 and a slightly increased immunoreactivity when treated with Y0Z0. Cultures treated with the endothelial isoform of agrin (Y0Z0) were reported to show no obvious difference in mRNA level of AQP4 and OAP frequency compared to control astrocytes grown without any agrin. Agrin Y0Z0 was regarded to influence the insertions and/ or aggregation of preexisting AQP4 into membranes, rather than the expression of AQP4. In contrast, the density of OAPs could be increased with the neuronal agrin isoform Y4Z8 (Fig. 31) (Noell et al., 2007). In the picture published by Noell et al. the enhanced staining of nuclei in treated cells is conspicuous and could be problematic. We could also detect an increased AQP4 reactivity similar to this when the contrast was enhanced during the process of taking pictures, but not as a result of agrin treatment on astrocytes.

Further on, recent data from the same laboratory of Dr. Hartwig Wolburg showed that the coating with agrin Y0Z0, Y4Z8 and extracellular matrix protein (ECM, from mouse sarcoma) could induce an increased AQP4 immunoreactivity in cultured astrocytes, fig. 32. They reported to detect the strongest increase of AQP4 staining in agrin- null mice and to a lesser extend in wild type mice when coated with ECM and agrin Y4Z8. No difference was detected when wild type and agrin- null mice were treated with Y0Z0 in comparison to untreated cells (Fallier-Becker et al., 2011).

In fig. 33 C to H there is no difference in AQP4 immunoreactivity between all treated astrocytes. The staining intensity of Y4Z8 and ECM treated cells is not increased in comparison to Y0Z0 as it was described. They all show a punctuated AQP4 staining with a patchy staining of the cell bodies and a stronger AQP4 staining at the borders of astrocyte processes of treated cells.

Further on, there is a striking difference between control cells and agrin Y0Z0 treated cells in the depicted examples. The authors characterized the untreated and Y0Z0 treated cells of wild type and agrin- null murine astrocytes to have the same immunoreactivity, which is not visible in the figure. These findings are also contradicting to our data. We could also see a punctuated AQP4 staining and a focal AQP4 upregulation at membrane borders in untreated cells rather than its induction by supplemental agrin.

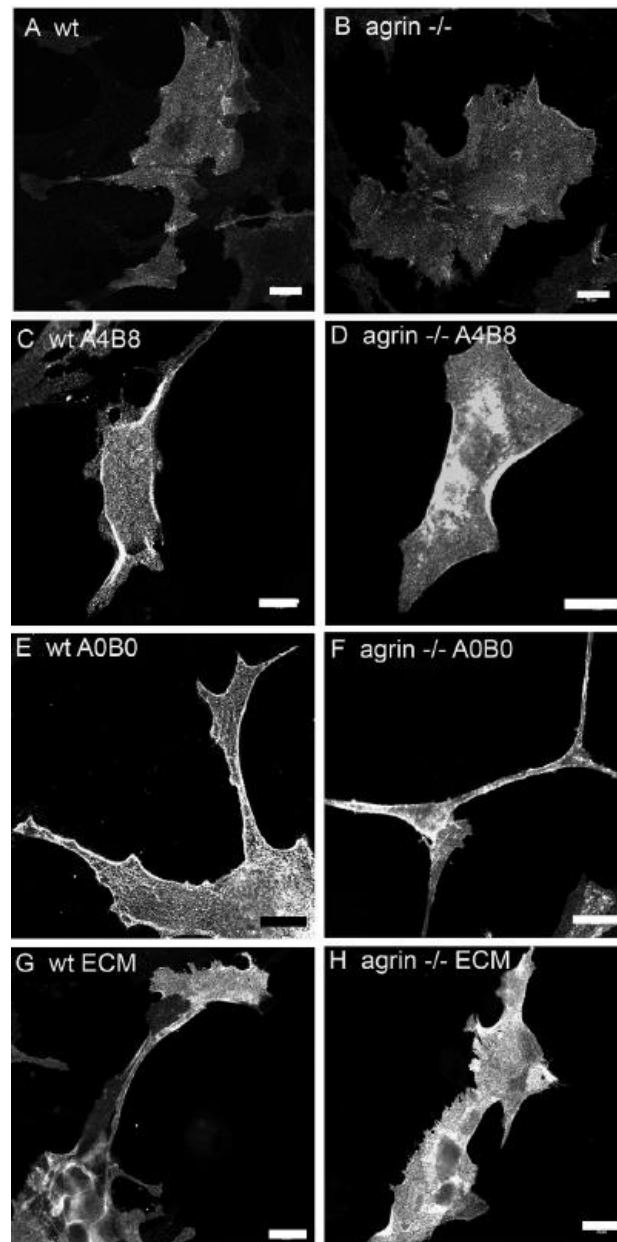


Figure 32: The impact of agrin and ECM on the AQP4 immunoreactivity in cultured astrocytes. A,C,E,G are wild type astrocytes; B,D,F and H agrin null mice. A and B did not have any treatment, C and D were treated with agrin isoform Y4Z8; E and F were treated with agrin isoform Y0Z0 and G and H were treated with ECM; Figure taken and legend adapted from (Fallier- Becker et al., 2011).

Our findings of agrin treatments of astrocytes might be supported by the findings of Noell et al. (2009) in regard of agrin dependent AQP4 clustering. The OAP polarity detected by freeze fracturing in astrocytic end feet was only given when agrin was present in the basal lamina of wild type mice. But, they found comparable subpial AQP4 immunoreactivity in histological stainings and no difference in the AQP4 immunoreactivity in cultured astrocytes of both, agrin- null mice and wild type mice. Additionally, no difference in the expression level of AQP4 mRNA and AQP4 protein level was detected when agrin was absent. They presumed that the localization and amount of AQP4, but not of OAPs, in astrocytes is independent on the presence of agrin. This is in agreement with our findings that agrin in the medium of astrocytes showed no effect on the distribution of AQP4.

This leads to the conclusion that there might be different mechanisms in OAP and AQP4 location and clustering. The question about the mechanisms that cluster AQP4 and about the function of AQP4 in OAPs still remains to be solved.

When agrin does not cause the clustering of AQP4 at the superficial and perivascular glia limitans, which candidates remain? Several studies suggest that the site specific anchoring is dependent on members of the dystrophin- dystroglycan- complex (DDC) (Neely et al., 2001; Amiry-Moghaddam et al., 2003; Amiry-Moghaddam et al., 2004; Bragg et al., 2006), because in dystrophin negative *mdx* mice AQP4 expression is markedly reduced and in α - syntrophin knockout mice, the perivascular and subpial pool of AQP4 is lost (Yokota et al., 2000; Neely et al., 2001).

α - syntrophin colocalizes with AQP4 in the brain and in muscle. Both proteins are detectable by co-immunoprecipitation (Inoue et al., 2002). Interestingly, in α - syntrophin- null mice, where the encoding gene was disrupted, other AQP4 pools than the perivascular or subpial were not altered in the brain (Neely et al., 2001) and AQP4 was redistributed on the whole cell membrane (Amiry-Moghaddam et al., 2003). This indicated a crucial role for members of the DDC in AQP4 localization.

This is also supported by findings in glioblastomas, where AQP4 is upregulated and at the same time OAPs decrease in number (Neuhaus, 1990).

2002 Rascher et al. reported for the first time that agrin is lost from endothelial basal laminae in glioblastomas. The underlying mechanism of this phenomenon is not known (Rascher et al., 2002).

In 2004 Warth et al. suggested that agrin might be important in AQP4 clustering, because OAPs were redistributed and no longer polarized at astrocytic end- feet when agrin was absent in perivascular basal lamina of central areas of glioblastoma, detected by means of immunohistochemistry. OAP density in the end feet of glioblastomas was even lower than in normal parenchymal astrocytes. AQP4 immunoreactivity in glioblastomas was increased and distributed over the whole cell surface

(Warth et al., 2004). This suggests that the loss of agrin results only in a loss of OAP polarization and AQP4 is still present in a non- OAP associated form.

But, agrin was not the only protein impaired. In central areas of glioblastomas, α - dystroglycan and α - syntrophin were redistributed and no longer polarized. It was not possible to determine the primary event or protein that caused the redistribution of OAPs over the cell membrane, because all of these proteins are involved in site specific anchoring.

5 years later Wolburg- Buchholz et al. (2009) analysed the distribution of AQP4 and members of the DDC in mice with experimental autoimmune encephalomyelitis (EAE). They found a loss of polarized AQP4 and the potassium channel Kir4.1 accompanied by edema formation and a loss of β - dystroglycan (β DG) (Agrawal et al., 2006) where CD45 positive cells were present in the perivascular space. The immunoreactivity of AQP4 was increased during the increase of severity of EAE, like it is the case in MS. The agrin staining in animals with severe EAE revealed that it was lost from basal laminae of endothelial cells, but not in the basal laminae of glial cells where it was stable.

When agrin was present and β DG was lost, AQP4 was redistributed over the cell surface of astrocytes. The number of OAPs did not decrease, but OAPs distributed over the whole cell. The authors concluded that agrin might be responsible for the aggregation of AQP4 in OAPs and that DG might be responsible for targeting the OAPs to astrocytic end feet (Wolburg- Buchholz et al., 2009).

It remains to be clarified whether agrin clusters AQP4 into OAPs and which protein causes the polarized distribution. There is much evidence that one or more than one protein of the dystrophin- dystroglycan- complex might be crucial for clustering AQP4 at the superficial and perivascular glia limitans.

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