MASTERARBEIT

Titel der Masterarbeit

„Amigo2 – a Novel Neuroprotective Molecule produced by Microglia Cells"

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Abstract

Microglia are the resident immunocompetent cells of the central nervous system (CNS) and their role is to support and maintain the proper function of neuronal cells. Inflammation is often considered as a destructive process and a mediator of neuropathology, but it is also needed to induce an appropriate healing response. During a TH1-cell driven inflammation, the proinflammatory cytokine Interferon-γ (IFN-γ) is released, which leads to an activation of microglia cells, which precedes the action of the other cell types.

Former reports have shown that a TH1-cell based inflammatory reaction has a beneficial effect on the recovery of spinal cord injuries (SCI). This raised the question, whether this positive outcome may be based on factors produced by IFN-γ activated microglia cells. To address this point, we treated microglia cell cultures with IFN-γ and looked for a differential expression of potential neuroprotective molecules. We found an adhesion molecule named Amigo2 to be upregulated. Coating of the recombinant extracellular domain of Amigo2 on culture dishes induced neurite outgrowth in hippocampal neurons, and in solution, the protein interfered with the adhesion properties of microglia cells. We also found Osteopontin (SPP1) to be prominently downregulated in IFN-γ primed microglia. The addition of Osteopontin into the medium of hippocampal cultures partly inhibited neurite outgrowth.

We therefore suggest that the differential expression of those two molecules in IFN-γ treated cells can contribute in promoting the regeneration of spinal cord nerves after injury.

Key words: Amigo2   Osteopontin   spinal cord injury   IFN-γ   microglia
              neurite outgrowth   inflammation   synapse stripping

Zusammenfassung

Mikroglia ist eine im zentralen Nervensystem (ZNS) ansässige, immunologisch aktive Zellgruppe, deren Aufgabe es ist, Nervenzellen zu schützen und funktionsfähig zu halten. Entzündungen im ZNS werden meist als ein zerstörerischer Prozess angesehen, der zu Schäden im Gewebe von Hirn und Rückenmark führt. Dabei wird häufig nicht berücksichtigt, dass eine Entzündungsreaktion von Nöten ist, um eine angemessene Heilung und Regeneration des Gewebes zu entfachen. Während einer durch TH1-Zellen ausgelösten Entzündung, kommt es zur verstärkten Ausschüttung des proinflammatorischen Zytokins Interferon-γ (IFN-γ), welches die Mikroglia Zellen aktiviert.

Frühere Literaturhinweise belegten, dass TH1-Zell vermittelte Entzündungsreaktionen positive Auswirkungen auf die Nervenregeneration nach Rückenmarksverletzungen haben. Können diese Auswirkungen auf Faktoren zurückgeführt werden, die von IFN-γ aktivierten Mikroglia Zellen produziert werden?

Um diese Frage zu beantworten, haben wir Mikroglia-Kulturen mit IFN-γ behandelt und nach einem veränderten Expressionsmuster von neuroprotektiven Molekülen gesucht. Dabei zeigte sich, dass ein Adhäsionsmolekül namens Amigo2 hochreguliert wird. Wir konnten zeigen, dass eine substratgebundene Form der extrazellulären Domäne des rekombinanten Proteins in hippokampalen Neuronen-Kulturen das Auswachsen von Neuriten induziert. Die Zugabe desselben rekombinanten Proteins in den Überstand von Mikroglia-Kulturen führt zu einer Veränderung der Adhäsionseigenschaften dieser Zellen. Eine Behandlung von Mikroglia Zellen mit IFN-γ führte auch zu einer geringeren Expression des Proteins Osteopontin. Zugabe rekombinanten Osteopontins in das Medium von Neuronen-Kulturen führt zu einer teilweisen Unterdrückung des Auswuchses der Neuriten. Diese beiden Befunde belegen eindeutig, dass IFN-γ behandelte Mikroglia Zellen dazu beitragen können, das Auswachsen von Nervenfasern nach Rückenmarksverletzungen zu fördern.

1   Introduction

Microglia cells are the primary immune effector cells of the central nervous system (CNS) and respond rapidly to any kind of CNS damage or disease. Up to 20% of the total glia cell population account for microglia, which are ubiquitously distributed in the CNS in non-overlapping territories (Lawson et al., 1990).

Microglia were first described under the name "Stäbchenzellen" (rod cells) by Franz Nissl in 1891. Pio Del Rio-Hortega first noticed their important role in CNS pathology and invented the name "microglia" (1932). The cells differentiate from hematopoietic stem cells and enter the CNS early in development. They populate the CNS in two different waves, the first consisting of microglia progenitor cells originating from the yolk sac, which then can be found in the brain rudiment very early during development (Alliot et al., 1999). The second consists of bone marrow-derived myeloid progenitor cells (Ritter et al., 2006), entering the brain via the *corpus callosum* and then further differentiate into microglia. But, to date it remains unclear, whether the second wave of progenitor cells can give rise to long lived microglia cells at all.

During adulthood, there is little or no replacement of parenchymal microglia in the CNS and further amplification is achieved by local proliferation. This self-renewal capacity is not a characteristic of fully differentiated cells, and microglia cells can be seen as partially, but not completely differentiated along the myeloid lineage. As uncommitted myeloid progenitors they can give rise to immature dendritic cells or macrophages, depending on the signals they receive from their environment (Santambrogio et al., 2001). Unlike this stable pool of parenchymal microglia, perivascular monocytes, which are located at the blood vessels and enclosed within the vascular basal lamina seem to have a much higher turnover rate with bone marrow derived cells as indicated by bone marrow chimera experiments (Lassmann et al., 1993; Hickey and Kimura, 1988). The perivascular monocytes are further differentiated, exhibit a high constitutive expression of major histocompatibility complex (MHC) class II antigens, are potentially phagocytic and can be seen as the first line of defense against any pathogens in the blood (Kreutzberg, 1996). Microglia are very sensitive and can get activated

even in response to minor pathological changes, like subtle alterations of the microenvironment in the CNS. They are capable of detecting changes in the extracellular ionic milieu, express receptors for signaling molecules like the neurotransmitter noradrenaline (Whittemore et al., 1993), ATP (Honda et al., 2001) or the calcitonin gene related peptide (CGRP) (Priller et al., 1995), all characteristics which may contribute to an early and rapid activation of microglia cells, that precedes the reaction of any other cell type in the CNS. Being activated, microglia undergo morphological and functional changes, which vary depending on the received stimulus.

In the healthy brain, when there are no foreign materials or dying cells, the ramified microglia are equally distributed. Although this type of cells is commonly referred to as "resting" microglia, they do show activity and continuously survey their environment with highly motile processes and thus are able to screen the complete brain parenchyma (Nimmerjahn et al., 2005). Microglia processes were also found to be in direct contact with astrocytes, neurons and blood vessels and they probably interact dynamically with other cortical elements in the healthy brain. When receiving any signal of brain damage or foreign material, ramified microglia become rapidly activated and respond with morphological and functional changes. Local laser lesions of capillaries causing a disruption of the blood-brain barrier (BBB) led to an activation of nearby microglia, characterized by a switch from undirected to a targeted movement of their processes towards the lesion site. The same behavior of microglia processes can be observed in response to a local lipopolysaccharide (LPS) application (Nimmerjahn et al., 2005). It has been shown in the intact mouse brain that this directional convergence of microglia processes towards the site of injury can be mimicked by a local injection of ATP and thus, extracellular ATP may be an important regulator of this rapid chemotactic response (Davalos et al., 2005). ATP can stimulate microglia via the purinergic receptor P2Y and can induce chemotaxis, membrane ruffling and ramification of cultured microglia (Honda et al., 2001).

Microglia's function is to monitor and protect the CNS. In order to fulfill these tasks, they have to be able to detect any abnormalities in their environment or to sense the normal activity of surrounding cells, like the firing activity of neurons and the corresponding release of neurotransmitter. The inhibition of neuronal firing can cause the upregulation of MHC class II molecules in microglia in the presence of IFN-γ, indicating that healthy neurons

participate in the suppression of MHC class II expression in microglia of the intact CNS (Neumann et al., 1996). Another form of cellular communication between neurons and microglia in the healthy brain is mediated by CD200, expressed in several cell types including neurons and its receptor CD200R present on microglia, whereby interactions are thought to provide a downregulatory signal (Broderick et al., 2002). These properties could therefore lead to an activation of microglia, if the neuronal activity is impaired or the cellular contact disrupted. Microglia also express receptors for neurotransmitters like the β_2-adrenoreceptor which may play a role in regulation of microglia cyto- and chemokine release (Prinz et al., 2001).

Microglia are among the first cells to react to CNS injuries and their reaction pattern shows a graded response. They are readily activated in the course of CNS infection, inflammation, traumatic, neoplastic, ischaemic and degenerative conditions. Activation becomes apparent by proliferation, recruitment to injury site, morphological, immunophenotypical and functional changes (Kreutzberg, 1996). Immunophenotypical changes include the expression of MHC class II, which is normally only found on perivascular macrophages outside of the CNS parenchyma (Hanisch, 2002). Activated microglia can release cytotoxic compounds like reactive oxygen species, which can be potent effectors of cellular injuries (Sankarapandi et al., 1998). Furthermore microglia are a major source and/or target of a huge variety of cytokines and chemokines (Hanisch, 2002). Their response to pathologic events is stimulus-specific and can vary from neuroprotective to toxic actions. Contact with bacterial material drives microglia to become phagocytic and to produce and release proinflammatory molecules (Hanisch et al., 2001). On the other hand, while removing apoptotic cells and debris, which might be harmful to the surrounding tissue, microglia cells decrease the release of proinflammatory cytokines (Magnus et al., 2001).

Excess glutamate has been shown to be an important neurotoxic substance in many neurodegenerative diseases. Under physiological conditions glutamate is predominantly removed by astrocytes, but under some pathological conditions, when astrocytic glutamate uptake is impaired, activated microglia can express the glutamate transporter GLT-1 and thus are able to protect neurons by reducing the excitatory stress (Persson et al., 2005).

Microglia cells express a variety of cytokines and growth factors, which play a role in tissue repair and promote the survival of neurons like Interleukin 6 (IL-6), IL-1 (Woodroofe et al., 1991), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-4/5 (Miwa et al., 1997).

CNS inflammation is often considered to be a mediator of secondary tissue damage and neuropathology and some reports showed a promoted recovery from spinal cord injuries (SCI) in rats in response to the antiinflammatory compound methylprednisolone (MP) (Constantini and Young, 1994). But there were also reports showing, that a well controlled local inflammatory response can have beneficial effects on the recovery of SCI. To review inflammation as an only positive or negative process is most likely an oversimplification regarding regeneration after SCI and probably different factors are beneficial at different time points after the injury. However, rats with a SCI, that were simultaneously injected with autoimmune T-cells against myelin basic protein (MBP) causing a local inflammation at the site of injury, showed a reduced spread of damage and significantly greater recovery (Hauben et al., 2000).

After an incomplete SCI, not only the directly injured neurons degenerate, but also the spared neurons suffer under secondary degenerative processes, caused by excess amount of secreted neurotransmitters and free radicals, as well as the lacking of neurotrophins and by poor regeneration. Recovery from those injuries largely depends on the protection of still viable neurons from secondary damage and the regeneration of severed processes (Hauben and Schwartz, 2003). Inflammation in rats caused by immunization with MBP or by the adoptive transfer of MBP specific CD4^{+} T-cells is mainly appending on TH1-cells, which are a major source of IFN-γ (Liblau et al., 1995). This leads to an activation of microglia cells at the lesion site. Immunohistochemical analysis of the spinal cord lesions showed, that enhanced recovery was correlated with an early rise in the numbers of activated microglia/macrophages (Hauben et al., 2003). Microglia might contribute to the neuroprotective effect by clearing dead cells and cell debris, buffering toxic compounds like abundant glutamate and secreting neurotrophic factors (Fig.1).
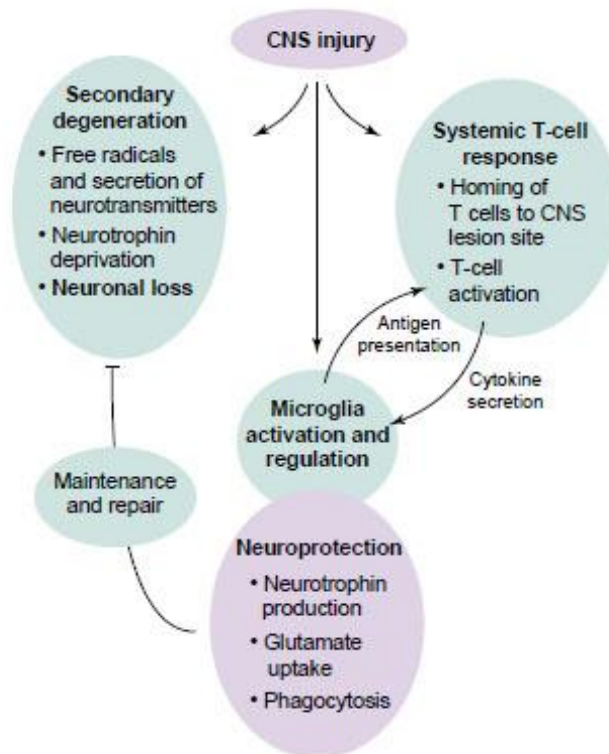
Figure 1: Cell-mediated immune responses affect the outcome of CNS injury. CNS injury activates both, protective and destructive mechanisms. Injury to CNS nerves induces secondary degenerative processes and a systemic T-cell response. T-cells home to the injury site, encounter the relevant antigen and are activated. Activated T-cells can regulate protective inflammatory processes at the injury site by secreting neurotrophic factors and cytokines that regulate microglia buffering activity. Regulation of both, the extent and the timing of the immune response results in less neuronal loss and improved functional outcome (Figure and legend taken from Hauben and Schwartz, 2003).

IFN-γ is a potent microglia activator and leads to a differential expression of many immunological relevant genes. Recent gene expression studies performed in our laboratory just before I started my master thesis revealed some genes, that might also contribute to the beneficial effect of a local inflammatory response on SCI. IFN-γ treated microglia showed a strong upregulation of a molecule called Amigo2 (14.8 fold), as well as a strong downregulation of Osteopontin (SPP1) (27.6 fold) when compared to untreated control cells (Fig.2).
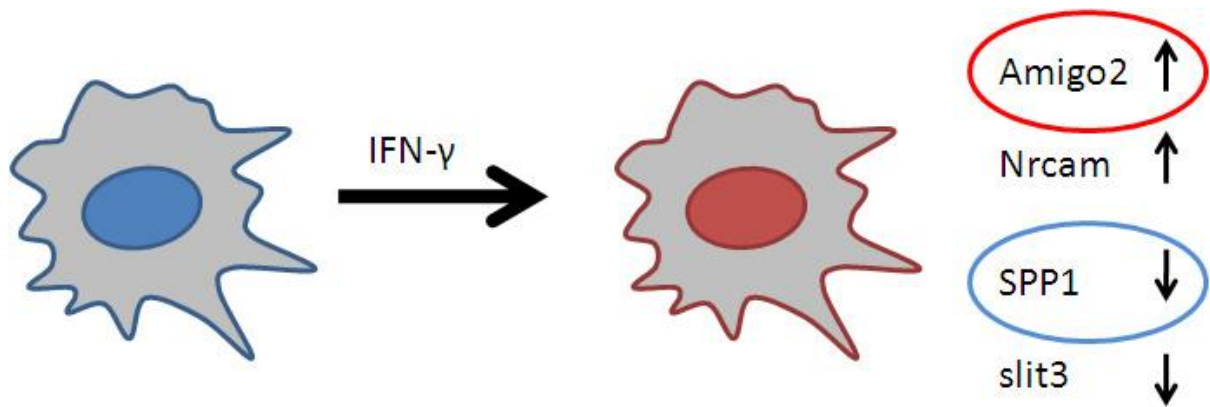
Figure 2: Differentially expressed genes in IFN-γ primed microglia cells.

Amigo2 is one of three members of a gene family called "amphoterin-induced gene and ORF" (AMIGO), induced in neurons by the neurite-promoting factor amphoterin (Kuja-Panula et al., 2003). Other synonyms referring to the same gene are "adhesion molecule with immunoglobulin (Ig)-like domain 2", "activity-dependent leucine-rich-repeat and Ig superfamily survival-related protein" (Alivin-1 or Ali-1) (Ono et al., 2003) or "differentially expressed in human gastric adenocarcinomas" (DEGA) (Rabenau et al., 2004). Amigo, Amigo2 and Amigo3 are all coding for a type1 transmembrane protein and share the same domain organization with a putative signal sequence for secretion, six leucine-rich repeats (LRRs), flanked by two cysteine rich LRR domains (LRRNT and LRRCT) and one immunoglobulin domain next to the transmembrane domain (Fig.3) (Kuja-Panula et al., 2003). In contrast to that, analyses of the AMIGO2 sequence done by Rabenau et al. resulted in five LRRs, whereas Ono et al. found seven (Rabenau et al., 2004; Ono et al., 2003). The cytosolic part does not contain any known protein domains, but contains a relatively high amount of serines or threonines, which might play a role in signal transduction mediated by phosphorylation (Rabenau et al., 2004).
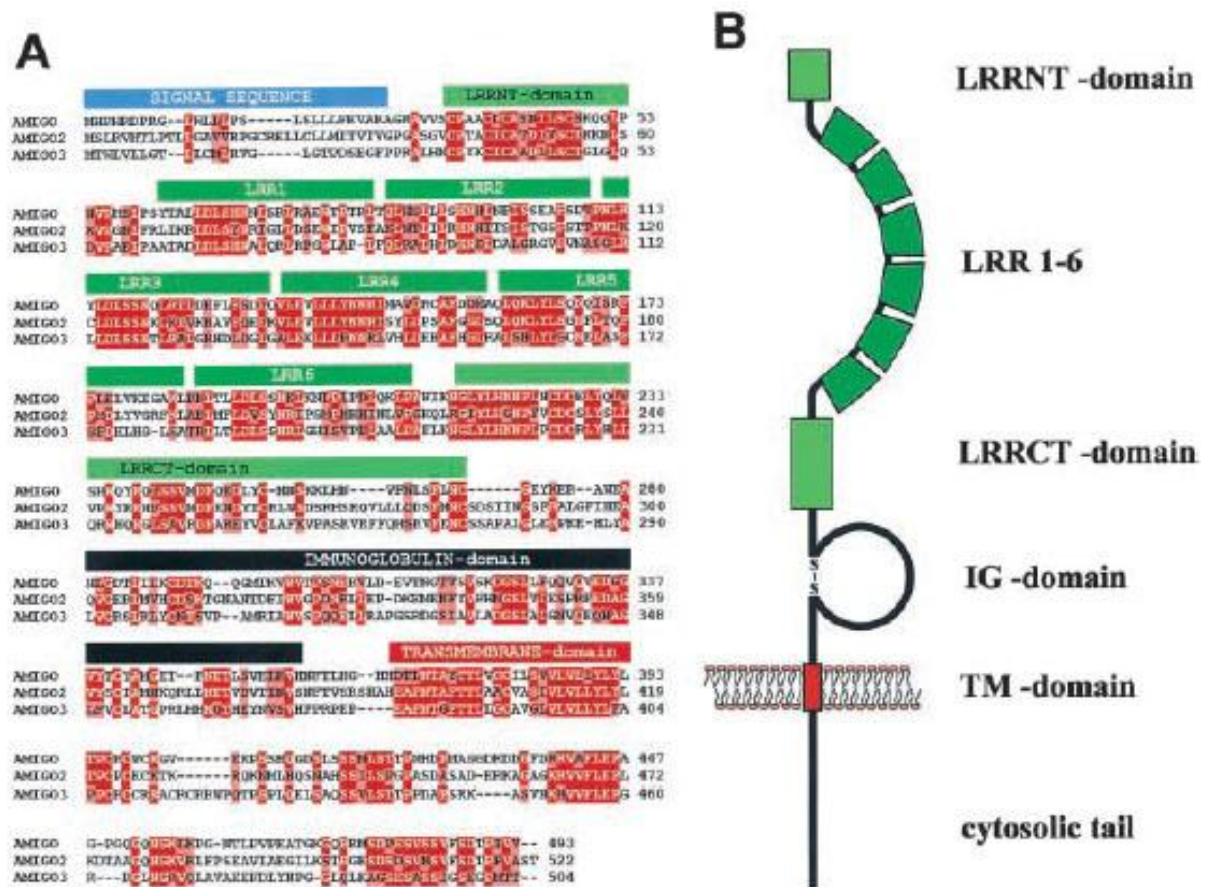
Figure 3: Primary structure of human Amigo, Amigo2 and Amigo3. A) Alignment of the three Amigo proteins. Identical amino acids are highlighted in red with white letters, similar amino acids are highlighted in red with black letters. B) Schematic view of the structure of the three Amigo proteins. (LRR, leucine-rich repeats; LRRNT, LRR NH$_2$-terminal domain; LRRCT, LRR COOH-terminal domain; IG, immunoglobulin; TM, transmembrane) (Figure and legend adapted from Kuja-Panula et al., 2003)

Amigo2 expression in adult mouse tissue is most prominent in cerebellum, retina, liver and lung, but also present in the cerebrum, e.g. in pyramidal and granule neurons of the hippocampus. In comparison to Amigo2, Amigo is even more restricted to the CNS where it is specifically expressed on neuronal fiber tracts, but Amigo3 expression shows no specific pattern (Kuja-Panula et al., 2003). Here we show that also microglia cells express Amigo2 and that this expression is upregulated by IFN-γ. Amigo2 expression in cortical neurons is activity dependent and promotes neuronal survival. Overexpression of Amigo2 in cerebellar granule neurons inhibited neuronal cell death in apoptosis-inducing conditions (Ono et al., 2003). For Amigo it has been shown that it promotes the outgrowth and can interfere with the fasciculation of neurites of hippocampal neurons (Kuja-Panula et al., 2003).

Amigo proteins are able to bind themselves through a homophilic binding mechanism and are also capable of binding the other family members in a heterophilic way (Kuja-Panula et al., 2003). These binding mechanisms may contribute to the possible role of Amigo in the fasciculation of neurites.

Proteins of the immunoglobulin superfamily, as well as extracellular proteins containing LRRs, have been shown to participate in neurite outgrowth, synaptic plasticity and axon guidance (Schachner, 1997; Battye et al., 2001). BLAST searches done by Kaju-Panula et al. show similarities of Amigo with axon guidance proteins of the Slit family (Slit1) and with the Nogo-66 receptor, especially in the LRR regions (2003). Slit as a repellent axon guidance cue is secreted by the floor plate and can bind to the midline receptor Roundabout (Robo). The binding of Slit to Robo and the corresponding repellent signal requires the LRR region of Slit (Battye et al., 2001). The extracellular domain of Nogo-A (Nogo-66) inhibits axonal extension by binding the brain-specific, GPI-linked Nogo-66 receptor which contains eight LRRs and a LRR C-terminal domain (Fournier et al., 2001). Similarities to the extracellular domain of Amigo2 are also found in proteins of the human Trk family, which are neurotrophin receptors (Ono et al., 2003).

Osteopontin is a secreted extracellular phosphoprotein which is expressed in many tissues during inflammation. Osteopontin can be seen as an adhesion molecule and as a pleiotropic cytokine. The protein acts on a number of different cell types by binding to several integrins α_v (β_1, β_3 or β_5) and (α_4, α_5, α_8 or α_9) β_1 as well as to different isoforms of the cell surface protein CD44 (Denhardt et al., 2001). Some of its features depend on the Arg-Gly-Asp (RGD)-containing motif, which is able to directly bind to multiple integrin receptors (Denhardt et al., 2001; Meller et al., 2005).

Osteopontin plays a role in diverse biologic functions including inflammation, recruitment of macrophages and leucocytes, regulation of T-cell response, cell survival and migration. But besides its proinflammatory actions, Osteopontin shows also antiinflammatory properties and its net effect depends on the environment.

Osteopontin affects T-cell survival and inhibits cell death by regulating the expression of proapoptotic proteins. In multiple sclerosis, an inflammatory and demyelinating disease in the brain and spinal cord, it is increased in the plasma of patients during relapses (Hur et al., 2007). Osteopontin deficient mice (OPN$^{-/-}$) exhibit less inflammation and produce less

proinflammatory cytokines like IL-6, TNF-α or IFN-γ (Hashimoto et al., 2007). Rats, that were paralyzed from experimental autoimmune encephalomyelitis (EAE) showed increased Osteopontin transcripts in spinal cord. Also, OPN$^{-/-}$ mice were resistant to progressive EAE and recovered more rapidly from the disease than the wild type (Chabas et al., 2001). But OPN$^{-/-}$ mice also showed a reduced spared white matter in comparison to wild type mice after SCI. This might rely on a potential neuroprotective role of Osteopontin (Hashimoto et al., 2007). Osteopontin can act via different pathways leading to enhanced cell survival, like the reduction of nitric oxide synthesis, activation of protein kinases like Akt, p42/p44 MAPK or the expression of prosurvival genes. (Hwang et al., 1994; Denhardt et al., 2001; Meller et al., 2005). Osteopontin is also upregulated in motorneurons in the spinal cord following spinal root avulsion where it has a neuroprotective effect (Fu et al., 2004).

Furthermore it seems to regulate the TH1 response by enhancing the expression of the cytokines IFN-γ and IL-12 and suppressing the expression of IL-10. Therefore it appears to polarize the T-cell response towards a TH1 cytokine response (Hur et al., 2007; Chabas et al., 2001; Ashkar et al., 2000).

It is known that axons can regenerate beyond the injury site in the peripheral (PNS), but not in the central nervous system. This difference regarding axon regeneration in the PNS and CNS is caused by the molecular environment of the cells, rather than by an intrinsic neuronal program. Transplantation of macrophages preconditioned with PNS tissue can promote regeneration of axons in the CNS (Lazarov-Spiegler et al., 1996). Osteopontin might contribute to this different regeneration capacity of neurons in the PNS and CNS. It is strongly expressed by macrophages at the injury site in the optic nerve (CNS), but not in the sciatic nerve (PNS) and in addition is able to inhibit the outgrowth of axons (Kury et al., 2005).

As mentioned above, recent microarray data from our laboratory showed a differential expression of Amigo2, Osteopontin and some other immunological relevant genes in IFN-γ primed cells. It was my project to investigate whether those two proteins have an effect on neuronal, as well as on microglia cells and to discuss, if they might also play a role in the beneficial effect of a local inflammatory response on the outcome of SCI.

2 Methods and Materials

2.1 Media

2.1.1 Microglia Medium

The following ingredients were added (vol/vol) to RPMI 1640 without L-Glutamine (Lonza Group):

- Fetal Bovine Serum (FBS; Lonza Biowhittaker)……………………………………………10 %
- Penicillin / Streptomycin (10000 u/ml; Lonza Biowhittaker)………………………1 %
- L-Glutamine (200 mM in 0,85 % NaCl; Lonza Biowhittaker)………………………..1 %

The MG medium was then filtered through a presterilized FAST PES Filter Unit (Nalgene) and kept at 4°C.

2.1.2 Neuronal Medium

The following ingredients were added to 1x MEM (Modified Eagle´s Medium) made from a 10x MEM stock (Invitrogen 21430-020) dissolved in ddH_2O:

- 26 mM $NaHCO_3$ (Merck 6329)
- 1 mM sodium pyruvate (Invitrogen 11360)
- 2 mM L-Glutamine (Lonza Biowhittaker)
- 33 mM D-glucose (Merck 8342)
- 2 % (vol/vol) B27 supplement (Invitrogen 17504044)

The medium was then filtered through ROTRAND filter and stored at 4 °C. Before using the medium, it was transferred to the incubator, where the pH adjusts automatically to 7.4 via the CO_2 concentration (5%).

2.1.3 Freezing Medium

The following ingredients were added to RPMI 1640 without L-Glutamine (Lonza Group AG):

- Fetal Bovine Serum (Lonza Biowhittaker)…………………………………………………..45 %
- DMSO (≥ 99 %; Sigma-Aldrich)…………………………………………………………………10 %

2.1.4 HBSS (Hank's Buffered Salt Solution)

Following ingredients were dissolved in ddH_2O and subsequently stored at 4 °C after sterile filtration through a filter:

- 20 mM HEPES
- 2 mM $CaCl_2$
- 5.4 mM KCl
- 1 mM $MgCl_2$
- 136 mM NaCl
- 1 mM Na_2HPO_4
- 5.6 mM D-glucose
- pH was adjusted to 7.3

2.1.5 Trypsin-EDTA Solution

- Use PAA Trypsin/EDTA 1x stock (0.05%/0.02%) and add
- 0.01% (w/vol) HEPES
- 0.1% (vol/vol) Pen/Strep (10000 u/ml; Lonza Biowhittaker)

The Trypsin-EDTA solution was sterile filtered and stored at -20 °C and thawed slowly at 4 °C before being used.

2.2 Animals

All procedures involving animals have been carried out in conformity with the institutional guidelines. Animals had free access to food and water all the time. For the primary mixed glia cell cultures, newborn to two days old Lewis rats were used (males and females). For the neuronal cultures, Sprague Dawley day 17 embryos were used.

2.3 Phase-contrast Microscopy and Imaging

The cells in the tissue culture flasks, dishes and 12-well plates were observed through 10X and 20X objective lenses and phase-contrast optics using an inverted microscope (DM IL, Leica® Microsystems) attached to an SLR CCD Camera (E-300, Olympus).

2.4 Cell Cultures

2.4.1 Microglia Cell Cultures

To prepare a mixed glia culture, newborn Lewis rats were quickly decapitated with a scissor and the heads were cleaned in 70% ethanol. The brains were removed from the head and placed in Dulbecco's Modified Eagle Medium (DMEM; Lonza Biowhittaker). After removing the meninges, the tissue was placed in 10 ml MG-medium and carefully homogenized by pipetting up and down, while trying to avoid air bubbles. The suspension was then transferred to a tissue culture flask, which was coated with Poly-L-Lysine (Sigma-Aldrich) before and placed in the incubator at 37 °C and 5% CO_2, so the cells could attach and later form a monolayer. Each flask contained 10 ml of a cell suspension composed of one brain. 24 h after plating the cells in the flask, the medium was carefully aspirated and replaced by 10 ml of fresh MG-medium. Before the first shake-off around day 8, the medium was again changed every second day. After this time, the cells have formed a confluent monolayer, where the microglia cells are loosely attached to (Fig. 4).
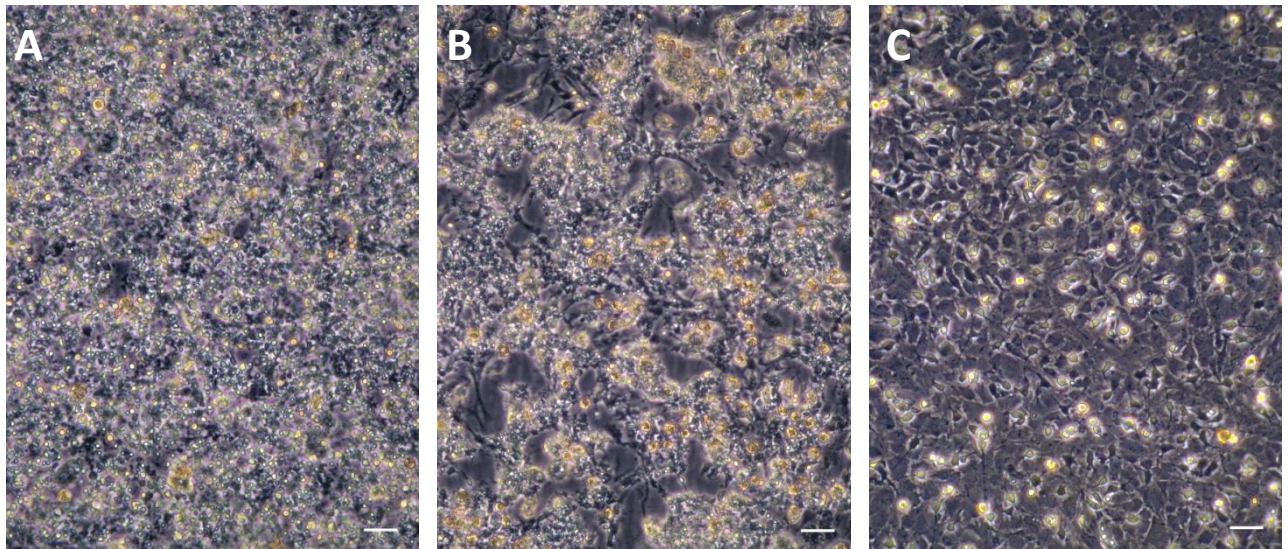
Figure 4: Mixed glia cell cultures in tissue culture flasks at different time points. Cell cultures after: A) 1 day, B) 4 days, C) 7days. Scale bars = 50 μm. Resting microglia cells appear as loosely attached, bright, spherical cells on top of the monolayer.

The microglia cells were then shaken off, by placing the flasks in a rotary shaker at 170 rpm for 4 – 6 h at 37 °C. The supernatant containing the microglia cells was collected in 50 ml falcon tubes and centrifuged at 300 g for 1 min/ml. The supernatant was then completely removed and the remaining cell pellet was redissolved in MG-medium and pipetted on an uncoated Petri dish. 7 ml of cell suspension were used for each dish. The cells were placed in an incubator (37 °C, 5 % CO_2) for 5 – 10 min to allow attachment of the microglia cells to the uncoated Petri dish. Afterwards the microglia culture was carefully washed 3 times with PBS or MG-medium and finally 5 ml of MG-medium per dish were added. Cells were again placed in the incubator overnight, so they could get used to their new environment, before treating them the next morning (Fig. 5).
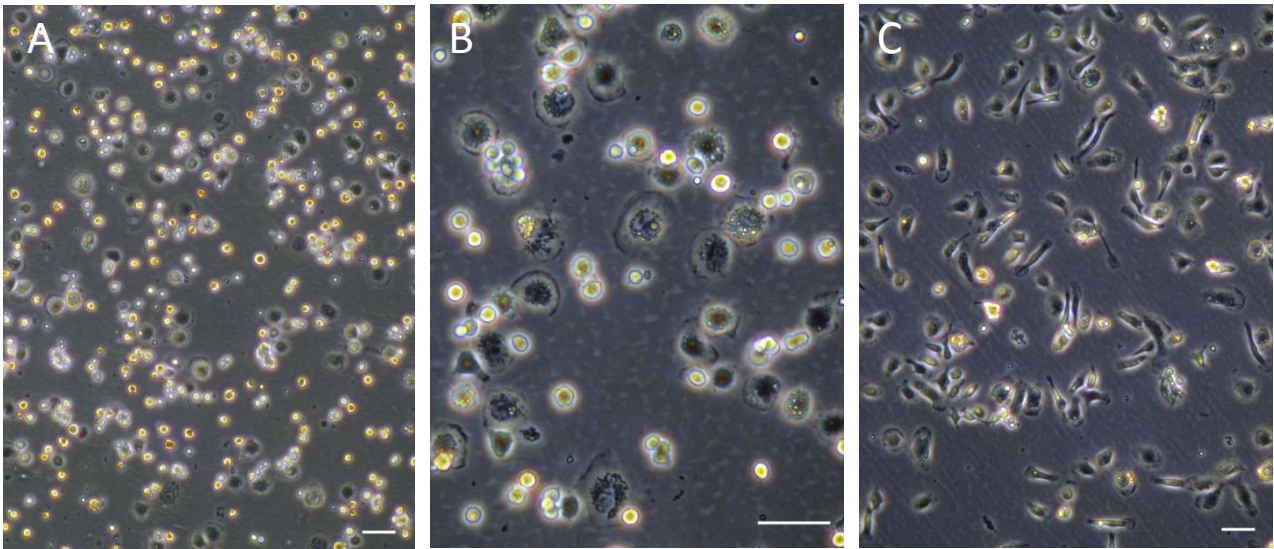
Figure 5: Isolated microglia cells from a mixed glia culture. Microglia cells are able to adhere tightly to the plastic dishes, while other cell types are removed by several washing steps. A) & B) Microglia cells immediately after plating, C) after 12 h in the incubator (37 °C, 5% CO_2). Scale bars = 50 µm

2.4.2 Neuronal Cell Cultures

For the preparation of neuronal cultures, we used dissociated E17 rat hippocampal neurons. Before anesthetizing the rat on day 17 of gestation with CO_2, HBSS (Hank´s Buffered Salt Solution) and trypsin-EDTA were prewarmed to 37 °C in a water bath. The anesthetized rat was killed by cervical dislocation and the abdomen was sterilized with Barrycidal 36. With a scissor, incisions in the skin along the midline were made and the skin was separated from the muscle. The abdomen was again sterilized with 70% ethanol, before it was cut in order to expose the uterus. The entire uterus containing the embryos was taken out and placed in a sterile 10 cm Petri dish. Embryos were removed, decapitated and the brains were placed in prewarmed HBSS. The brain hemispheres were dissected from the brain stem, the meninges were removed and the hippocampi were carefully dissected while trying to avoid removing surrounding tissue. The isolated hippocampi were collected in a 15 ml falcon containing new prewarmed HBSS. After collecting all hippocampi, HBSS was removed and replaced with prewarmed trypsin-EDTA solution and incubated at 37 °C for 10 min. Afterwards, the trypsin-EDTA solution was removed and the tissue was washed 2 times with HBSS before the

hippocampi were dissociated by gently pipetting up and down with a Pasteur pipette. The hippocampi were then further dissociated by pipetting up and down a second time for 1 min with a Pasteur pipette which has had its opening reduced to half of the original diameter. The size of the opening can be reduced by flaming the tip of the pipette for a short time. The cells were counted and the appropriate suspension volume was added to a 6 cm Petri dish containing up to 8 cover slips in 5 ml neuronal medium and placed in the incubator (37 °C, 5% CO_2). 160.000 cells per Petri dish were added (Fig. 6). Alternatively, the neuronal single cell suspension was added to a 12-well plate, which was coated with the proteins of interest before, containing 1 ml/well of neuronal medium.

If the cells are cultured for a longer time period than 7 days, the neuronal medium should be changed after one week, or even better, the cells should be kept together with a glial support culture.
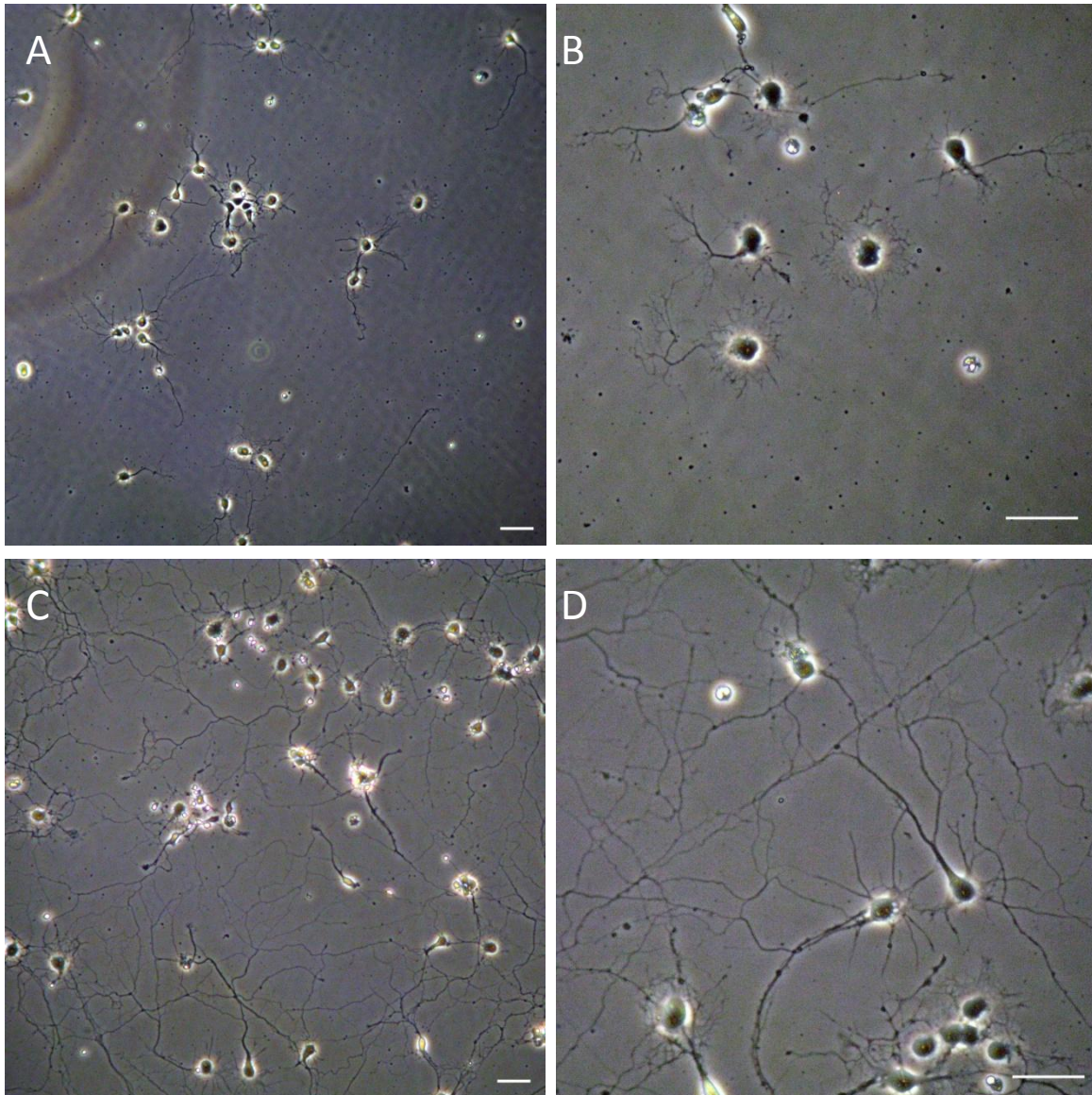
Figure 6: Neuronal cells plated on PLL coated cover slips after: A) & B) 3 days, C) & D) 7 days in culture. Scale bars = 50 μm

2.5   Culture Treatment

2.5.1   Microglia Cell Cultures

Microglia from primary glia cultures were plated on uncoated Petri dishes as described before and placed in the incubator (37 °C, 5% CO_2) overnight, allowing the cells to

acclimatize to their new environment. Cultures were then treated with recombinant Rat IFN-γ (R&D Systems, Cat.nr. 585-IF) in a final concentration of 100 ng/ml for 24 or 48 h. After treatment the medium was aspirated and the cells were washed with PBS at RT to get rid of any remaining culture medium. The cells were then processed for RNA isolation or for protein isolation, both described further on.

Additionally microglia cells were treated with the extracellular domain of recombinant Amigo2 protein in different concentrations, as well as with the recombinant protein Osteopontin (R&D Systems, Cat.nr. 441-OP). Pictures from those treated cultures were taken at different time points before and after treatment, and cells were also processed for RNA isolation. Microglia cells plated on cover slips were also used for fluorescent staining and microscopy.

2.5.2   Neuronal Cell Cultures

Neuronal cultures from embryonic day 17 rat hippocampi were cultured on PLL coated cover slips and also stained for fluorescent microscopy after day 7 in vitro. Additionally the neurons were seeded on recombinant Amigo2 coated plastic wells with different concentrations and the number of outgrowing neurites per cell, as well as their length was determined. Neuronal cultures grown on PLL coated, as well as on Amigo2 coated plastic wells were also treated with SPP1 in different concentrations and again, the number and length of their neurites were determined.

2.6   Neurite Outgrowth Assay

For counting of outgrowing neurites, cells were cultured for 24 h, images were taken from living cells using randomly selected microscopic fields and the extensions, which were twice the length of the cell soma, were considered as neurites. For quantification of neurite outgrowth induced by Amigo2, we used 48 images of every concentration to evaluate the effect on neurite outgrowth. Data were pooled from 3 independent experiments.

For determining the length of the neurites, cells were cultured for 48 h before taking the images. For quantification, 6 images of each concentration were evaluated and the data belong to one experiment.

For determining the effect of Osteopontin on the number of outgrowing neurites, cells were cultured for 24 h on Amigo2 (50 µg/ml) or PLL (100 µg/ml) coated dishes in the presence of soluble Osteopontin in different concentrations (0 – 1000 ng/ml). For quantification, 12 images of each concentration were evaluated and the data was pooled from two independent experiments.

2.7 Preparation of Poly-L-Lysine (PLL) coated Cover Slips

Before starting the procedure, a borate buffer solution was prepared by adding 1.24 g Boric Acid and 1.9 g Borax in 400 ml of ddH$_2$O. PLL hydrobromide powder was dissolved in Borate buffer to a concentration of 1 mg/ml and stored at -20 °C, if not used immediately.

The glass cover slips were placed in a glass beaker containing 1 M HCl and kept for 12 h at 50-60 °C. After the acid cooled down to RT it was rinsed out with ddH$_2$O. The beaker was refilled with fresh ddH$_2$O and sonicated in a water bath for 1.5 h, whereby every 30 min the water was replaced by fresh ddH$_2$O. Afterwards, the container was filled with 50% ethanol and sonicated for 30 min, followed by 30 min sonication in 70% ethanol and finally 30 min sonication in 90% ethanol.

The cover slips were then placed in sterilized plastic racks in a sterile glass tray and put in an oven at 70 °C for at least 1h to dry. Up to 6 dried cover slips were placed in a 60 mm Petri dish. The paraffin beads were melted in a small glass beaker placed on a heating plate at 70°C. The tip of a short Pasteur pipette was dipped into the liquid wax and then quickly touched each cover slip 3 times, leaving a droplet of paraffin each time. Cover slips were coated by adding approximately 100 µl of 1 mg/ml PLL over each and placing the dishes in the incubator overnight. Next day, they were washed with ddH$_2$O once for 5 min and once for 2 h. Dishes were then filled with culture medium and stored in the incubator.

2.8 Coating of 12-Well Plates

To test the ability of Amigo2 to induce the outgrowth of neurites from rat hippocampal neurons, 12-well polystyrene dishes were coated with the extracellular domain of recombinant rat Amigo2 in different concentrations. The dishes were coated with the test protein (0 – 100 µg/ml) in PBS overnight at 4 °C in a wet chamber. 100 µl of each concentration of Amigo2 in PBS was added into the 12-well plate as a drop in the middle of each well. An uncoated cover slip with paraffin dots was mounted on top of the drop (dots pointing down) to ensure an equal distribution of the protein solution in the well and also to slow down evaporation. The next day, the plates were washed 2 times with 1 ml/well PBS, the cover slips were removed and the plates were blocked with 1 ml/well 1% BSA in PBS at RT for 1 h. After washing again 2 times with PBS, 1 ml of neuronal medium was added in each well and the plates were placed in the incubator (37°C, 5% CO_2) at least 1 h before adding the cells.

2.9 Western Blot

2.9.1 Membrane Protein Isolation

Homogenization Buffer: 10 mM Hepes (pH = 7.5)

1 mM EDTA

300 mM Succrose

Dissolve in Milli-Q water

Add 1 tablet Protease Inhibitor Cocktail per 10 ml

Wash Buffer: 10 mM Hepes (pH = 7.5)

1 mM EDTA

Dissolve in Milli-Q water

Add 1 tablet Protease Inhibitor Cocktail per 10 ml

2.9.1.1 Cell Cultures

For microglia cultures, 1 ml of homogenization buffer was pipetted on a 94 mm Petri dish and the dish was placed for 10 min at 4°C on a rotary shaker, before the cells were collected with a cell scraper. Eventually, the leftover cells were collected with additionally 1 ml of homogenization buffer. Cell suspension was transferred into a 2 ml Eppendorf tube and centrifuged for 20 min at full speed. All centrifugation steps were processed in a precooled centrifuge at 4°C. After discarding the supernatant, 1 ml of homogenization buffer was added to the pellet, followed by a short sonication step to redissolve the pellet. The sample was again centrifuged with the same settings and the supernatant was discarded. The pellet was redissolved in 1 ml homogenization buffer by vortexing, followed by another centrifugation step. Afterwards the pellet was dissolved in wash buffer and the protein concentration was determined. After a last centrifugation step, the pellet was dissolved in an appropriate amount of 1x LDS loading buffer.

2.9.1.2 Whole Tissue (Cerebellum)

After removing the meninges, approximately half a cerebellum was transferred into an Eppendorf tube and 750 µl of homogenization buffer was added. The tissue was homogenized by using the sonicator. Subsequent steps were identical with the treatment of cultured cells.

2.9.1.3 Protein Estimation

Protein concentration was determined using the BCA assay kit by mixing the BCA assay reagents A and B in a ratio of 50 : 1 (A : B). We then transferred 2 x 20 µl of each sample (duplicates), and also 20 µl of twelve standards with a given protein concentration, into a 96-well plate and added 200 µl of the BCA assay reagents mix to each well. Before estimating

the protein concentration in a photodetector (PowerWave X 340), samples were incubated for 30 min at 37 °C.

2.9.1.4 Protein Precipitation

To precipitate the protein, samples were diluted to a final volume of 500 µl with Milli-Q water (Millipore) and 500 µl Methanol, as well as 250 µl Chloroform were added and the content was mixed by vortexing briefly. While the samples were centrifuged at 13000 rpm for 5 min at RT, another Eppendorf centrifuge was precooled to 4 °C. After centrifugation the upper phase of the fluid was removed, while paying attention not to disturb the interface region. Before the second centrifugation at 14000 rpm for 30 min at 4 °C, 1000 µl of Methanol were added and the tube was vortexed again. After removing the supernatant, the tube was again centrifuged for a short time and all the remaining fluid was removed, before the pellet was kept at RT for drying. The pellet was then dissolved again with 1x LDS (NuPAGE Lithium Dodecyl Sulfate sample buffer; Invitrogen) to a final concentration of 1 µg/µl.

2.9.2 Gel Electrophoresis

For casting the polyacrylamide gel, two separate gel layers had to be prepared. First the separating gel was prepared by mixing the following ingredients in the respective volumes and order:

10% Seperating Gel (Bis-Tris-Gel)	10 ml
Milli-Q H_2O	4,0 ml
4x Separating-Buffer	2,5 ml
30% Acrylamide/Bis	3,3 ml
10% SDS	100 µl
TEMED	10 µl
10% APS	100 µl

During casting, all ingredients were kept on ice and after adding the 10% APS, the solution was mixed shortly before the separating gel was filled in empty NOVEX gel-cassettes, which were fixed in the mountings (3/4 of the cassette volume was filled; ~7 ml). Gels were covered with 0,1 % SDS (spray bottle) and left at RT for 4 h to polymerize. Before storing the gel at 4 °C in a vacuum bag for later use, the top was filled up again with 0,1 % SDS.

For casting the stacking gel layer the following ingredients were mixed in the respective volumes and order:

5% Stacking Gel (Bis-Tris-Gel)	5 ml
Milli-Q H$_2$O	2,8 ml
4x Stacking-Buffer	1,25 ml
30% Acrylamide/Bis	850 µl
10% SDS	50 µl
TEMED	5 µl
10% APS	75 µl

Before adding TEMED and 10% APS to the stacking gel, the 0,1% SDS on top of the separating gel was removed by washing several times with the 1x Stacking-Buffer and any remaining liquid was removed by using a vacuum pump. After adding TEMED and APS, the solution was shortly mixed in a beaker by pipetting up and down, before the stacking gel was loaded on top of the separating gel layer. Immediately afterwards a 10- or 12-well comb was put in the stacking gel and it was left at RT for at least 30 min to polymerize. After polymerization, the comb was removed carefully and each slot was washed accurately several times with 1x MOPS before removing the remaining liquid with a vacuum pump.

Samples were prepared by adding NuPAGE 4x LDS sample buffer (Invitrogen) and NuPAGE 10x Reducing Agent (Invitrogen) to final concentrations of 1x. Final protein concentration was 1 µg/µl. Samples were heated to 70°C for 10 min and spinned for 2 min at 13000 rpm before finally loading.

The gel setup was then placed in the electrophoresis unit and 250 ml of 1x MOPS running buffer was mixed together with NuPAGE Antioxidant (Invitrogen) to a final concentration of 0,25% . The inner chamber was then filled with this 1x MOPS/Antioxidant and simple 1x MOPS was poured into the outer chamber. Samples were loaded alongside MagicMark

Western Protein Standard (Invitrogen) and the gel was run for 60 min at constant voltage 200 V and approximately 125 mA (per gel).

2.9.3 Immunoblotting

After protein electrophoresis, the separating gel was removed from its encasing cassette and placed in a transfer buffer bath for 15 min. A PVDF/Nitrocellulose membrane (Immobilon-P) was cut to the approximate size of the gel and equilibrated by dipping in Methanol for about 30 s and then in Milli-Q water for 2 min, after which the membrane was placed in transfer buffer for 10 min. Also 2 filter papers cut to the approximate size were soaked in transfer buffer.

The blot was setup according to Figure 7 and ran at maximum of 20 V for 60 min with 58 mA (per gel). After transfer, the membrane was blocked for 60 min in blocking buffer and incubated overnight at 4 °C on a rocking platform with the primary antibody (polyclonal goat antibody, R&D Systems) diluted in blocking buffer at a concentration of 1 µg/µl.
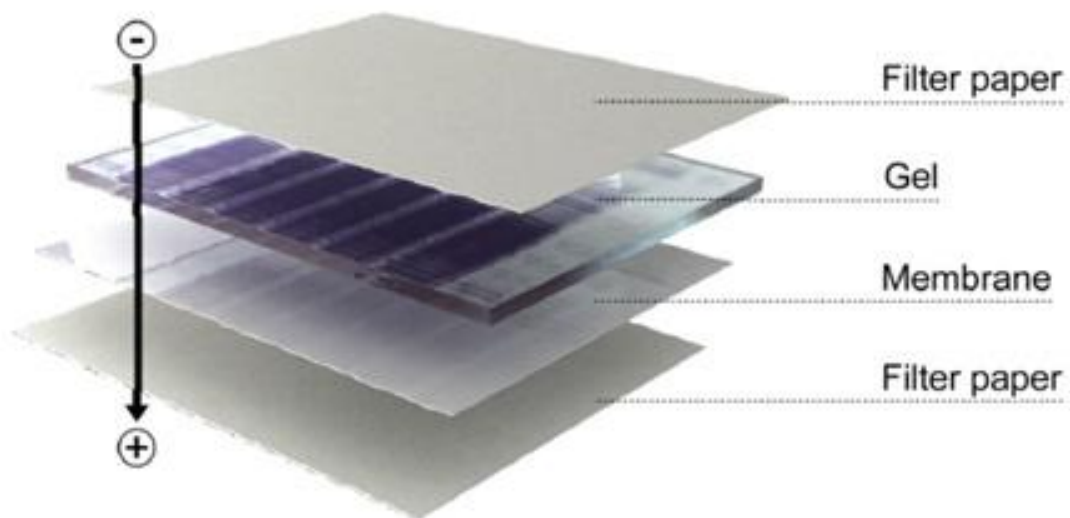
Figure 7: Stack setup for transferring proteins from gel to membrane. Picture taken from Rui Martins.

The membrane was then washed three times with blocking buffer for 15 min at RT and a 1:2000 dilution of the secondary antibody (alkaline phosphatase conjugated anti-goat antibody, Jackson ImmunoResearch) in blocking buffer was prepared. Incubation lasted for 60 min at RT, after which the membrane was washed again three times for 15 min in blocking buffer at RT and then three times for 5 min in CDP-Star Assay buffer. The substrate for detection of alkaline phosphatase was prepared by mixing 10 µl CDP-Star Reagent (New England Biolabs, Inc.) in 1 ml CDP-Star Assay buffer. The membrane was then placed between two plastic foils, and the substrate was pipetted over the whole membrane. After removing the surplus liquid by pressing the plastic foil over the membrane, results were captured using a chemodetector (Fluor-S Multi-Imager).

Blocking Buffer: 15 g milk powder

                      1 litre 1x PBS

                      1 ml TWEEN

Assay Buffer: 250 ml Milli-Q water

                      2,4 ml Diethanolamin

                      250 µl 1M $MgCl_2$

                      Adjust to pH=10 with HCl

2.9.4 Stripping Off

5x Stripping Buffer: 33,5 g Tris

                      150 g SDS

                      Dissolve in 1 l Milli-Q H_2O

                      Adjust pH to 6,8

                      Store at RT

1x Stripping Buffer: 60 ml 5x Stripping Buffer

240 ml Milli-Q H_2O

2310 mg DTT

Mix at RT

Washing Buffer: 1x PBS

0,1% TWEEN-20

Mix at RT

For stripping off the antibodies, the membrane was washed 2 times for 30 min in 1x Stripping buffer in a water bath at 55 °C. Afterwards, the membrane was washed 4 times for 10 min in washing buffer at RT. The membrane was then again blocked for 1 h at RT with blocking buffer, before it was incubated with the new primary antibody overnight at 4°C. For loading control we used mouse-monoclonal antibody to β-actin (Abcam) in a ratio of 1:500, diluted in blocking buffer. For secondary antibody we used alkaline phosphatase conjugated anti-mouse antibody (Jackson ImmunoResearch) diluted 1:2000 in blocking buffer.

2.10   Total RNA Isolation, DNA Digestion & RNA Cleanup

2.10.1 Total RNA Isolation

For the total RNA Isolation of microglia cells plated on a 94 mm Petri dish, the MG-medium was aspirated and the cells were washed with PBS once. After aspirating the PBS, 600 µl of RLT lysis buffer containing β-mercaptoethanol (β-ME) were pippeted onto the plate and the crude cell lysate was then transferred directly into a QIAshredder spin column placed in a 2 ml collection tube. All following steps were performed using the Quiagen RNeasy Mini Kit according to the manufacturer's instructions.

For purification of total RNA of rat cerebellum, 600 µl of RLT lysis buffer containing β-ME were added to 30 mg of tissue, which was then homogenized by using a homogenizing drive.

The lysate was transferred into a QIAshredder spin column and all following steps were identical with those from the RNA Isolation of microglia cells.

2.10.2 DNA Digestion

To eliminate any genomic DNA contamination, a DNase digestion step was performed by adding 7 µl 10x DNase I Buffer (Fermentas), 2 µl Ribolock RNase Inhibitor (40 u/µl, Fermentas), 7 µl DNase I (1 u/µl, Fermentas) to the 50 µl total RNA sample and filling it up to a total volume of 70 µl with RNase-free water, followed by an incubation period of 30 min at 37 °C. Afterwards, 7 µl EDTA were added and the sample was put at 65 °C for 10 min to stop the reaction.

2.10.3 RNA Cleanup

The total RNA was then purified using the Quiagen RNeasy Mini Kit RNA Cleanup protocol according to the manufacturer's instructions. To elute the RNA during the final step, 30 µl of RNase free water were added.

2.11 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.11.1 RT-PCR

For the reverse transcription (RT) step, 2 µl T7-N7 primer were added to each 30 µl total RNA sample and placed for 5 min at 70 °C and afterwards 5 min in ice. Meanwhile, the MasterMix (10x stock) was prepared by mixing the following components (amounts for 1 volume):

- 10 µl of M-MLV 5x RT Buffer (Promega Corporation)

- 3 µl PCR grade dNTPs (Roche Applied Science)

- 3 µl Ribolock RNase Inhibitor (40 u/µl, Fermentas)

- 5 µl ddH$_2$O

The total RNA samples were removed from ice and 20 µl MasterMix were added to each probe and placed for 2 min at 40 °C in a thermoblock. 2 µl M-MLV RT RNase H(-) Point Mutant (200 u/µl, Promega Corporation) were added and samples were led for 10 min at RT, before placing them for 50 min at 40 °C. Finally, samples were heated up to 70 °C for 15 min, before storing at -20 °C for later use.

For the polymerase chain reaction (PCR) step, the MasterMix was prepared freshly from Roche Applied Bioscience´s FastStart Taq DNA Polymerase, dNTPack in the following amounts (for 1 volume):

- 5 µl PCR Reaction Buffer (10x conc. with 20 mM MgCl$_2$)

- 1 µl PCR Grade Nucleotide Mix

- 1 µl Forward Primer

- 1 µl Reverse Primer

- 0,4 µl FastStart Taq DNA Polymerase (5 u/µl)

- 41,6 µl nuclease free water

Reverse Transcription Polymerase Chain Reactions (RT-PCR) were performed using a Biometra TPersonal or a Bio-Rad MyCycler thermal cycler. The protocols start with denaturing the cDNA products of the RT for 11 min at 95 °C. Amplification steps were set for 30 s denaturation at 95 °C, annealing for 30 s and extension at 72 °C for 30 s (or 2 min for longer PCR-products). Primer sequences and further experimental conditions are given in Table 1. All primers were designed using the Web Primer3Plus interface for the Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

	Primersequence	Product size	Annealing temp.	PCR-cycles	Accesion number
AMIGO2	5´-AGTTGATTGGAACGAAAGCAAT-3´ 5´-CAGTGCGTTTTTCTGTCTCTTG-3´	1460 bp	59°C	40	NM_182816.2
AMIGO2	5´-GAACTGTTCTCACAGCGTCATC-3´ 5´-TCTATAACCAGGCTTCCGTTGT-3´	213 bp	53°C	40	NM_182816.2
AMIGO2	5´-CGTGGTCAGTATCGTCCTAGTG-3´ 5´-TAGGGAGAGGTCAAATCACGAC-3	391 bp	55°C	40	NM_182816.2
GAPDH	5´-GGCATTGCTCTCAATGACACC-3´ 5´-TGAGGGTGCAGCGAACTTTAT-3´	310 bp	53°C	40	NM_017008.3
SPP1	5´-CAAACACTCAGATGCTGTAGCC-3´ 5´-GAGTTCACAGAATCCTCGCTCT-3´	216 bp	55°C	40	NM_012881.2

Table 1: Experimental conditions used for RT-PCR and primer sequences.

2.11.2 Gel Electrophoresis

Loading dye was prepared by mixing the following ingredients:

- 4 g sucrose

- 1,25 ml (2%) bromophenol blue

- 1,25 ml (2%) xylene cyanol

- Fill up to a total volume of 10 ml with ddH$_2$O

3% agarose gels were prepared by mixing agarose with 1x Tris-Acetate-EDTA (TAE) buffer (50 mM Tris-Acetate [pH 8,0]; 1 mM EDTA [pH 8,0]) and heating it up in a microwave, until the agarose is completely dissolved. After cooling the solution for a short time, 0,4 µg/ml ethidium bromide (Sigma-Aldrich) was added, gels were filled into the chambers and led at RT for polymerization.

The RT-PCR products were mixed with 15 µl loading dye before loading the samples alongside 100 bp ladder (1 µg/µl; Invitrogen). Electrophoresis was performed in PerfectBlue Gelsystem Mini L 'Revolution' horizontal gel chamber (Peqlab Biotechnologie GmbH) filled with 1x TAE buffer to cover the gel, at a constant voltage of 90 V for 60 min. Separated PCR-products were visualized by using Gel Doc 2000 Transiluminator (λ = 302 nm; Bio-Rad Laboratories) together with a CCD Gel Doc MZL camera.

2.11.3 RT-PCR Products Sequencing

After gel electrophoresis, the RT-PCR products were extracted from the gels or directly from the RT-PCR reaction using Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Cleaned-up samples were sent to VBC-BIOTECH Service GmbH Vienna for sequencing.

2.12   Immunofluorescent Staining

2.12.1 Staining of Tissue Slices

For immunofluorescence staining of rat spinal cord tissue slices, tissue sections were first deparaffinized by washing them 2x 20 min in Xylol. Afterwards slices were rehydrated in a 96% (2x), 70%, 50% ethanol series and rinsed in ddH$_2$O. For antigen retrieval, slides were immersed in Citrate buffer working solution and placed in a steamer for 60 min. Afterwards slides were removed from the steamer and allowed to cool for 30 min at RT, before being washed 3-5 times with TBS (Tris-Buffered Saline). From this point on, slices were always placed in a wet chamber to prevent them from drying out. To minimize unspecific background reaction, the tissue sections were blocked for 20 min with 10 % FCS/DAKO buffer. Primary antibodies were diluted with 10% FCS/DAKO buffer and slices were incubated at 4 °C overnight (polyclonal rabbit antibody against rat Amigo2 (1:2500), Lifespan Bioscience; monoclonal mouse antibody against rat homologue of human CD68 (ED1)

(1:4500), Serotec). Slides were again washed 3-5x in TBS. Secondary antibodies were diluted in 10% FCS/DAKO and slides were incubated at RT for 1 h, whereby the wet chamber was placed on a shaker (donkey anti mouse antibody conjugated with Cy3 (1:200); biotinylated donkey anti rabbit antibody (1:2000), Jackson). Secondary antibodies were protected from light the whole time. Slides were again washed 3-5x in TBS. Streptavidin conjugated to Cy2 (Sigma) was diluted in 10% FCS/DAKO (1:75) and added on top of the sections for detection of the biotinylated secondary antibody. Finally, slides were again washed 3-5x in TBS and mounted with Gallate/Geltol, paying attention to avoid any air bubbles and placed at 4 °C over night for hardening.

2.12.2 Staining of Cell Cultures

For the staining of microglia cells grown on Poly-L-lysine (PLL) coated cover slips, microglia derived from mixed glia cultures were separated from other cells by placing the supernatant of the mixed glia cultures in uncoated plastic Petri dishes, as described before. The microglia which adhered to the plastic were washed 2x with PBS (or medium) to remove other glia cells. The cells were then again removed from the dish, by placing it on ice for 10 min and pipetting cold microglia medium onto the dish several times. The medium containing the cells was collected and centrifuged with 300 g at RT. The pellet was resuspended and the cell suspension was pipetted in a Petri dish containing PLL coated cover slips and placed in the incubator (37 °C, 5% CO_2) overnight. The cover slips were transferred to a 12-well plate afterwards.

After the treatment, the medium was removed from the wells and the cells were washed 2x with 1 ml PBS before adding 4% PFA (1ml/well) at RT for 15 min. After fixation, cells were washed 2x with TBS. For permeabilization, TBS containing 0,5% Triton X-100, 5% BSA, 5% donkey serum was added and left at RT for 30 min, followed by 2 washes with TBS. Primary antibodies were diluted 1:100 in TBS containing 1% donkey serum (polyclonal goat anti Amigo2 antibody, R&D Systems; monoclonal mouse antibody against rat homologue of human CD68 (ED1), Serotec) and 100 µl of the antibody solution were provided as a drop on

each cover slip. The 12-well plate was placed in a wet chamber at 4 °C and antibodies were incubated overnight. Cover slips were washed 3x for 5 min with TBS. Secondary antibody was also diluted 1:100 in TBS containing 1% donkey serum and provided in the same way as the primary antibodies, but with a 60 min incubation time at RT (donkey anti goat Cy5, Jackson Immuno Research Lab; donkey anti mouse antibody conjugated with Cy2). Cover slips were washed 2x with TBS.

After removing the paraffin dots, cover slips were mounted on a drop of Gallate/Geltol on a slide and left overnight at 4 °C and additionally for some more time at RT for drying, before cells were observed with a confocal microscope.

Additionally also neuronal cultures grown on PLL coated cover slips were stained for immunofluorescent microscopy. Cells were stained after 7 days in vitro. Instead of the microglial marker ED1, the neuronal markers SMI 311 and SMI 312 were used.

2.13 Cloning, Expression & Purification of the Extracellular Domain of Amigo2

The pTrcHis2 TOPO TA Expression Kit (Invitrogen) was used for insertion of the *Taq* polymerase-amplified PCR product into a plasmid vector and the subsequent expression in *E.coli*. Finally the recombinant Amigo2 protein was purified via a NiNTA column with the help of a six tandem histidine tag.

2.13.1 Production of PCR Products

Following primers were used to amplify the PCR product of interest:

Sense primer Amigo2:   5`- ATG TCG TTA AGG TTC CAC ACA CTG C-3`
Antisense primer Amigo2:   5`- CAG TGC GTT TTT CTG TCT CTT GTC TC- 3`

For the PCR reaction the following ingredients were mixed together:

 4 µl cDNA from microglia cells (see section **2.11.1**)

 0,5 µl sense primer Amigo2

 0,5 µl antisense primer

 0,5 µl dNTPs

 2,5 µl *Taq*-Buffer

 1,2 µl Titanium *Taq* DNA Polymerase (Clontech)

 16 µl nuclease free water

The protocols start with denaturing the cDNA products for 1 min at 95 °C. Amplification steps were set for 30 s denaturation at 95 °C, annealing for 45 s at 64 °C, and extension at 68°C for 1 min. The PCR was run for 28 cycles, before the PCR product was analyzed by gel electrophoresis on a 1% agarose gel. For purification of the DNA fragment from the gel, the Mini Elute Gel Extraction protocol from Quiagen was performed according to the manufacturer's instructions.

2.13.2 TOPO Cloning and Transformation

To 4 µl of fresh PCR sample, 1 µl TOPO vector was added, mixed gently and incubated for 5 min at RT. 2 µl of this TOPO Cloning reaction were added into a vial of One Shot cells, mixed again gently and incubated on ice for 30 min. Afterwards the cells were heat shocked for 30 s at 42 °C without shaking and subsequently the tubes were transferred to ice. 250 µl of SOC medium were added and the tubes were shaken at 37 °C for 30 min. 20 µl of the transformation were spread on a pre-warmed selection plate (containing 100 µg/ml ampicillin) and were incubated overnight at 37 °C. Colonies were picked for analysis.

A single colony was picked from a freshly streaked selective plate and a starter culture of 2,5 ml LB medium containing ampicillin (100 µg/ml) was inoculated. The cells were incubated overnight at 37 °C while shaking (250 rpm). The next morning, the bacterial cells were harvested by centrifugation at 8000 rpm for 5 min. The supernatant was discarded and the

pellet was resuspended in 300 µl P1 buffer (Resuspension-Buffer). Next, 300 µl of P2 Lysis-Buffer were added, the tubes were inverted and incubated for 5 min at RT. Afterwards, 300 µl of P3 Neutralizing-Buffer were added and the tubes were incubated for 15 min at 4 °C before being centrifuged at 12000 rpm for 15 min.

800 µl of the supernatant were added into a new tube, which already contained 700 µl of Isopropanol. The tube was inverted and incubated for 15 min, before centrifuging again at 12000 rpm for 15 min. The supernatant was discarded. 500 µl of 70% ethanol were added to the pellet, mixed and centrifuged at 12000 rpm for 10 min. After discarding the supernatant, the pellet was dried for 1 min, before being dissolved in 30 µl TE buffer.

10 µl were then used for restriction enzyme cleavage to determine the orientation of the cloned insert. As a restriction enzyme Bgl1 was used:

  3 µl Mini pTrcHis2-Amigo2

  2 µl restriction enzyme Bgl1 (BioLabs, R0143S)

  2 µl Buffer 3

  13 µl ddH$_2$O

Cleavage was performed for 2 h at 37 °C and the restriction products were analyzed on a 1% agarose gel. The Amigo2 insert sequence with the correct orientation was verified by DNA sequencing (Microsynth Balgach, Switzerland).

A glycerol stock solution was prepared by adding 100 µl glycerol to 100 µl Amigo2 expressing *E.coli* cells (=Mini), and stored at -80 °C.

2.13.3 Expression of the PCR Product (Amigo2)

40 µl of the glycerol culture (Amigo2 expressing *E.coli* cells) were inoculated with 50 ml LB medium, containing 100 µg/ml ampicillin and shaken overnight at 37 °C at 250 rpm. The next day, 4x 250 ml LB medium (without ampicillin) were inoculated with 10 ml of the overnight culture respectively, using 4 separate Erlenmeyer flasks. The cultures were grown for 2 h at 37 °C.

Amigo2 expression was induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 1 mM. The cells were grown further for 4 h more at 37 °C with shaking. Afterwards, the cultures were poured into microcentrifuge tubes and the cells were harvested by centrifugation for 20 min at 6000 rpm at 4 °C. The supernatants were removed and all pellets were dissolved in 15 ml Buffer B (pH=8.0) per tube (60 ml in total) and 2% Triton X-100 and 20 mM 2-Mercaptoethanol were added. The suspension was first frozen at -20 °C and subsequently incubated at 4 °C overnight with shaking. Finally the lysate was stored at -20 °C.

Buffer B: 8 M Urea

100 mM NaH_2PO_4 x H_2O

10 mM Tris

Dissolved in ddH_2O

Sterile filtration

Adjust pH to 8.0 or 6.3 or 4.5

2.13.4 Purification of Amigo2 via NiNTA Column (Quiagen) / Affinity Chromatography

The NiNTA Purification System is designed around the high affinity and selectivity of NiNTA agarose for recombinant proteins that are tagged with six tandem histidine residues.

The NiNTA agarose was resuspended in its bottle by inverting and gently tapping the bottle repeatedly. 4 ml of the resin were poured into a 10 ml purification column (Matrix: NiNTA Superflow Agarose, Fa. Quiagen), and the resin was settled by gravity overnight. The lysate was thawn at RT and sonicated 6 times on ice with 10 s bursts at high intensity and a 10 s cooling period between each sonication step. Subsequently, the lysate was centrifuged at 10000 rpm for 30 min to remove any cellular debris and the supernatant was used for purification of the extracellular domain of Amigo2.

The column was equilibrated with Buffer B (pH=8.0) with a flow through velocity of 1 ml/min for approximately 20 min. The supernatant containing the Amigo2 extract was diluted 1:2 using Buffer B (pH=8.0), loaded on the column and was washed with Buffer B (pH=8.0) with a

velocity of 1 ml/min until the peak on the chromatogram disappeared. The column was then again washed with Buffer B (pH=6.3) until the peak disappeared (flow through velocity 1 ml/min), followed by a washing step with 60% Isopropanol / 10 mM Tris (pH=8.0) until the peak disappeared or rather until the OD-level was stable (flow through velocity 1 ml/min).

60% Isopropanol / 10 mM Tris: 300 ml Isopropanol

                                            200 ml ddH$_2$O

                                          0,605 g Tris

                                          Adjust pH=8.0

The Amigo2 protein was eluted using Buffer B (pH=4.5) and the eluat was collected until the Amigo2 peak on the chromatogram disappeared. The pH level was adjusted to 7.5 and half of the eluat was frozen at -20 °C.

The remaining eluat was dialyzed using the dialysis membrane from Spectra/Por (Roth 6-8MW) for 1 h at RT against 1 l of PBS, followed by an overnight dialysis at 4 °C using 1 l of fresh PBS.

The recombinant Amigo2 protein was then concentrated using Vivaspin2 (Sartorius) 10000 MWCO according to the manufacturer's instruction. The Amigo2 concentration was determined using the BCA method and by photometric measurement (Nanodrop).

The protein was then analyzed by Western Blot, using an anti-His antibody (mouse antibody, Quiagen) diluted 1:5000, followed by an alkaline phosphatase conjugated secondary antibody (goat anti mouse IgG antibody), also diluted 1:5000.

2.14 Flow Cytometry Analysis

2.14.1 Staining Procedure

For flow cytometry analysis of microglia cells, a microglia cell culture was established as described before (see section **2.4.1**). To harvest the cells from the Petri dishes, they were washed with 10 ml PBS and cold shocked for 15 min, by placing the dishes on ice. The cells

then rounded up and were easier to detach afterwards. The microglia were then detached by taking up the 10 ml cold PBS and pipetting it vigorously several times over the hole plate. The cell suspension was transferred into a 50 ml falcon and was centrifuged at 300 g for 1 min/ml. The supernatant was aspirated, the cell pellet was resuspended in Pharmingen Stain Buffer (BSA; Becton Dickinson) containing 10% rat serum and left for 30 min at 4 °C for blocking.

Afterwards the cell suspension was transferred into a 96 well plate (number of wells depending on number of antibodies used) and washed by filling the wells up to a total volume of 100 µl with BSA and subsequent centrifugation with 1300 rpm for 5 min at 4 °C. The supernatant was discarded by decanting the well plate and the pellets were resuspended in 100 µl BSA. The primary antibodies were added in a ratio of 1:100 and incubated for 30 min on ice (only unlabelled antibodies were added).

The cells were again washed by adding 100 µl of BSA and being centrifuged at 1300 rpm for 5 min at 4 °C. The pellet was resuspended in 100 µl of BSA and the secondary antibodies were added in a ratio 0f 1:100 (all labeled antibodies). The microglia cells were again washed (adding 100 µl BSA, centrifugation at 1300 rpm, 4 °C, 5 min).

Finally the cells were resuspended in 200 µl of BSA and transferred into FACS tubes and kept on ice until performing flow cytometry analysis within the next few hours.

2.14.2 Anibodies

Polyclonal goat antibody against Amigo2 (R&D Systems)

Goat antibody against GFAP (SantaCruz; neg control for Amigo2)

Mouse antibody against CD45 (Serotec)

Mouse IgG 1 (Dako; neg control for CD45)

3   Results

Former experiments done in this laboratory showed a differential expression of many immunological relevant genes in IFN-γ treated microglia in comparison with untreated microglia. The total RNA from IFN-γ primed cells was extracted using the Quiagen RNeasy Mini Kit (see Section 2.10). Gene Expression profiling of stimulated and control cultures with Agilent Multiplex 4x44K *Rattus norvegicus* Arrays was performed by ImaGenes GmbH, Berlin (www.imagenes-bio.de). Among those genes, an upregulation of Amigo2 mRNA (14.8 fold) and a strong downregulation of Osteopontin mRNA (27.6 fold) in IFN-γ primed cells were most noticeable.

The first aim of the hereby presented thesis was to confirm the different expression of those two molecules in the primed cells versus the untreated controls.

The second part of my thesis was to study the effect of recombinant Amigo2 and Osteopontin on neurite outgrowth of neuronal cultures, as well as the effect of these molecules on microglia cultures.

3.1   RNA Isolation

To confirm the effect of IFN-γ on the Amigo2 expression, microglia cells were isolated and treated with 100 ng/ml IFN-γ for 24 h, as described before. After this time period, the cells were processed for RNA Isolation and RT-PCR was performed for Amigo2 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as a housekeeping gene. The Amigo2 PCR-product covered the part of the mRNA coding for the extracellular and transmembrane domain. Separation of the products by gel electrophoresis showed a clear upregulation of Amigo2 mRNA in the treated microglia (Fig. 8). As a positive control, isolated total RNA from rat cerebellum was used, because the Amigo2 protein is known to be prominently expressed in this tissue.

To be sure that microglia produce the whole length mRNA and not only a truncated version, another PCR was run, producing a PCR-product that matches to the sequence of the mRNA coding for the intracellular domain of the Amigo2 protein. For this PCR, cells were processed

that have been sorted via fluorescent activated cell sorting (FACS) before by using a CD45 antibody conjugated to FITC. Using sorted microglia cells for the RNA Isolation, it became certain that the Amigo2 mRNA was produced by microglia and not by any possible contaminating cell types.
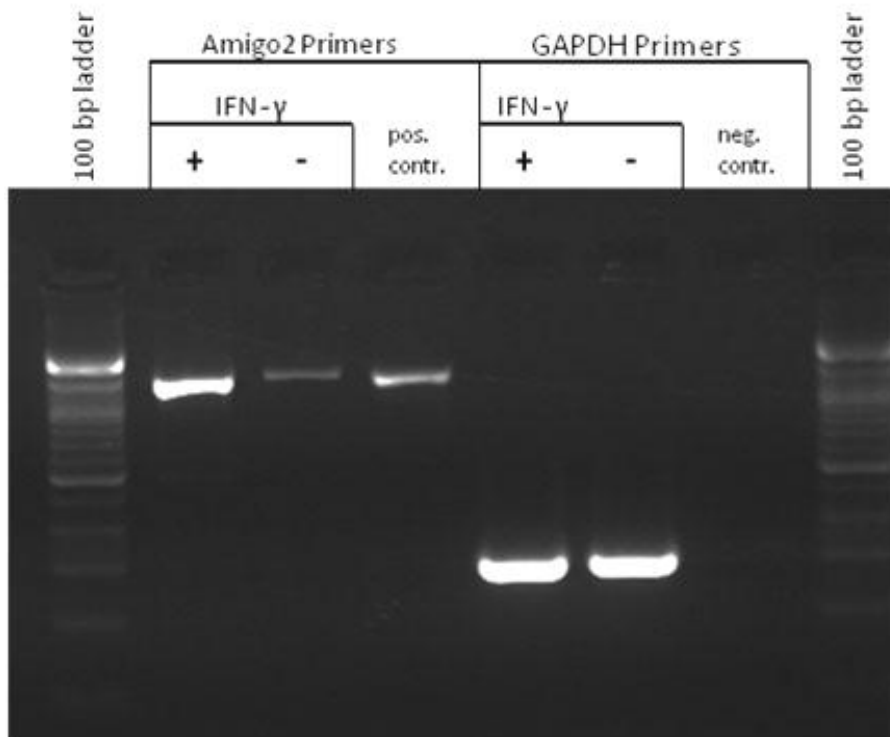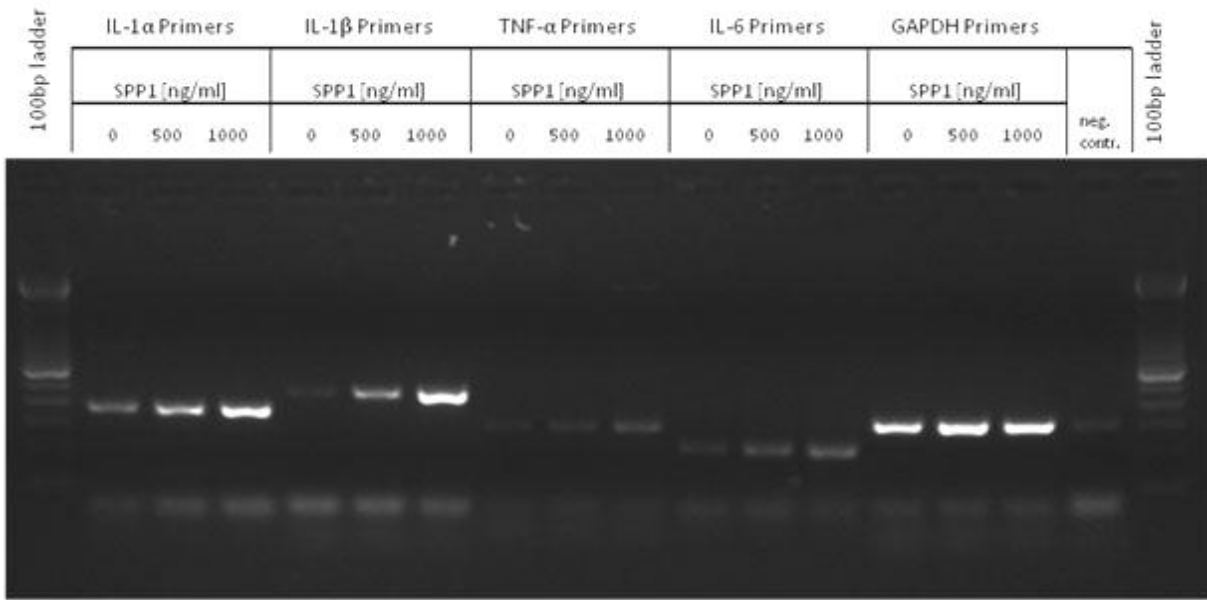

Figure 8: Gel electrophoresis of the Amigo2 products (1460 bp long) and the GAPDH products (311 bp long). Total RNA isolated from rat cerebellum was used as a positive control for Amigo2 expression. The equal portion of GAPDH products indicate an evenly loading of the samples.

We also looked at the expression of Osteopontin mRNA in IFN-γ treated and untreated microglia and again, the RT-PCR confirmed the data from the arrays and showed a clear downregulation of Osteopontin mRNA in the treated cells compared with the untreated control (Fig. 9).

Because in literature, Osteopontin is often described as a proinflammatory cytokine, we tested its effect on cultured microglia. RT-PCR indicated that Osteopontin treated microglia expressed significantly more interleukin-1α (IL-1α), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) mRNA compared with the untreated cells (Fig. 10). The upregulation of those cytokines indicates that soluble Osteopontin in the medium can be perceived by microglia, leads to activation and drives the cells into a proinflammatory direction.
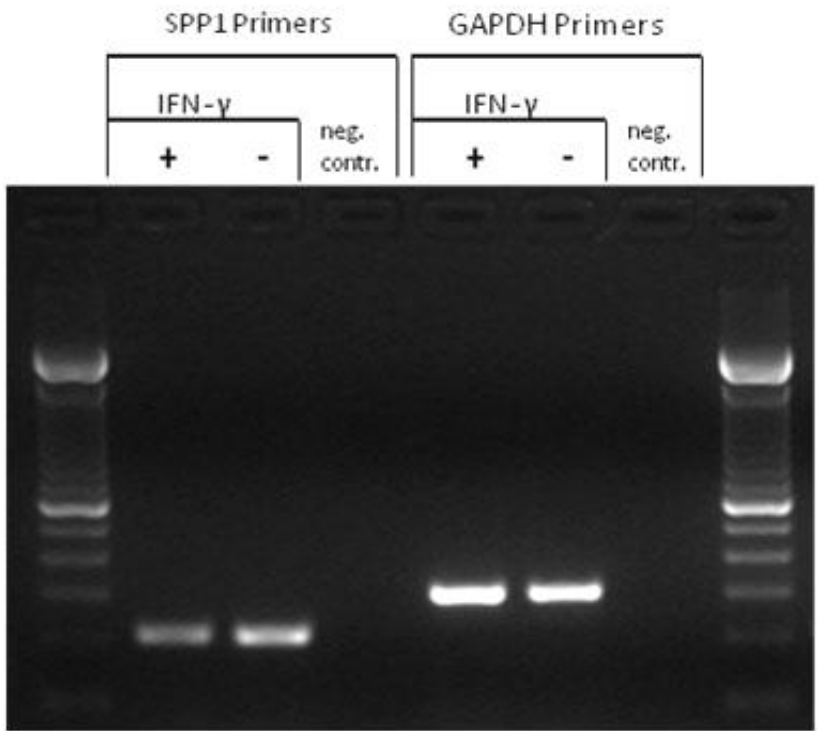
Figure 9: Gel electrophoresis of the Osteopontin (SPP1) products (216 bp long) and the GAPDH products. Microglia treated with IFN-γ downregulate the expression of Osteopontin mRNA. The equal portion of GAPDH products indicates an evenly loading of the samples.

Figure 10: Gel electrophoresis of different products of proinflammatory cytokines (IL-1α, IL-1β, TNF-α, IL-6). Microglia treated with Osteopontin for 24 h in a concentration of 500 or 1000 ng/ml upregulate the expression of the mRNAs of all four tested cytokines.

3.1.1 Sequencing

After extracting the RT-PCR products from the RT-PCR reaction, the samples were sent to VBC-BIOTECH for sequencing. The sequencing analysis confirmed that the product matches to the sequence of the Amigo2 mRNA. The product shows 94% identity to the Amigo2 mRNA sequence (Fig. 11). The majority of the mismatches are due to mixed base sites results during sequencing. According to the IUB code for mixed base sites, nearly all of them fit to the original sequence. (Use IUB code for mixed base sites: K= G,T / S= G,C / W= A,T / M= A,C/ Y= C,T / R= A,G. Mismatches that still fit according to this aspect are highlighted in red.) Those inaccuracies in the sequence of the PCR-product are probably caused by inaccuracies of the Taq-Polymerase used for the PCR reaction, which is missing a proofreading function.

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PCR-Product  1     CTCTAAGGGGTT-ACTAGCTGGCAGTTAACTTGTACTTCTCTCCCGTTTTTAAATGTTGT  59
                   |||||||||||| ||||| |||||||||||||||||||||||||||||||||||||||||
Amigo2       574   CTCTAAGGGGTTAACTAG-TGGCAGTTAACTTGTACTTCTCTCCCGTTTTTAAATGTTGT  632

PCR-Product  60    TACCAGATTGGTGGGAGGCTGTGCTGGTGAGAAGCGACTGGCAAGAGGCTCAGAGGCGAC  119
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       633   TACCAGATTGGTGGGAGGCTGTGCTGGTGAGAAGCGACTGGCAAGAGGCTCAGAGGCGAC  692

PCR-Product  120   CATAATGTCGTTAAGGTTCCACACACTGCCCACCCTGCCTAGAGCTGTCAAACCGGGTTG  179
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       693   CATAATGTCGTTAAGGTTCCACACACTGCCCACCCTGCCTAGAGCTGTCAAACCGGGTTG  752

PCR-Product  180   CAGAGAGCTGCTGTGTCTGTTGGTGATCGCAGTGATGGTGAGCCCCAGCTCCTCAGGACT  239
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       753   CAGAGAGCTGCTGTGTCTGTTGGTGATCGCAGTGATGGTGAGCCCCAGCTCCTCAGGACT  812

PCR-Product  240   GTGCCCTACGGCTTGCATCTGTGCCACTGACATTGTAAGCTGCACCAACAAAAASCTGTC  299
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Amigo2       813   GTGCCCTACGGCTTGCATCTGTGCCACTGACATTGTAAGCTGCACCAACAAAAACCTGTC  872

PCR-Product  300   TAARGTGCCTGGGAACCTTTTCCGACTGATTAAAAGACTGGATCTGAGCTATAACAGAAT  359
                   |||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       873   TAAGGTGCCTGGGAACCTTTTCCGACTGATTAAAAGACTGGATCTGAGCTATAACAGAAT  932

PCR-Product  360   CGGACTCCTGGATGCCGACTGGATTCCGGTGTCGTTCGTCAAGCTGAGCACCCTGATTGT  419
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       933   CGGACTCCTGGATGCCGACTGGATTCCGGTGTCGTTCGTCAAGCTGAGCACCCTGATTGT  992

PCR-Product  420   TCGCCACAACAACATCACCAGMATTTCCACGGGCAGCTTCTCCACCACTCCAAATTTAAA  479
                   |||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||
Amigo2       993   TCGCCACAACAACATCACCAGCATTTCCACGGGCAGCTTCTCCACCACTCCAAATTTAAA  1052

PCR-Product  480   GTGTCTCGACTTATCGTCCAATAGGCTGAAGTCGGTGAAAAGTGCCATGTTCCAGGAGCT  539
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       1053  GTGTCTCGACTTATCGTCCAATAGGCTGAAGTCGGTGAAAAGTGCCATGTTCCAGGAGCT  1112

PCR-Product  540   AAAGGTTCTGGAAGTGCTCCTGCTTTACAACAACCACATTTCCTATCTGGACCCGGCAGC  599
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       1113  AAAGGTTCTGGAAGTGCTCCTGCTTTACAACAACCACATTTCCTATCTGGACCCGGCAGC  1172

PCR-Product  600   GTTCGGAGGGCTCTCCCACTTGSAGAAACTCTACCTGAGTGGGAACTTCCTCACGAA-TT  658
                   |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||| ||
Amigo2       1173  GTTCGGAGGGCTCTCCCACTTGCAGAAACTCTACCTGAGTGGGAACTTCCTCACGAAGTT  1232

PCR-Product  659   CCCTATGGATT-GTATGTCNG-ARGTTCAAGCTGGCTGATCTGACATTTGAAGATGTTTC  716
                   ||||||||||| |||||||| | | ||||||||||||||||||||||||||  |||||||||
Amigo2       1233  CCCTATGGATTTGTATGTCGGGAGGTTCAAGCTGGCTGATCTGACATTTTTAGATGTTTC  1292
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PCR-Product   717   GTACATTCRAATTGCTTCCATACCAATGCACCWCATAAATTTAGTGCCGGGGAAAGCAGC   776
                    ||||| || |||||||||||||||||||||||| |||||||||||||||||||| ||||||||
Amigo2       1293   GTACAATCAAATTGCTTCCATACCAATGCACCACATAAATTTAGTGCCGGG-AAAGCAGC   1351

PCR-Product   777   TGAGGGGMATCTTCCTTCACGGGAACCCTTTMGTCTGTGACTGKTCCCTGTASTCCTTGC   836
                    ||||||| |||||||||||||||||||||||| ||||||||||| ||||||||| |||||||
Amigo2       1352   TGAGGGGCATCTTCCTTCACGGGAACCCTTTCGTCTGTGACTGTTCCCTGTACTCCTTGC   1411

PCR-Product   837   TGACSTTCTGGTASCGTAGGCACTTTAACTCTGTGASGGAWTTCAWGCATGACTACACCT   896
                    |||| |||||||| ||||||||||||||||||||||| ||| |||| |||||||||||||||
Amigo2       1412   TGACCTTCTGGTACCGTAGGCACTTTAACTCTGTGACGGATTTCAAGCATGACTACACCT   1471

PCR-Product   897   GTCGCCTGTGGWTGGACTCCMSGCACTCCCACCAGACTGTTGCTGMTCCAGGATAGCTTT   956
                    ||||||||||| ||||||||  ||||||||||||||- |||||||||| |||||||||||||
Amigo2       1472   GTCGCCTGTGGTTGGACTCCAGGCACTCCCACCAG-CTGTTGCTGCTCCAGGATAGCTTT   1530

PCR-Product   957   CTGAACTGTTCTCACAGCKKCATCAACGGMTCCTTSCACRMASTTGSCKTTATCCWYKAG   1016
                    ||||||||||||||||| ||||| || |||||| |||  | ||| | |||||| ||
Amigo2       1531   CTGAACTGTTCTCACAGCGTCATCAATGGCTCCTTCCACGCACTTGGCTTTATCCACGAG   1590

PCR-Product  1017   GCWCAGRTTGRRRAAAGGGMKATTRTCCACTGTRACRKCNAAAYCNKKAACGSSAACASC   1076
                    || ||| ||| |||     ||||| |||||||| |||| | |||| ||| |||| ||| |
Amigo2       1591   GCTCAGGTTGGGGAAAGGGCGATTGTCCACTGTGACGGCAAAACCGGTAACGGGAACACC   1650

PCR-Product  1077   GATTTCATCTGGGTGGGTCCCGACAACASGCTGWTGGAKCCSSATAANGACACSGSSAAC   1136
                    |||||||||||||||||||||||||||| |||| |||| || |||| ||||| |   |||
Amigo2       1651   GATTTCATCTGGGTGGGTCCCGACAACAGGCTGTTGGAGCCGGATAAAGACACGGGGAAC   1710

PCR-Product  1137   TTTCGAGTGTTTTACANCKSAASCCTGGTTATAKAGAACCSTGGTTTTGAKGACGCCRGR   1196
                    |||||||||||||| |  ||  || ||||||||| |||||| ||||||||| |||||| |
Amigo2       1711   TTTCGAGTGTTTTACAACGGAAGCCTGGTTATAGAGAACCCTGGTTTTGAGGACGCCGGG   1770

PCR-Product  1197   GKTTACWCCTGTATCAACATGAACAGGCWSCGSCWGTTRAATGAGACGGWGGACATCATG   1256
                    | |||| |||||||||  |||||||||| || | ||| |||||||||||| ||||||||||
Amigo2       1771   GTTTACTCCTGTATCGCCATGAACAGGCAGCGCCTGTTGAATGAGACGGTGGACATCATG   1830

PCR-Product  1257   ATTAACRYGAGCAATTTC-CCATAAASRGATCCCACCACKNCCAMRAGGYMTTYAACAYG   1315
                    || |||  |||||||||| ||||||| | |||||||||| ||| | ||| ||  ||| |
Amigo2       1831   ATAAACGTGAGCAATTTCACCATAAACAGATCCCACCACGCCCACGAGGCATTTAACACG   1890

PCR-Product  1316   GCTWWCACCACCCTGACTGCCWRCGTGNTCAGTATCNTCCWA-TGCWACTGTACCTGNAC   1374
                    ||| | |||||||||||| ||||| |||| ||||||||| ||| | ||| ||||||||| ||
Amigo2       1891   GCTTTCACCACCCTGGCTGCCTGCGTGGTCAGTATCGTCCTAGTGCTACTGTACCTGTAC   1950

PCR-Product  1375   CTGACACCGTGTCCCTGCAAATGTAGAGACAAGAGACAGAAAAACGCACTGAACCAAAGC   1434
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       1951   CTGACACCGTGTCCCTGCAAATGTAGAGACAAGAGACAGAAAAACGCACTGAACCAAAGC   2010

PCR-Product  1435   AACGCCCATTCGTCCATTCTCAGTCCCGGCCCCACCCGGGATGCCTCTGCTGAGGATCGG   1494
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       2011   AACGCCCATTCGTCCATTCTCAGTCCCGGCCCCACCCGGGATGCCTCTGCTGAGGATCGG   2070

PCR-Product  1495   AAGGCAGGTAAAAGAGTGGTGTTTTTGGAGCCCCTGAAGGATCCAGCGGTGGGACAGAAT   1554
                    ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Amigo2       2071   AAGGCAGGTAAAAGAGTGGTGTTTTTGGAGCCCCTGAAGGATCCAGCAGTGGGACAGAAT   2130

PCR-Product  1555   GGAAAAGTCAAGCTTGTCCCCAGTGAGACCGTTATAGCCGAGGGCATCTTAAAGTCCTCC   1614
                    ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Amigo2       2131   GGAAAAGTCAAGCTTGTCCCCAGTAAGACCGTTATAGCCGAGGGCATCTTAAAGTCCTCC   2190

PCR-Product  1615   AGGGCAAAATCTGACTCAGACTCGGTCAATTCCGTGTTCTCAGACACCCCCTTCGTGGCA   1674
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       2191   AGGGCAAAATCTGACTCAGACTCGGTCAATTCCGTGTTCTCAGACACCCCCTTCGTGGCA   2250

PCR-Product  1675   TCCACTTAACCGTGTGCCTGTGTTCGTCTGGTGTCGTGATTTGACCTCTCCCTA        1728
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Amigo2       2251   TCCACTTAACCGTGTGCCTGTGTTCGTCTGGTGTCGTGATTTGACCTCTCCCTA        2304
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Figure 11: Analyzed sequence of the PCR-product in comparison to the original Amigo2 mRNA sequence. Mismatches that, in consideration of the IUB code, still fit to the original sequence are highlighted in red. IUB code for mixed base sites: K= G,T / S= G,C / W= A,T / M= A,C / Y= C,T / R= A,G.

Two different PCRs were run with two different primer pairs both matching to the Amigo2 mRNA. The first cloned PCR-product covers the whole part of the original sequence which is coding for the extracellular and the transmembrane domain of the Amigo2 protein. The PCR-product ends at the corresponding amino acid 434, meaning that the majority of the cytosolic tail is excluded.

We also sequenced the cloned PCR-product coding for the intracellular domain of the protein and the analysis confirmed the correct sequence. Figure 4 shows the alignment of both sequences and therefore covers the whole coding region of the Amigo2 mRNA. Hence, microglia cells express the Amigo2 mRNA in an IFN-γ regulated manner and the cells express the whole mRNA sequence and not only a truncated version.

Also the PCR-product gain from the Osteopontin primers was sent in for sequencing and was verified as the correct sequence.

3.2   Western Blot

For confirmation of the upregulation of Amigo2 expression on the protein level, the membrane proteins of IFN-γ treated and untreated microglia were isolated, separated by gel electrophoresis and stained for Amigo2 protein (Fig. 12). As a positive control, the recombinant extracellular part of the Amigo2 protein was loaded, as well as the total protein isolated from rat cerebellum.

The western blot pictures showed a strong band occurring around 50 kDa, which was upregulated in the IFN-γ stimulated sample but also present in the untreated one, as well as in the protein lysates from rat cerebellum. The antibody also recognized the recombinant extracellular domain of Amigo2. This band appeared slightly beneath the other bands. The molecular mass of Amigo2 based on the number of amino acids should be around 57 kDa and based on literature, western blotting of Amigo2 should give a band around 65 kDa.
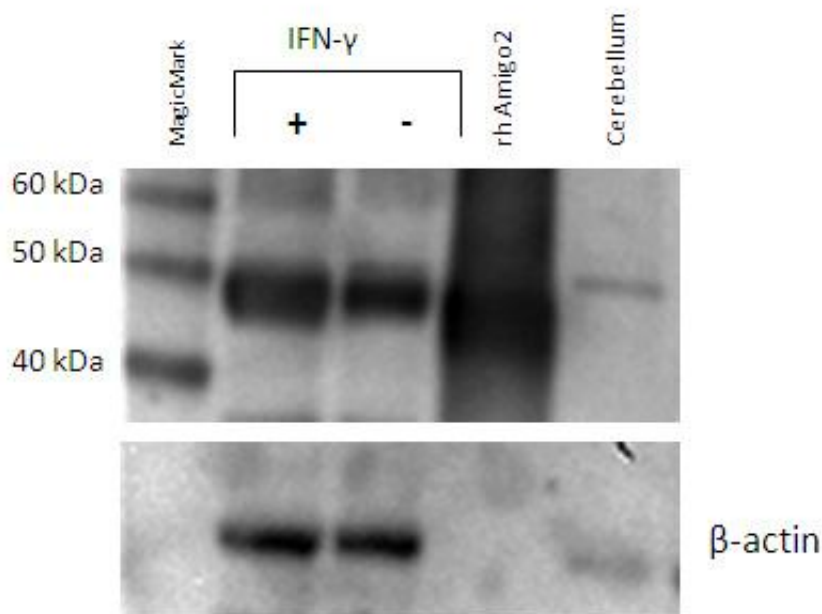
Figure 12: Protein lysates from IFN-γ treated and untreated microglia, the recombinant extracellular part of Amigo2 and protein lysates from rat cerebellum were immunoblotted with goat anti-Amigo2 antibodies. The equal bands of β-actin in the protein lysates of the microglia cultures indicate an evenly loading of those two samples. The loading control for the cerebellum sample appears only very weak and of course no β-actin expression can be seen in the rhAmigo2 sample.
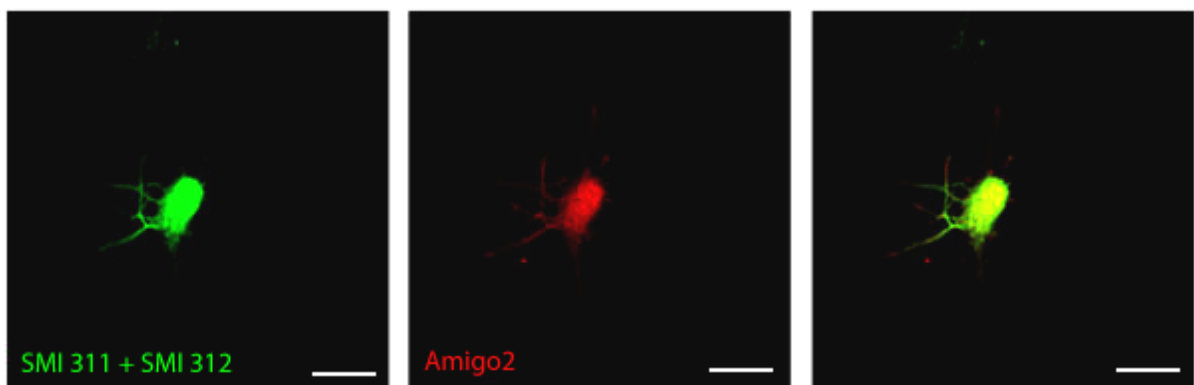
3.3 Immunofluorescent Staining of Microglia and Neuronal Cultures

Primary microglia cultures were treated for 48 h with IFN-γ and stained for Amigo2, together with the microglia marker ED1 (against the rat homologue of human CD68) and monitored with a confocal microscope. The microglia cells revealed a strong signal for ED1, but only very few cells also showed a strong signal for Amigo2. Maybe only few microglia express higher rates of Amigo2, while most microglia express only low amounts of the protein. The culture also contained a low amount of other cell types, which by morphology seemed to be neurons. Those contaminating cells were negative for ED1 and positive for Amigo2, which according to literature would also make sense for neuronal cells (Fig. 13).
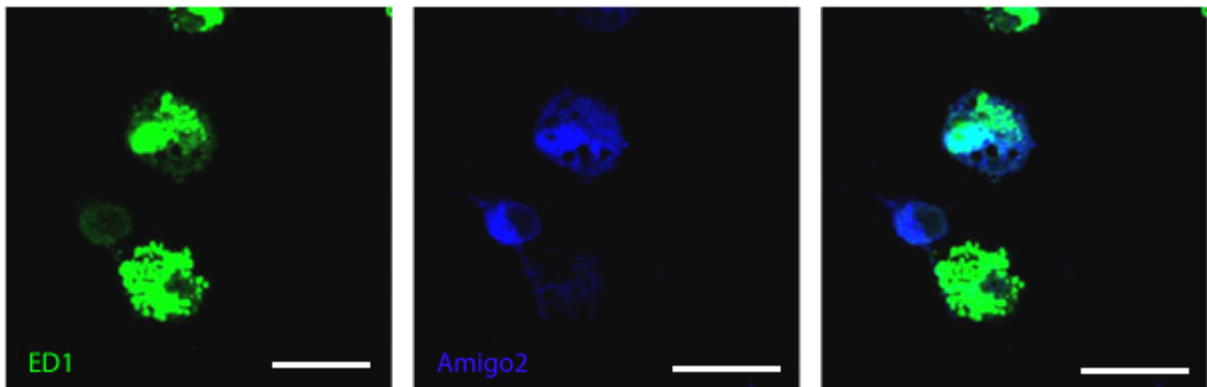
Figure 13: Immunofluorescent staining of microglia cells using the antibody ED1 as a microglia marker together with a secondary antibody conjugated to Cy2 (green) and an antibody against Amigo2 together with a secondary antibody conjugated to Cy5 (blue). Scale bars = 20 µm.

Neuronal cultures grown on PLL coated cover slips were also stained for Amigo2 together with the neuronal markers SMI311 and SMI312 (against phosphorylated and unphosphorylated neurofilaments) after day 7 in culture. The cells seemed to be positive for Amigo2, revealing a strong signal (Fig. 14).

Figure 14: Immunofluorescent staining of neuronal cells using the antibodies SMI311 and SMI312 as a neuronal cell marker together with a secondary antibody conjugated to Cy2 (green) and an antibody against Amigo2 together with a secondary antibody conjugated to Cy3 (red). Scale bars = 20 µm.

3.4   Immunohistochemistry

We also stained spinal cord slices from wild type Lewis rats and from transgenic Lewis rats overexpressing the proteolipid protein (PLP) in oligodendrocytes, which leads to a myelin degenerative CNS. Hemizygous PLP transgenic animals are viable and do not show any obvious clinical signs based on external inspection. But, as a result of accumulation of PLP in the oligodendrocytes, the cells become stressed and apoptotic, which in turn leads to infiltration of T-cells and to the activation of microglia cells in the CNS (Bradl et al., 1999). The homozygous PLP transgenic animals suffer under severe seizures.

Spinal cord slices were stained for Amigo2 and counterstained with haematoxylin for visualizing the nuclei. Because no specific microglia marker was used, microglia cells could only be identified based on their morphology. Although we cannot be certain, some cells that are positive for Amigo2 do show characteristic features of microglia cells like an elongated nucleus, some even in a triangular form, or processes with a typical shape. Another cell type which is also positive for Amigo2 exhibits a bigger and rounded up nucleus and is most probably a neuron. Additionally, we stained spinal cord slices of 10-12 month old Lewis wild type rats as a normal control. Amigo2 positive microglia already appeared in the control rats, but only in the white matter (Fig. 15 A). In heterozygous PLP transgenic rats of the same age, Amigo2 producing microglia cells were also restricted to the white matter, but were present in higher numbers when compared to control rats (Fig. 15 B). In homozygous rats with a spinal cord injury, also Amigo2 positive macrophages appear in the parenchyma next to the hemorrhages (Fig. 15 C). During inflammation the number of Amigo2 positive cells increases and the distribution changes. In heterozygous PLP transgenic rats that had been simultaneously injected with MBP specific T-cells causing an inflammatory response, positive microglia and macrophages can be found in the white matter, as well as in the grey matter of the spinal cord (Fig. 15 D and E).
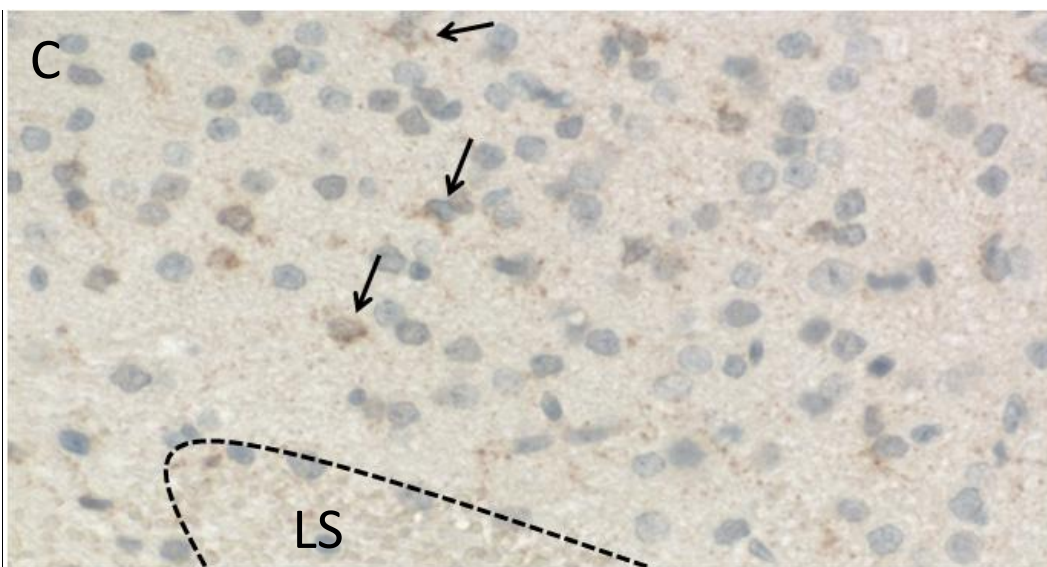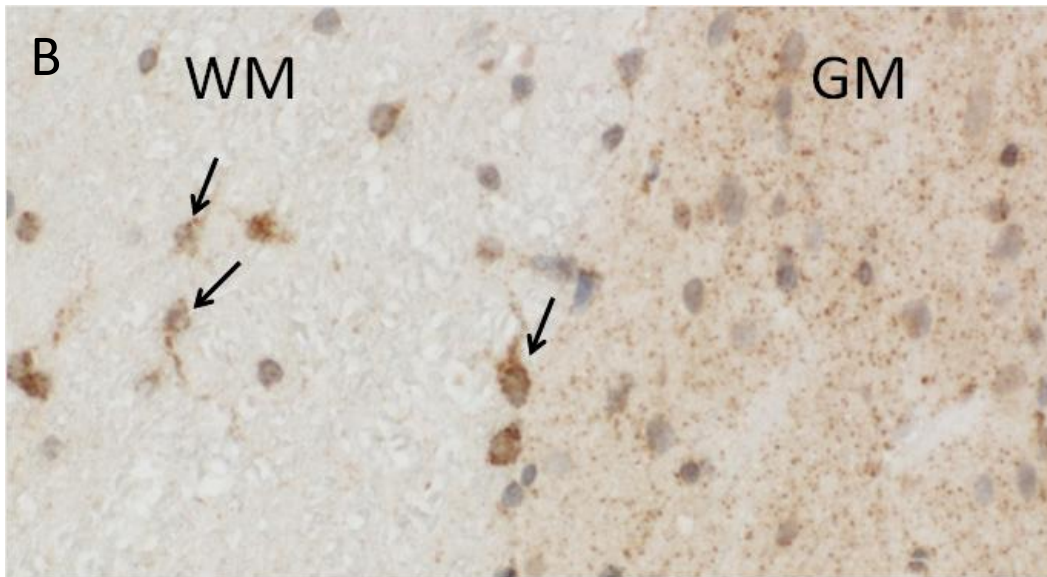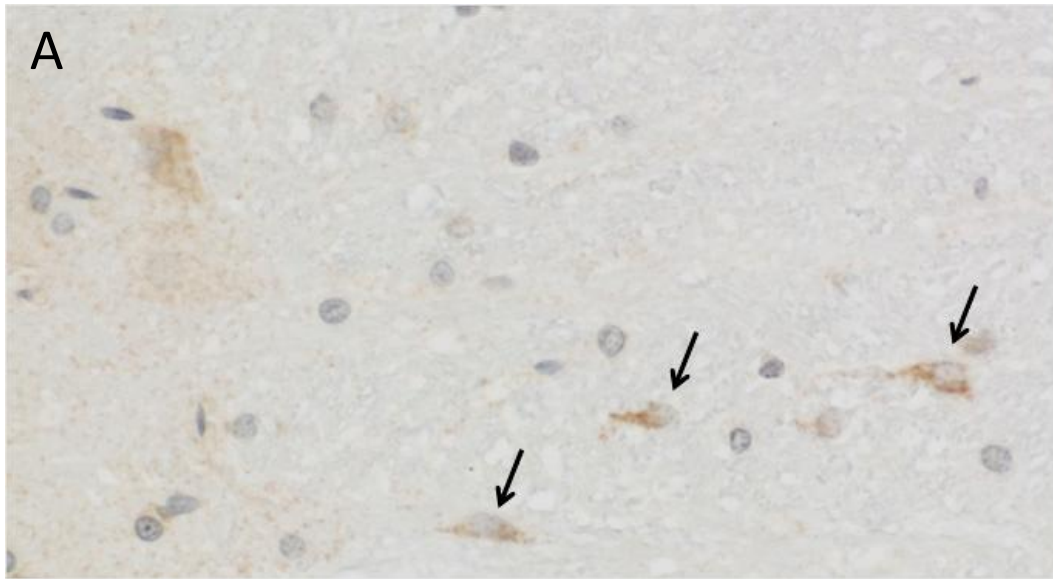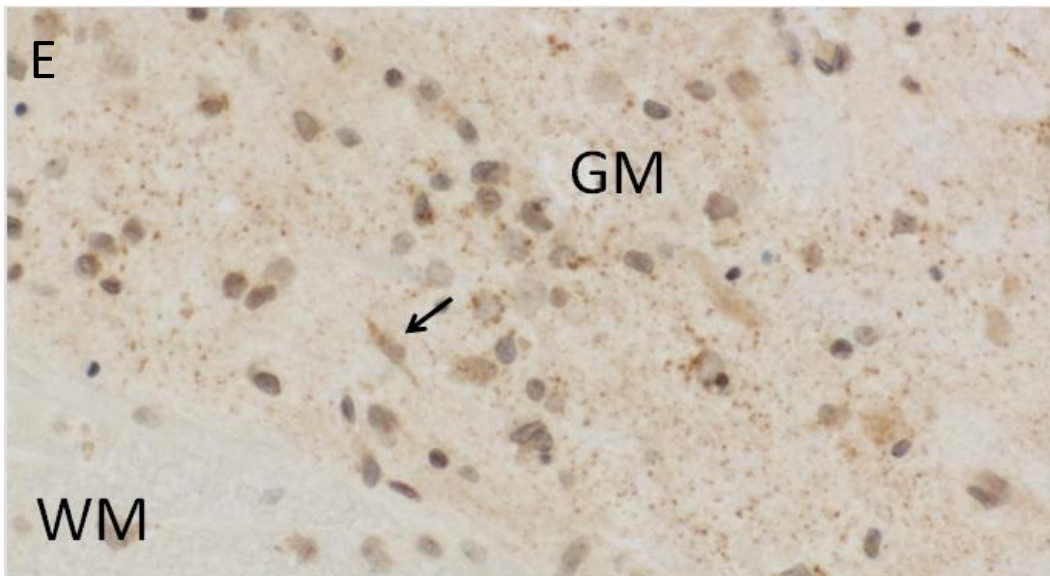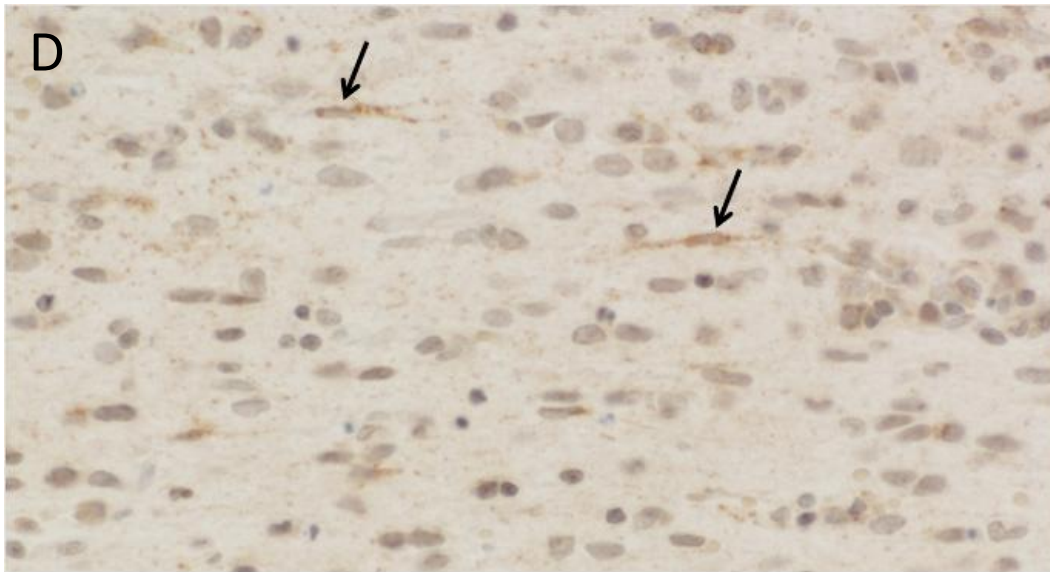
Figure 15: Spinal cord slices from PLP transgenic Lewis rats and wild type stained with haematoxylin (blue) and with Amigo2 (brown). Cells that, according to their morphology, look like microglia are marked with a black arrow. A) wild type, B) heterozygous PLP, C) homozygous PLP with a SCI, where the dotted line encloses the lesion site, D) and E) heterozygous PLP transgenic rats, which had been simultaneously injected with MBP specific T-cells. WM = white matter, GM = grey matter, LS = lesion site.

3.5 Flow Cytometry Analysis

To further verify the effect of IFN-γ on the Amigo2 expression in microglia, the cells were incubated with IFN-γ at a concentration of 100 ng/ml for 48 h and flow cytometry analysis was performed, showing that the cells express Amigo2.

The cells were stained for CD45 to prove, that the culture consisted only of CD45 expressing cells and does not contain any other contaminating cells. We stained two samples independently for CD45 and for Amigo2 and one sample was used for a double detection of both antibodies. Also isotype matched goat and mouse antibodies were used, which were not supposed to bind to microglia cells, as a negative control for the Amigo2 and CD45 staining. Another sample omitting any antibodies was analyzed as a general control. The primary antibodies were detected by secondary antibodies conjugated to FITC (CD45) or to PE (Amigo2).

Because microglia cells are known to express Fc receptors on their surface and therefore are able to bind antibodies in an unspecific manner, we blocked the cells with rat serum prior to the antibody staining. But nevertheless, the negative controls demonstrated that the cells, in a lower amount, were still capable of unspecific antibody binding, causing a shift between the unlabeled cells and the negative controls towards higher intensities (Fig. 16 D and 17 D).

Nearly all the cells were positive for CD45 and a strong shift in the fluorescence value peak of FITC labeled antibodies could be detected between the CD45 sample and the corresponding negative control (Fig. 16 A, B and D).

The fluorescent shift caused by the antibody against Amigo2 was much smaller, but appeared in all four independent experiments (Fig. 17 A). Taking a look at the forward and sideward scatters of the Amigo2 stained cells (Fig. 17 C), the positive cells are evenly distributed in the whole microglia population. This indicates a relative low expression of the protein in all cells and not only in a subpopulation.

The double staining revealed that the cells were double positive for CD45 and for Amigo2 (data not shown).
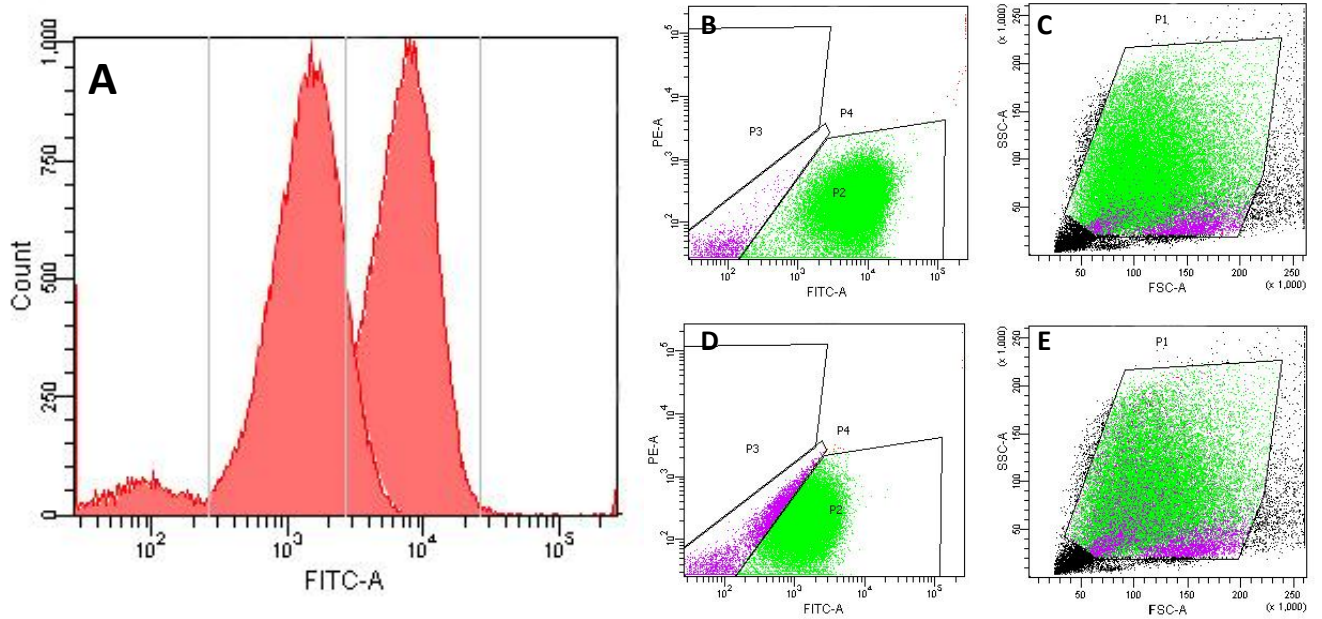
Figure 16: A) Histogram showing the expression of FITC labeled CD45. Between the control culture stained with a mouse IgG antibody as a negative control and the culture stained with the CD45 antibody, a clear shift of the fluorescence value peak from 1500 to 8000 is observable. B) & C) Dot plot presentation of the FITC labeled CD45 antibodies fluorescence intensities. D) & E) Dot plot presentation of the FITC labeled IgG antibodies fluorescence intensities. The shift in the intensity level of the negative control in comparison to unlabeled cells is due to unspecific binding of the antibody by the microglia cells (D).
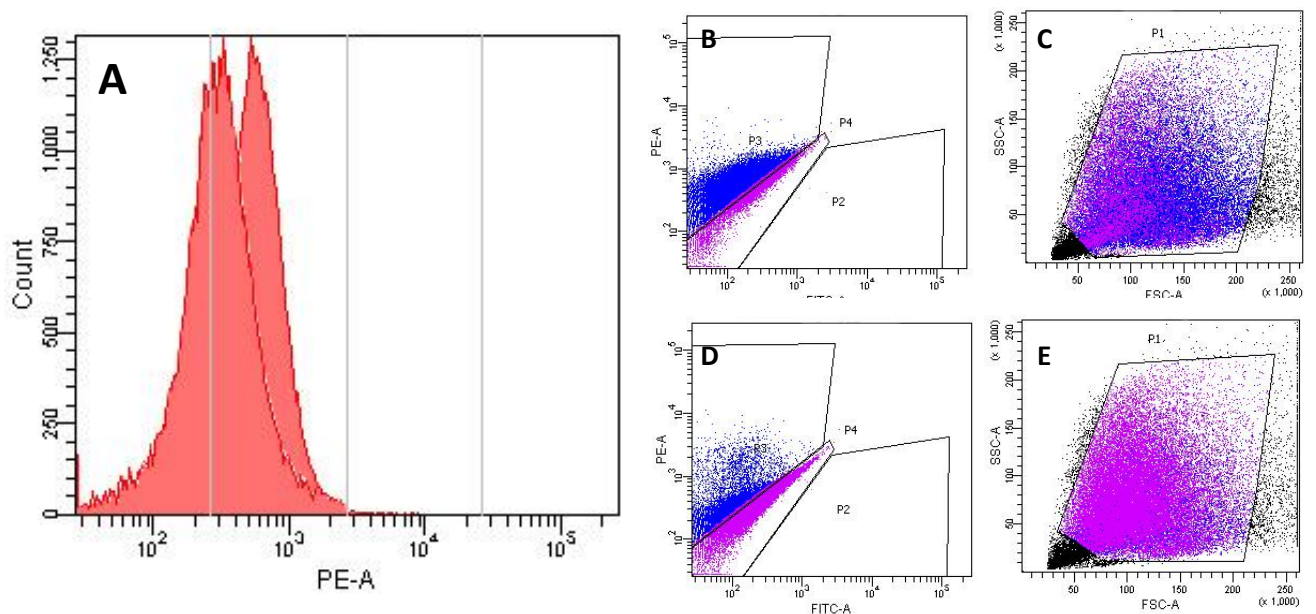

Figure17: A) Histogram showing the expression of PE labeled Amigo2. Between the control culture stained with a goat IgG antibody as a negative control and the culture stained with the Amigo2 antibody, a small shift of the fluorescence value peak from 300 to 550 is observable. B) & C) Dot plot presentation of the PE labeled Amigo2 antibodies fluorescence intensities. D) & E) Dot plot presentation of the PE labeled IgG antibodies fluorescence intensities.

3.6   Neuronal Cultures grown on Amigo2 coated dishes

It was shown that Amigo1 is an adhesion molecule favoring neuronal outgrowth and survival (Kuja-Panula et al., 2003). This raised the question whether Amigo2 has a similar function. To test this, neurons were cultured on Amigo2 coated dishes.

Neuronal cells were gained from dissected hippocampi from embryonic day 17 Sprague Dawley rats and cultured in 12-well plates, each well containing 1 ml of prewarmed neuronal medium. The wells were coated before with the recombinant extracellular domain of Amigo2 in different concentrations (0/ 3,125/ 6,25/ 12,5/ 25/ 50/ 100 µg/ml in PBS) (see section 2.4.2 and 2.8). To each well, the same amount of neuronal cells was added. The cells were seeded with a density of 50.000 cells/well. Pictures were taken from randomly selected microscopic fields after 24 h or 48 h and the cells and their neurites were counted.

After 24 h the neurons were able to adhere to the Amigo2 coated dishes and to induce the outgrowth of their neurites (Fig. 18).
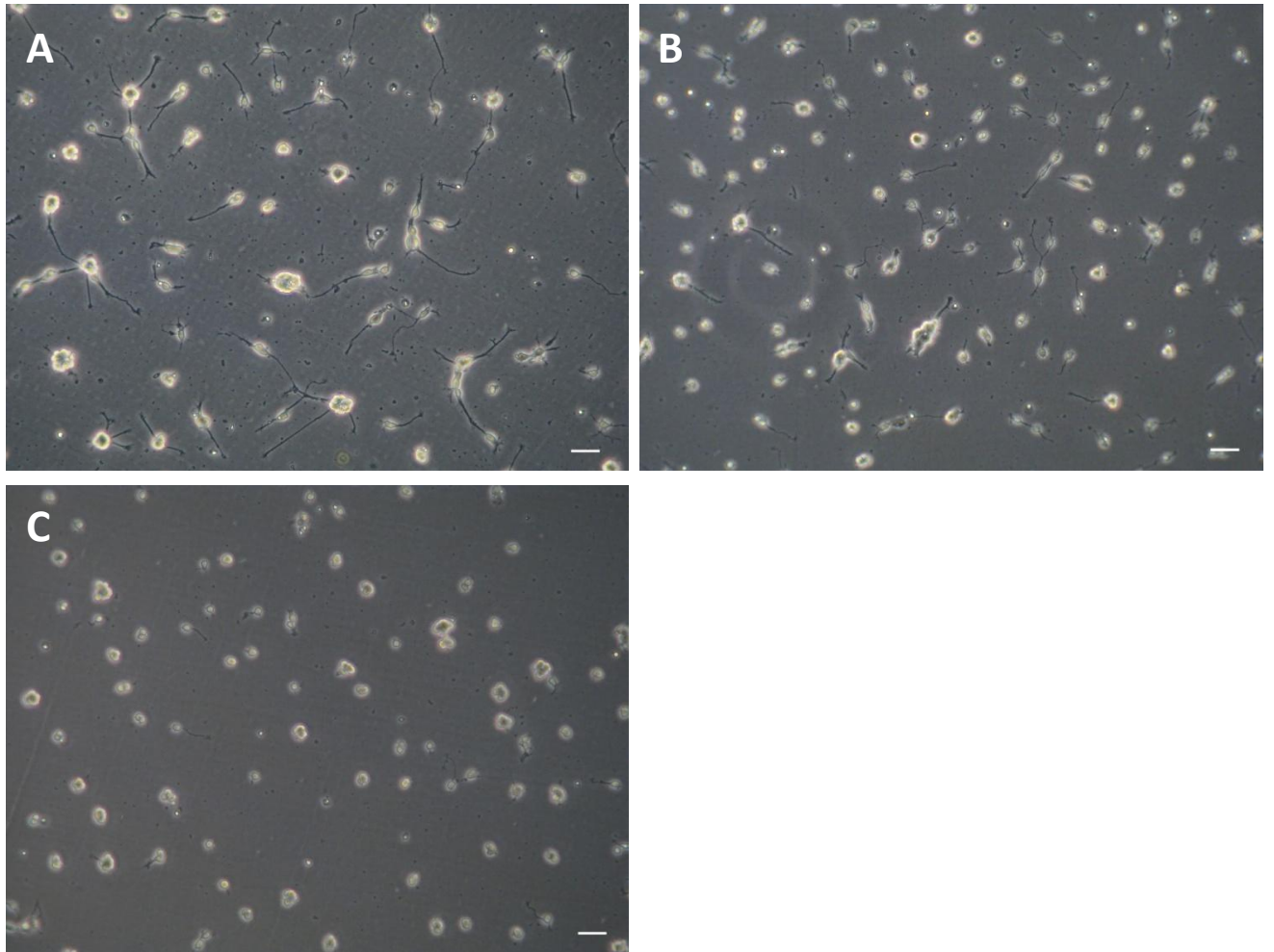
Figure 18: Pictures taken from randomly selected microscopic fields of neuronal cultures seeded on differentially coated plastic dishes 24 h after plating. A) Neurons plated on dishes coated with 100 µg/ml of the extracellular domain of Amigo2 are able to adhere and to differentiate, showing more and longer neurites than the other cultures. B) Neurons grown on dishes coated with 50 µg/ml or C) without any coating. Neurons growing on uncoated dishes develop only very short and very few neurites. Scale bars = 50 µm.

The wells coated with higher concentrations of the recombinant protein provided a more efficient substrate for the neurons to adhere to and contained more firmly attached cells and also more cells with processes (Fig. 19). In the well which was not coated with any proteins at all (0 µg/ml), only very few cells were able to adhere to the plastic surface and to induce neurite outgrowth. Also, those cells were not as firmly attached as the ones grown on coated dishes and detached easier.
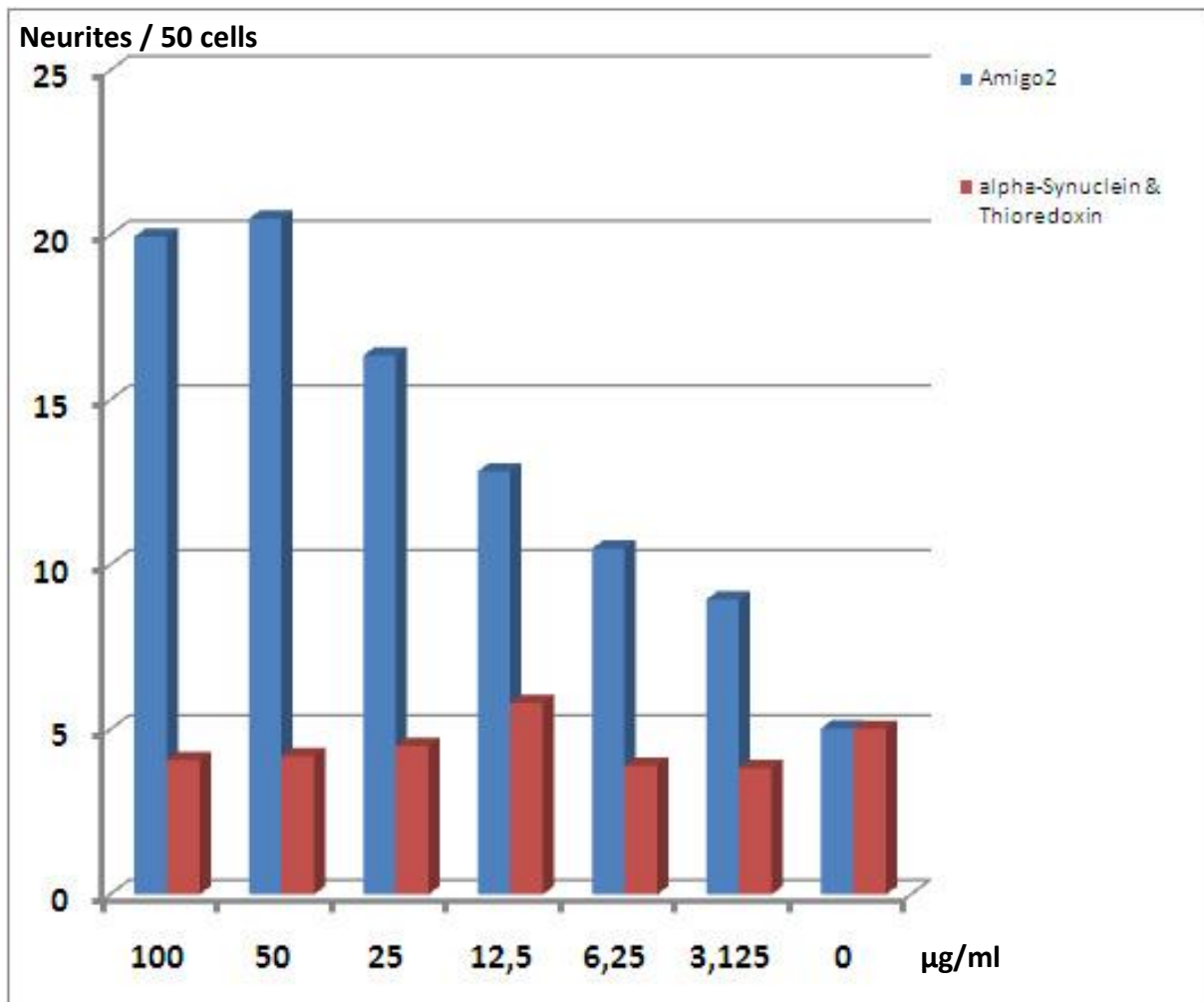
Figure 19: The recombinant Amigo2 protein induced the outgrowth of hippocampal neurites after 24 h of culture (blue bars). The control proteins, which were expressed and purified in the same way as Amigo2, did not induce any neurite outgrowth (red bars). As control proteins α-Synuclein and Theoredoxin were used and the data of both samples were pooled together. The data of the uncoated dishes (0 µg/ml) were pooled from all samples.

As a negative control, 12-well plates were coated with recombinant α-Synuclein or recombinant Thioredoxin, both expressed and purified in the same way as Amigo2 was, in the same concentration range (0–100 µg/ml). The neuronal cells were only very poorly able to adhere to the surface and to induce neurite outgrowth and no significant difference could be observed between the different concentrations, nor in comparison to the uncoated negative control (Fig. 19 and 20).
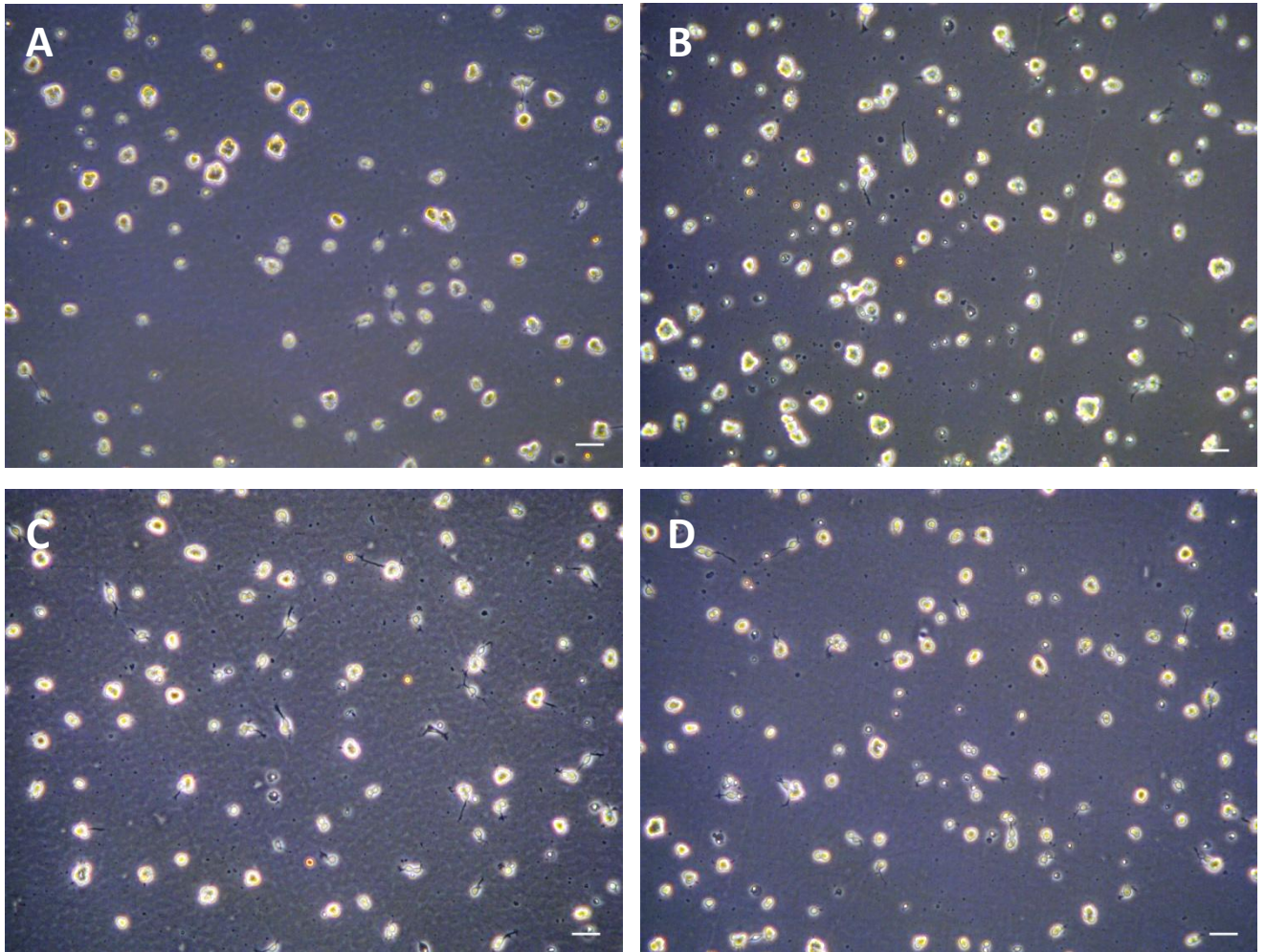
Figure 20: : Pictures taken from randomly selected microscopic fields of neuronal cultures seeded on differentially coated plastic dishes 24 h after plating. A) Neurons plated on dishes coated with 100 μg/ml or B) 50 μg/ml of α-Synuclein. C) Neurons plated on dishes coated with 100 μg/ml or D) 50 μg/ml of Thioredoxin. No significant differences can be observed between the different concentrations of the coated proteins, nor to the uncoated negative control (compare to Fig. 18 C).

Pictures were also taken 48 h after seeding the cells and the length of the outgrowing processes was determined. Only processes with the minimal size of twice the length of the cell soma were considered as neurites.

The neurons grown in wells coated with high concentrations of Amigo2 not only exhibit a higher number of processes, their neurites were also longer than the ones of the cells grown on lower concentrations of Amigo2 or in uncoated wells. In comparison to this, the cells grown on dishes coated with α-Synuclein or Thioredoxin showed no differences in the length of their processes regarding the different concentrations of the proteins used for coating. In

these control cultures, the processes had in general the same size as the ones from the cells grown on uncoated dishes (Fig. 21)
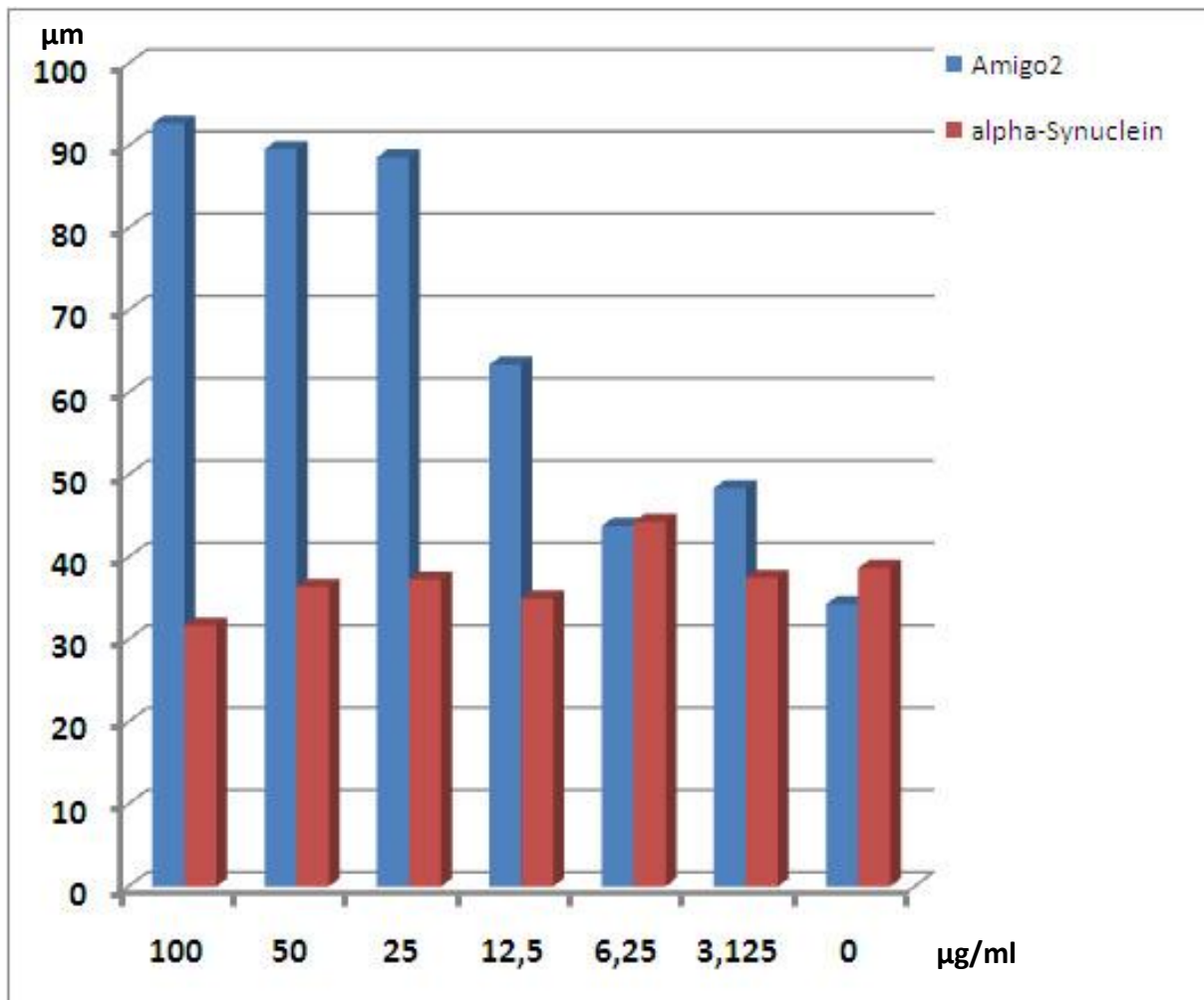

Figure 21: The neurites from the cells grown in wells coated with higher concentrations of Amigo2 were in general longer than those of the wells coated with lower concentrations (blue bars) or of the uncoated ones or of those coated with α-Synuclein as a negative control (red bars).

3.7 The Effect of Osteopontin on Neuronal Cultures

As described before, the treatment of microglia cultures with IFN-γ led to an upregulation of Amigo2 expression and to a downregulation of the expression of Osteopontin. Therefore, it was important for us, to also examine the effect of Osteopontin on neuronal cultures. 12-well plates were coated with Amigo2 (25 µg/ml in PBS) or with PLL (100 µg/ml in PBS) and used for culturing hippocampal neurons. Additionally, Osteopontin was added in different concentrations (0–1000 ng/ml) to the supernatant of the cultures and the cells were

photographed 24 h after seeding and treatment. Osteopontin seemed to have an effect on the cultures grown on Amigo2 coated wells, whereby the neurons grown in presence of high concentrations of the protein had less neurites than the ones grown in absence or in the presence of only low amounts of Osteopontin (Fig. 22).
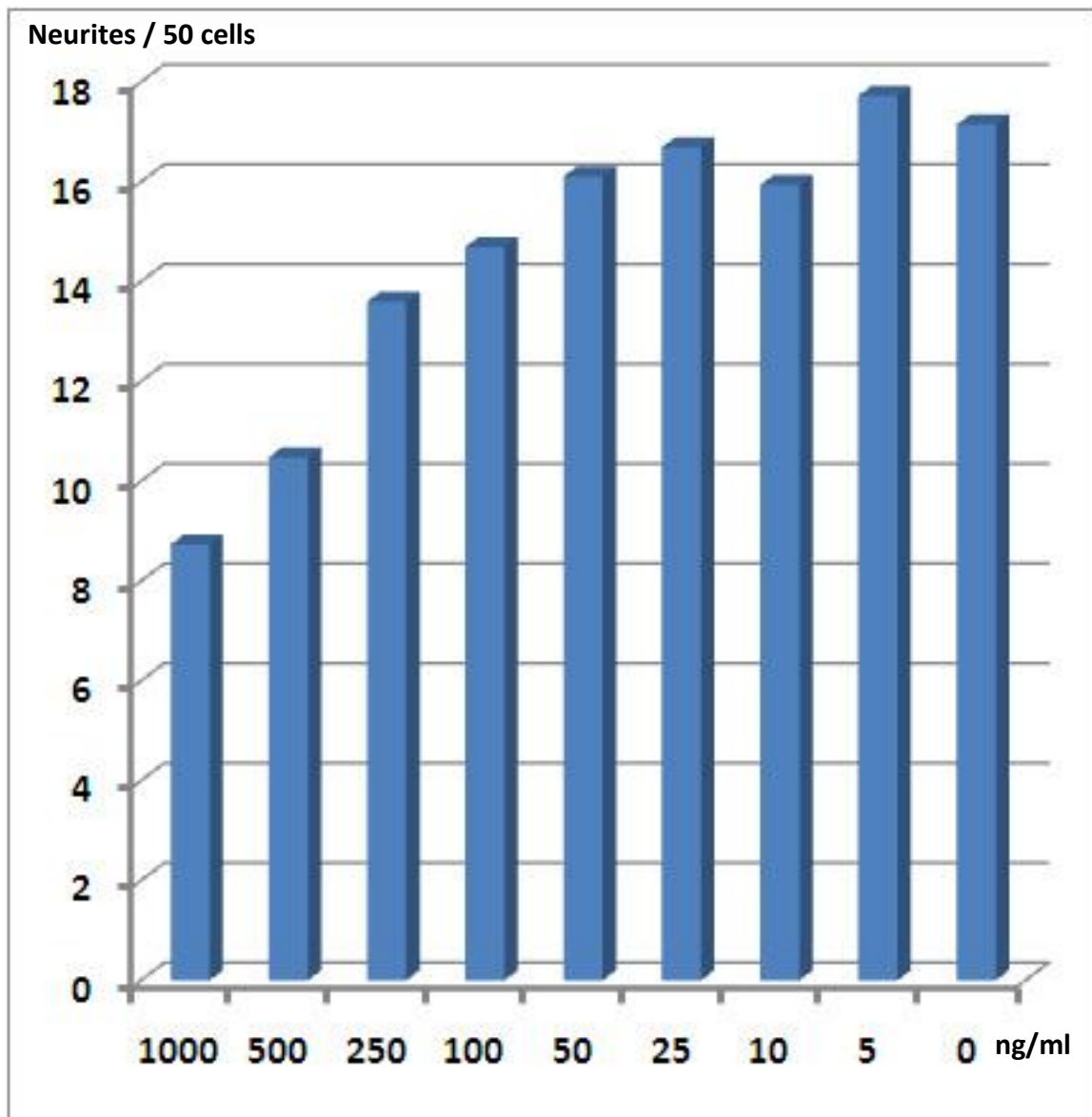
Figure 22: The outgrowth of neurites from hippocampal neurons grown on Amigo2 coated dishes (substrate coated with 25 µg/ml) in the presence of soluble Osteopontin in the medium in different concentrations (0 – 1000 ng/ml). Osteopontin in high concentrations inhibits the outgrowth of hippocampal neurites.

The processes of the cells grown in the presence of high Osteopontin concentrations also seemed to be shorter than the others (Fig. 23).
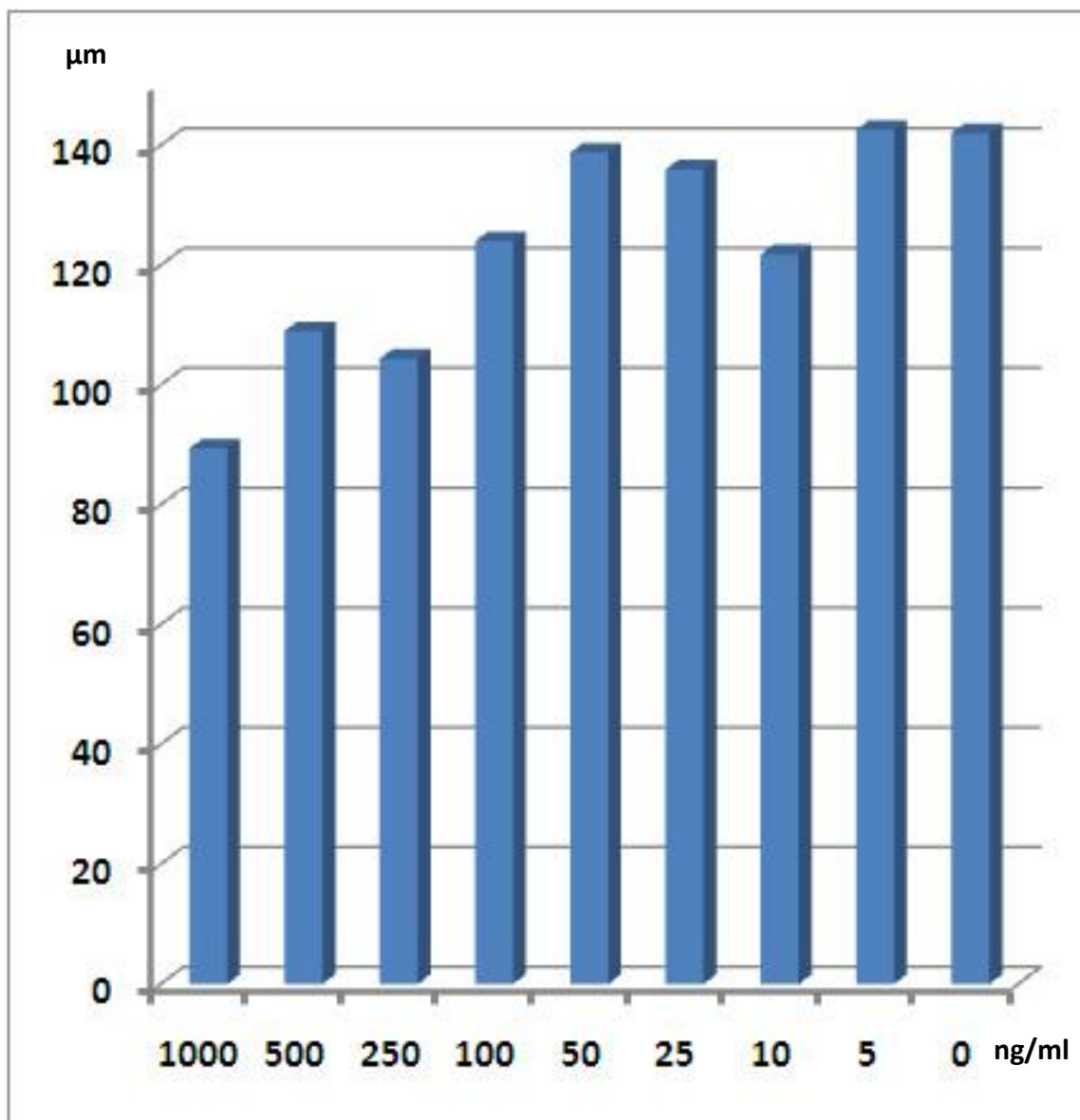
Figure 23: The length of the neurites grown on Amigo2 coated dishes (25 µg/ml) in the presence of high concentrations of soluble Osteopontin is shorter than the length from those grown in the absence of the protein.

No visible effects could be observed during the treatment of neuronal cultures grown on PLL coated dishes with different concentrations of Osteopontin (Fig. 24). Coating dishes with PLL is commonly used for neuronal cultures and provides optimal conditions for the cells to adhere to the substrate and to induce the outgrowth of their processes. In general, the neurons adhered more firmly to the PLL coated dishes and the adherence was more stable when compared to the cells grown on Amigo2 coated dishes. The neuronal cultures grown on PLL coated dishes exhibit a strong neurite outgrowth and form a neuronal network by building connections with each other (Fig. 6).
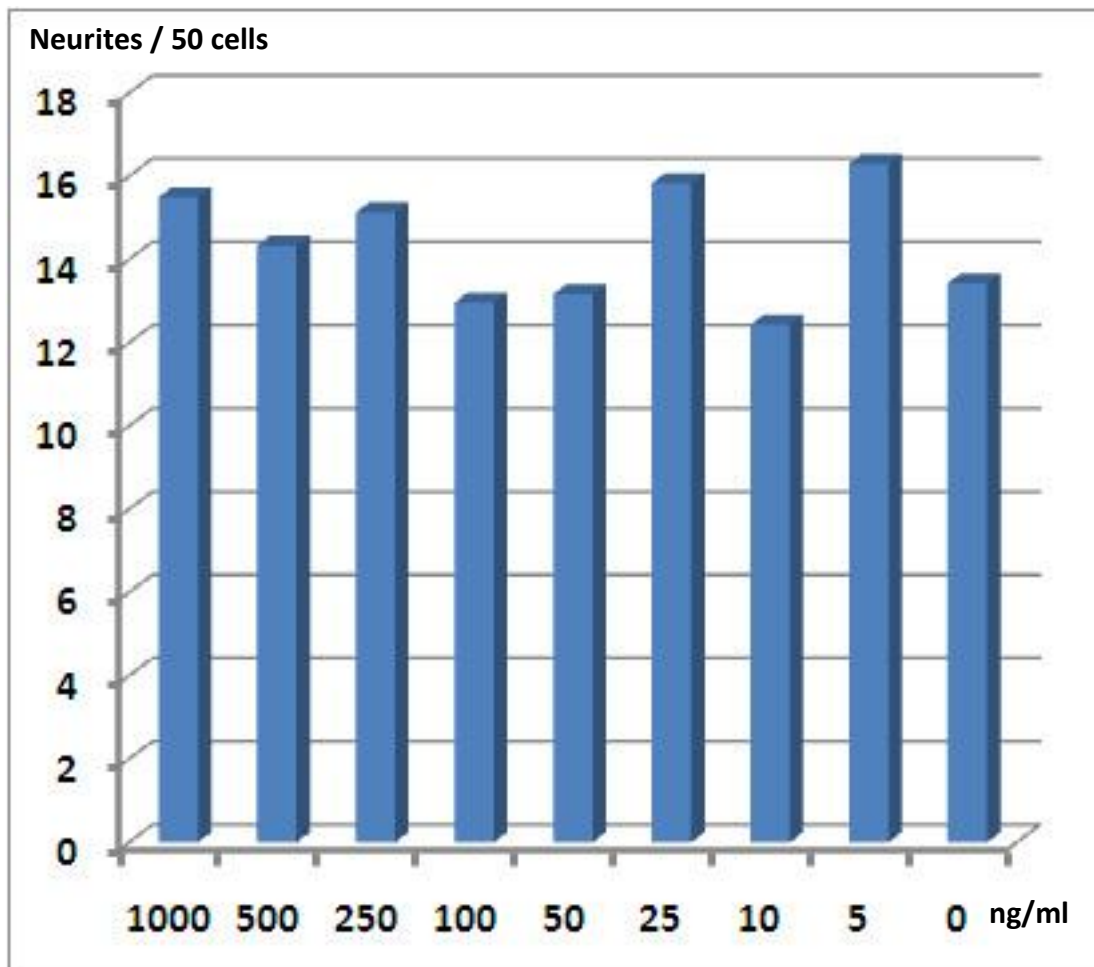
Figure 24: The outgrowth of neurites from hippocampal neurons grown on PLL coated dishes (substrate coated with 100 µg/ml) in the presence of soluble Osteopontin in the medium in different concentrations (0 – 1000 ng/ml). No effect of soluble Osteopontin on the outgrowth of neurites from neuronal cultures grown on PLL coated dishes can be observed.

3.8 Microglia Cultures treated with the extracellular domain of Amigo2

Because Amigo2 is able to bind itself and the other members of the Amigo protein family by hemophilic and heterophilc binding (Kuja-Panula et al., 2003), it might also influence the behavior of microglia cells.

To study any possible effect of the Amigo2 protein on microglia cells, primary microglia cultures were incubated with the recombinant extracellular domain of Amigo2 in a concentration of 10 µg/ml for 24 h (Fig. 25). After plating, the cells were placed in the incubator overnight before adding the protein to the medium the next morning.

Already after 5 h of incubation, a clear effect of the Amigo2 protein on the microglia cells became apparent, based on external inspection. The treated cells seemed to adhere much tighter to the bottom of the culture dishes than the control cultures did. This became apparent by the morphology of the cells, being shallower and therefore appearing darker under the microscope than the control cells, which had a more rounded up and spherical shape and appeared brighter. However, the opposite effect was seen at later time points.

After a 24 h period many of the treated cells had completely lost contact to the bottom of the culture dish and were floating in the medium. In contrast to this, the majority of the control cells did not detach and were still adherent to the surface.

During the whole 24 h period, no strong differences in the morphology of the control cells could be observed, whereby the treated cells first seemed to be strongly adherent, before losing the ability to adhere to the substrate.
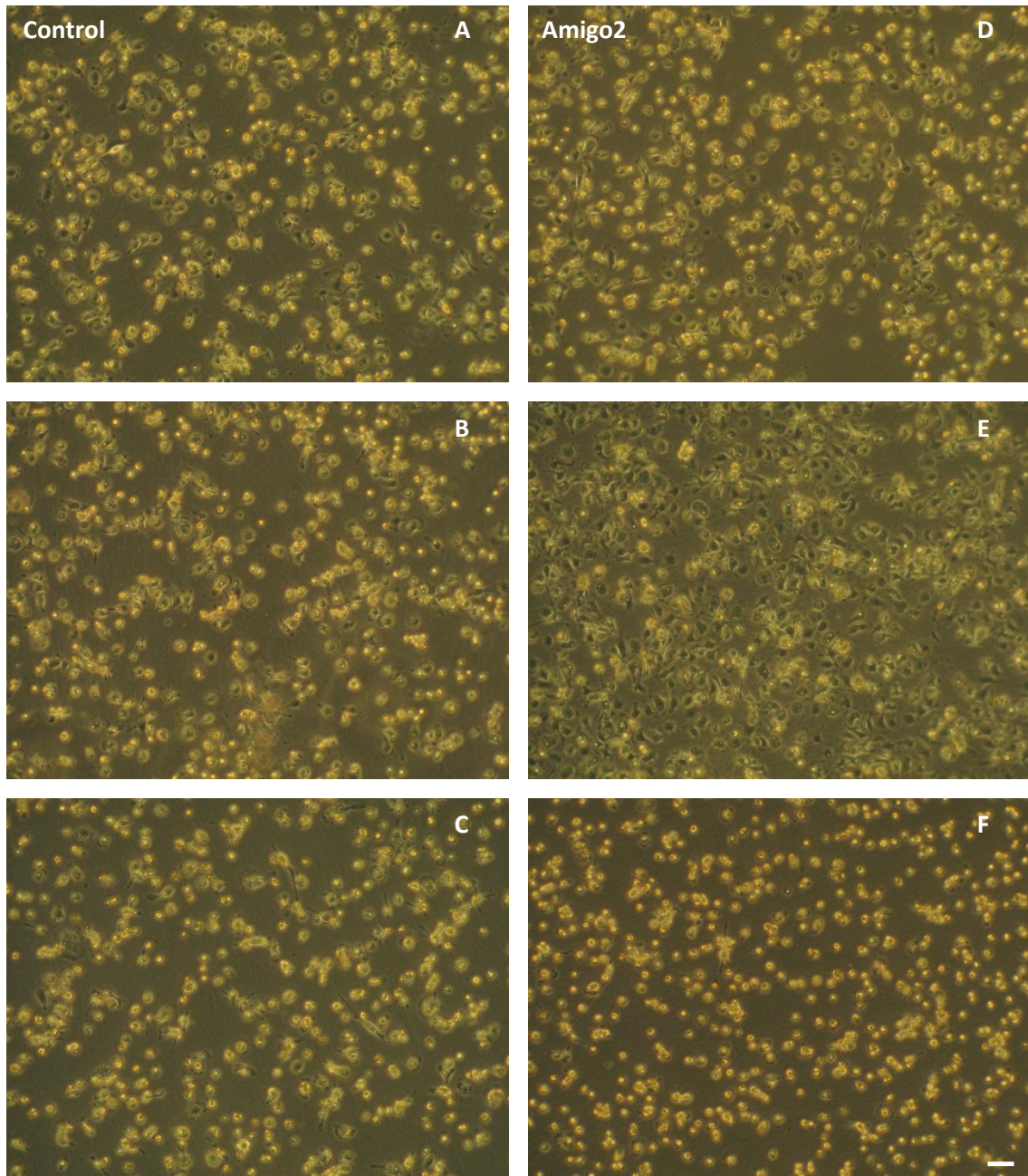
Figure 25: Primary microglia cultures incubated with 10 µg/ml of the extracellular domain of recombinant Amigo2. The Amigo2 protein was dissolved in 8 M urea and therefore the same amount of 8 M urea (14 µl) was added to the control culture. A) Control culture before, B) 5h and C) 24 h after the treatment. No drastic changes of the cell morphology could be observed. D) Amigo2 treated culture before, E) 5h and F) 24 h after the treatment. The cells first flatten and therefore appear darker (E), before they detach after 24 h (F). The control cells have a more rounded up and spherical shape (A-C). Scale bar = 50 µm.

4   Discussion

Tissue injury triggers an inflammatory reaction leading to the recruitment and infiltration of macrophages and/or microglia to the injury site, which is a necessary reaction for a proper healing response. Microglia and macrophages do play a critical role in tissue restoration, by removing dead cells, buffering toxic compounds and secreting signaling and neurotrophic factors. Therefore, the restricted CNS recruitment of macrophages and the limited activation of macrophages/microglia may be linked to the inability of the CNS to regenerate (Lazarov-Spiegler et al., 1998). The failure of neuronal regeneration is probably caused by the non-permissive postinjury environment of the CNS and not due to an intrinsic inability of the neurons, as it has been shown that some CNS axons were able to regrow, if provided with a permissive environment (David and Aguayo, 1981). There were reports, showing that the injection of autoimmune T-cells against MBP in rats with a SCI, cause a local inflammatory response at the injury site, that is beneficial for the recovery (Hauben et al., 2000). During this inflammation, TH1-cells secrete many different cytokines, including IFN-γ, and therefore lead to the recruitment and activation of other leucocytes, macrophages and microglia.

During my thesis, we wanted to investigate, whether microglia cells may contribute to an enhanced recovery of tissue injury following inflammation. Therefore we looked for a differential expression of immunological relevant genes in microglia treated with IFN-γ. We found Amigo2 to be upregulated in response to the cytokine, whereas Osteopontin expression is downregulated. Furthermore, we could show that the coating of Amigo2 protein bears a substrate for neurons to adhere to and induces the outgrowth of their processes. The addition of Osteopontin partly inhibited this effect.

Amigo2 is upregulated in microglia cells as a response to the proinflammatory cytokine IFN-γ. The protein, as well as its family member Amigo1, is also expressed in neurons (Ono et al., 2003; Kuja-Panula et al., 2003). The extracellular part of the Amigo proteins contains many motifs that are typically found in adhesion molecules and are needed for protein-protein interactions. Although there are no known domains in the intracellular part of the protein, it cannot be ruled out that it is also able to transduce a signal by inducing an intracellular

cascade (Rabenau et al., 2004). However, we could show that Amigo2, used as a substrate for hippocampal neurons, is sufficient to induce the outgrowth of neurites. Neuronal cells were able to adhere to the substrate and to produce processes. This neurite outgrowth could be partly inhibited by the addition of Osteopontin to the medium. Amigo2 is expressed in neurons in an activity dependent manner, inhibits apoptosis and promotes the survival of the cells (Ono et al., 2003).

Maybe the upregulation of Amigo2 in microglia during inflammation helps to establish a closer contact of microglia cells and neurons, which might be beneficial. Microglia can be neuroprotective and enhance nerve repair (Streit, 2005). Activated microglia can possibly also protect neurons by removing synaptic input from the cells, a process known as synaptic stripping, which might be helpful in promoting regrowth and remapping of neuronal connections. The underlying mechanism of this process is still unknown, but activated microglia and possibly astrocytes are thought to play a major role. It has been shown, that activated microglia are able to strip synapses from cerebral cortex neurons (Trapp et al., 2007). A possible reason for stripping off synapses from injured or stressed neurons is to reduce potential excitotoxic influence, by removing more excitatory than inhibitory synapses (Linda et al., 2000). The close physical proximity of microglia and neurons after a lesion may therefore be neuroprotective by shielding the cell from excitatory stress and facilitating an exchange of signaling or trophic molecules (Cullheim and Thams, 2007). The upregulation of Amigo2 protein in activated microglia cells may contribute to this process by serving as a membrane bound adhesion molecule, promoting the interplay between neurons and microglia.
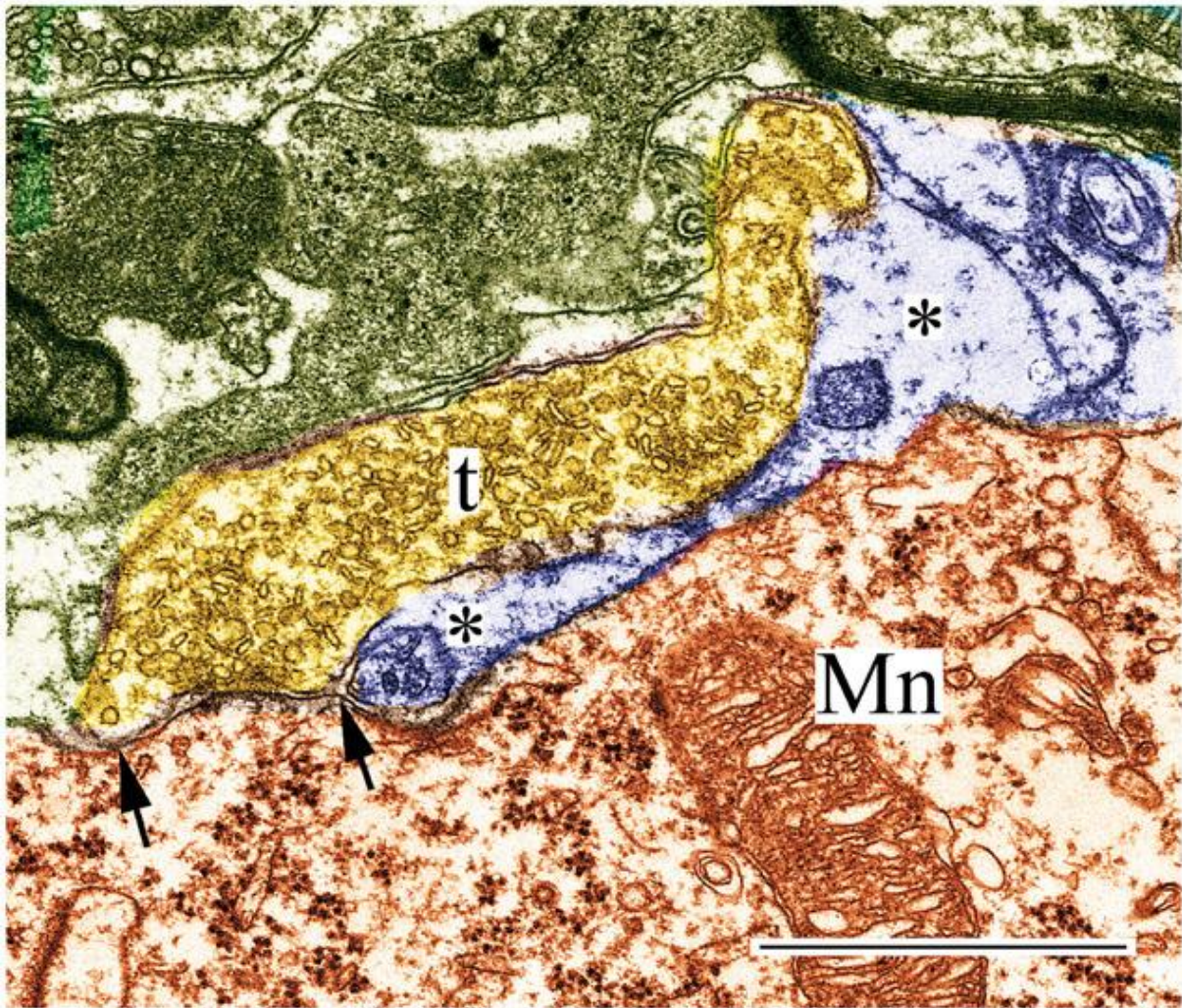
Figure X: Example of ongoing "synaptic stripping" after axon lesion. Electron micrograph showing a partially detached nerve terminal (t) on an axotomized sciatic motorneuron (Mn) in the spinal cord at 1 week after sciatic nerve transection. Note the presence of a finger-like glia process (*) between the motorneuron membrane and a large part of the terminal. The arrows denote the borders for the apposition of the terminal to the motorneuron. Scale bar = 1 μm (reproduced from Cullheim and Thams, 2007).

Immunoblotting of total microglia cell proteins with anti-Amigo2 antibody revealed a band around 50 kDa, which is lighter than the expected weight of the protein of 65 kDa. Maybe this is due to the existence of a truncated product. The protein might be cleaved and the extracellular domain secreted. Based on the weight of Amigo2 that is given in literature (65 kDa) and the assumption, that a secreted extracellular domain would still contain all the important binding domains, like the Ig- and LRR-domains, the weight of a possibly secreted form would be around 50 kDa. But of course, we cannot rule out that this result is based on unspecific binding of the antibody. The strong and specific band in the positive control containing the recombinant protein reveals that the antibody does recognize the

extracellular domain of Amigo2, like it is supposed to do. The interplay of microglia and neurons may involve either the membrane bound or a secreted form. Also in the pictures taken with the confocal microscope, it appears more as if the protein is located intracellular than on the cell surface. It is always a problem to stain microglia cells because of their Fc-receptors, which are located at the cell surface and are able to unspecifically bind antibodies. However, if the binding would be due to unspecific binding, you would expect all microglia cells to be positive and not only a part of the total cell population.

The immunohistochemistry staining of spinal cords from different rat models with Amigo2 antibodies showed that Amigo2 positive microglia can already be found in the normal control rats, but only in the white matter of the spinal cord. In the spinal cord of a PLP transgenic rat, which shows signs of microglia activation, Amigo2 positive microglia were also located in the white matter, but in higher numbers. Homozygous PLP transgenic rats with a spinal cord injury showed Amigo2 positive macrophages, which have infiltrated the tissue at the lesion site. PLP transgenic rats which also suffered under CNS inflammation induced by the injection of MBP specific T-cells not only show higher numbers of Amigo2 positive microglia/macrophages, but also a different distribution, where cells can be found in the white and grey matter of the spinal cord. Those pictures indicate, that microglia in the spinal cord do express Amigo2 and that the number of Amigo2 positive cells increases during inflammation or general microglia activation, like it is the case in the PLP transgenic rats. Also macrophages that infiltrate the tissue at the lesion site express the Amigo2 protein.

We also looked for an effect of the extracellular domain of Amigo2 on microglia cells. Therefore microglia cultures were treated with 10 µg/ml of recombinant Amigo2 protein in the medium. The protein can bind itself and its family members via a homo- or heterophilic binding mechanism (Kuja-Panula et al., 2003) and thus should be able to influence microglia cells that express Amigo2 in a membrane bound form. We observed a strong effect of the protein on the binding properties of the cells. After 5 h of incubation, the cells appeared shallower and seemed to have tightened their adherence to the substrate. This may be caused by the extracellular domain binding to the microglia cells and connecting them to the ground of the cell culture dish or to other microglia cells. However, after 24 h the opposite effect occurs and the cells start to detach. Maybe the soluble recombinant protein starts to build aggregates and therefore promotes the detachment of the cells.

Here we show that Osteopontin, besides its neuroprotective actions, exhibits an inhibitory function when it comes to axon outgrowth. Hippocampal neurons grown on Amigo2 coated dishes in the presence of recombinant Osteopontin had less outgrowing neurites than those cells growing on the same substrate in the absence of the protein.

Osteopontin is expressed in many tissues suffering from injury and after SCI, it is thought to be upregulated in microglia cells (Hashimoto et al., 2007). In vitro experiments done in our laboratory have shown that microglia cells treated with IFN-γ downregulate the expression of Osteopontin mRNA. But, although IFN-γ is one of the key molecules for microglia activation during inflammation, the cells are also influenced by many other factors in vivo and therefore their response in the living organism might differ. The effect of Osteopontin after injury is a double-edged sword. On the one hand its neuroprotective effect might be beneficial in SCI, on the other hand its inhibitory effect on outgrowing axons might suppress the regeneration process. On microglia itself the protein has a proinflammatory effect, as we could show that it upregulates the expression of the cytokines IL-1α, IL-1β, IL-6 and TNF-α on the mRNA level. This finding is in agreement with the downregulation of IL-1β, IL-6 and TNF-α in OPN^{-/-} mice shown in other reports (Hashimoto et al., 2007).

Additional to the limited involvement of macrophages in the postinjury processes of the CNS, it was also shown that macrophage activation during inflammation differs in PNS and CNS. The implantation of macrophages prestimulated with PNS tissue into completely transected spinal cords led to a partial locomotor recovery (Rapalino et al., 1998; Schwartz et al., 1999). A possible role in causing those differences between the CNS and PNS plays Osteopontin, which is strongly expressed by macrophages at the lesion site of crushed optic nerves (part of the CNS), but not at the sciatic nerve (PNS) (Kury et al., 2005).

Passive immunization with MBP specific T-cells into spinal cord injured rats led to an infiltration of the specific T-cells into the spinal cord and caused a significant decrease in the lesion volume (Lu et al., 2008). This finding is in perfect agreement with the "protective immunity" hypothesis of Schwartz and colleagues. In contrast to this, other groups, using the same animal models and vaccine protocols, were not able to reproduce these beneficial effects (Ankeny and Popovich, 2007). So, besides those reports indicating a positive effect of a boosted immune response in the functional recovery of SCI, there are also controversial

opinions, interpreting a neuroinflammatory response in SCI as an amplifier of tissue injury. Transgenic mice enriched in MBP reactive T-cells suffered from exacerbated axonal injury, demyelination and functional loss after SCI (Popovich and Jones, 2003). However, IFN-γ, which is released during neuroinflammation, has been recently shown to decrease the chondroitin sulfate proteoglycan (CSPG) expression in reactive astrocytes, which is a critical axon outgrowth inhibitor (Fujiyoshi et al., 2010). Here we have shown that IFN-γ upregulates the expression of Amigo2 in microglia, a protein with the ability to induce neurite outgrowth in neuronal cultures. Furthermore, the cytokine downregulates the expression of Osteopontin, a protein which was shown to inhibit axon outgrowth.

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