The Role of the p75 Neurotrophin Receptor in Experimental Inflammation of the Central Nervous System

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Declaration

I hereby declare that I have written my Ph.D. thesis entitled “The Role of the p75 Neurotrophin Receptor in Experimental Inflammation of the Central Nervous System” independently and with no other sources and aids than quoted. This thesis has not been submitted elsewhere for any academic degree.

Tobias Dallenga
Göttingen, October 2010
Abstract

The extent of the permanent clinical disability in multiple sclerosis patients emerges from axonal damage and loss. In this study, we show a major role of the low affinity neurotrophin receptor p75\textsuperscript{NTR} for axonal damage in experimental autoimmune encephalomyelitis (EAE). After EAE induction by active immunization with the myelin oligodendrocyte glycoprotein peptide MOG\textsubscript{35-55}, p75\textsuperscript{NTR} -/- mice suffer from a more severe disease course, demyelination, and axonal loss. To elucidate whether the increased disability derives from a more aggressive inflammation or a more vulnerable central nervous system (CNS), I examined both, the immune system during peripheral disease generation and the overt disease phase, and cells of the CNS in vivo, ex vivo, and in vitro. No difference with regard to the quality of inflammation was found using immunohistochemical, flow cytometric, ELISA, and mRNA analysis, ruling out a major role of the immune cell populations examined. However, constitutive expression of p75\textsuperscript{NTR} by B cells suggests a role for p75\textsuperscript{NTR} during immune response generation within lymph nodes, since p75\textsuperscript{NTR} -/- mice show increased disease scores from the very beginning on.

To circumvent the effects of p75\textsuperscript{NTR} deficiency during peripheral priming, EAE was also induced by adoptive transfer of an encephalitogenic MOG\textsubscript{35-55}-specific T cell clone in p75\textsuperscript{NTR} -/- and wild type (wt) animals with similar disease incidence, onset and kinetics. Comparable degree and quality of inflammation were found by immunohistochemical and mRNA analysis in both strains at the peak of disease. However, p75\textsuperscript{NTR} deficient animals suffered from significantly more severe disease in the chronic disease stage due to increased axonal damage and loss. This suggests a protective role for p75\textsuperscript{NTR} within the CNS. In this work it is shown that astrocytes, but not microglia, express the p75\textsuperscript{NTR} constitutively. However, no p75\textsuperscript{NTR} mediated regulation of cytokine/chemokine and respiratory oxygen species (ROS) production by astrocytes was found in vitro. These data lead to a neuroprotective role of p75\textsuperscript{NTR} on neurons and axons in inflammatory CNS disease.

Active immunization of bone marrow chimeric mice, in which only immune or CNS parenchymal cells, respectively, carry a functioning p75\textsuperscript{NTR} confirm these results. However, while both, p75\textsuperscript{NTR} -/- → wt and wt → p75\textsuperscript{NTR} -/- chimeras suffer from a more severe disease course than wt → wt at peak of disease, only wt → p75\textsuperscript{NTR} -/- animals exhibit increased early axonal damage and loss.

In summary, our data suggest a role of p75\textsuperscript{NTR} towards a more aggressive inflammation within the peripheral immune system (namely mediated by B cells) as well as...
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>allopheocyanin</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>BAX</td>
<td>B cell lymphoma 2-associated X protein</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>Bel-xL</td>
<td>B cell lymphoma-extra large</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow derived macrophages</td>
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<tr>
<td>BME</td>
<td>basal medium eagle</td>
</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
</tr>
<tr>
<td>CCL</td>
<td>CC motif ligand</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund's adjuvant</td>
</tr>
<tr>
<td>CIS</td>
<td>clinically isolated syndrome</td>
</tr>
<tr>
<td>CM-H₂DCFDA</td>
<td>5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>1. cerebrospinal fluid 2. colony stimulating factor</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC motif ligand</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<tr>
<td>dATP</td>
<td>desoxyriboadenosinetriphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>desoxyribocytidinetriphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>desoxyriboguanosinetriphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>desoxyribonucleotide</td>
</tr>
<tr>
<td>Dpi</td>
<td>days post immunization</td>
</tr>
<tr>
<td>dTTP</td>
<td>desoxyribothymidinetriphosphate</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>Fig.</td>
<td>figure</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FoxP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphat dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>GTPase</td>
<td>guanosine triphosphatase</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HE</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HEPES</td>
<td>hydroxyethyl piperazine-ethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IPA</td>
<td>isopropanol</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kB</td>
<td>kilo bases</td>
</tr>
<tr>
<td>LFB/PAS</td>
<td>Luxol fast blue / periodic acid-Schiff</td>
</tr>
<tr>
<td>LINGO-1</td>
<td>leucine rich repeat and Ig domain containing 1</td>
</tr>
<tr>
<td>LNGFR</td>
<td>low affinity nerve growth factor receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAG</td>
<td>myelin associated glycoprotein</td>
</tr>
<tr>
<td>MAGE</td>
<td>melanoma antigen gene</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>matrixmetalloprotease</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>N</td>
<td>nitrogen</td>
</tr>
<tr>
<td>Na</td>
<td>sodium</td>
</tr>
<tr>
<td>Na-P</td>
<td>sodium pyruvate</td>
</tr>
<tr>
<td>NADE</td>
<td>p75NTR-associated cell death executor</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
</tr>
<tr>
<td>NF-H</td>
<td>heavy neurofilament</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NGF-R</td>
<td>nerve growth factor receptor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>Nogo</td>
<td>neurite outgrowth inhibitor</td>
</tr>
<tr>
<td>NogoR</td>
<td>neurite outgrowth inhibitor receptor</td>
</tr>
<tr>
<td>NOS2</td>
<td>nitric oxide synthase 2</td>
</tr>
<tr>
<td>NRAGE</td>
<td>neurotrophin receptor-interacting MAGE homolog</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrophin 3</td>
</tr>
<tr>
<td>NT-4/5</td>
<td>neurotrophin 4 / 5</td>
</tr>
<tr>
<td>NT-R</td>
<td>neurotrophin receptor</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 low affinity neurotrophin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>penicillin / streptomycin</td>
</tr>
<tr>
<td>PerCP</td>
<td>peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetat</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PP-MS</td>
<td>primary progressive multiple sclerosis</td>
</tr>
<tr>
<td>PR-MS</td>
<td>progressive relapsing multiple sclerosis</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family member A</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RR-MS</td>
<td>relapsing remitting multiple sclerosis</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SP-MS</td>
<td>secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>TBE</td>
<td>tris/borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethyle benzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFRSP</td>
<td>tumor necrosis factor receptor superfamily</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomyosin-related kinase</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>WM</td>
<td>white matter</td>
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</table>
1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS), first described by Jean Martin Charcot in 1868, is thought to be an autoimmune, chronic inflammatory, demyelinating disease of the central nervous system (CNS). Besides destruction of myelin sheaths and loss of oligodendrocytes resulting in circumscribed demyelinated areas of the white matter (WM), another hallmark of MS pathology is axonal damage and subsequent loss of axons as well as neuronal cell death (Sospedra & Martin, 2005). Various neurological symptoms can be observed in MS patients depending on the localization of these lesions within the brain and/or spinal cord.

MS is the most common neurological disease with persistent disability in young adults with an incidence of 60 to 200 per 100,000 people in industrialized countries. Women are more often affected than men (Sanders & De Keyser, 2007).

Even though the cause of disease has not been clarified yet, among others potential roles of genetic predisposition, environmental influences, changes in the hormonal balance, viral infections and other pathogens are discussed (Sospedra & Martin, 2005).

The disease course is highly heterogeneous from patient to patient and not predictable. However, four different prototypic disease courses are distinguished (Lublin & Reingold, 1996):

1) Relapsing remitting MS (RR-MS)

About 80 to 85% of MS patients suffer from unpredictable relapses followed by periods of remission.
2) Secondary progressive MS (SP-MS)

RR-MS usually transforms into SP-MS with a steady worsening of symptoms without clear phases of remission.

3) Primary progressive MS (PP-MS)

PP-MS is characterized by a steady progression of disability from the onset.

4) Progressive relapsing MS (PR-MS)

The disease course of PR-MS is the same as PP-MS but with superimposed attacks and partial remissions.

A typical MS disease course (Fig. 1.1) begins with an initial episode of neurological symptoms, the so called clinically isolated syndrome (CIS), with first symptoms like changes in sensation and impaired vision. After the attack the deficits completely resolve followed by a symptom-free period. During the subsequent relapsing remitting disease stage and due to
several attacks the CNS takes lasting damage resulting in permanent neurological disabilities like paresis, disturbances of sensation or vision or bladder dysfunction. After several years and relapses the majority of patients enters the secondary progressive disease phase which is characterized by a steady worsening with our without superimposed relapses. During the disease course the cerebral volume is decreasing while the total lesion volume is increasing.

1.1.1 Pathogenesis of MS

Even though the exact etiology of MS is still unknown, it is thought that it emerges on the cellular level from autoreactive cluster of differentiation 4 positive (CD4⁺) T helper cells that recognize self antigens of the CNS, mainly the myelin sheath (Sospedra & Martin, 2005), e.g. myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG). This is confirmed by findings of oligoclonal CD4⁺ T cells in lesions and in the cerebrospinal fluid (CSF) of MS patients (Sospedra & Martin, 2005). However, the mechanisms of generation of autoreactive T cells are still unknown.

Potential autoreactive T cells could possibly be activated in the peripheral immune system, e.g. by viral infection. The cell adhesion molecules (CAMs), like integrins, on the surface of T cells allow them to adhere to endothelial cells and subsequently pass the blood brain barrier (BBB) to infiltrate the brain and spinal cord parenchyma. Within the CNS the T cells receptors (TCRs) interact specifically with the autoantigen presented by perivascular cells. Consecutively secretion of proinflammatory cytokines like interleukin-2 (IL-2), tumor
necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Navikas & Link, 1996) and chemokines like CC motif ligand 2 (CCL2) and CXC motif ligand 10 (CXCL10) recruit more resident cells (microglia, astrocytes) as well as cells from the peripheral immune system (macrophages, T and B cells) (Lassmann et al., 2007). Matrix metalloproteases (MMPs), reactive oxygen species (ROS) and nitric oxides (NO) are produced, which open the BBB. In this inflammatory milieu damage to myelin, oligodendrocytes and axons occur. Excitotoxicity, myelin phagocytosis and apoptosis result in demyelinated axons, damage to axonal transport, axonal transections, loss of neurons and oligodendrocytes, and proliferation of astrocytes and autoreactive immune cells (Sospedra & Martin, 2005).

In RR MS, after the inflammatory episode a shift to an antiinflammatory T helper cell 2 (Th2)-mediated immune response occurs, including production of IL-10, TGF-β and growth factors, clearance of debris, activation of oligodendrocyte precursor cells (OPCs) and remyelination (Blakemore & Keirstead, 1999; Sospedra & Martin, 2005).

Involvements of potential autoreactive antibodies by B cells, complement factors (Parker, 1993) and cytotoxic CD8+ T cells are also discussed (Babbe et al., 2000; Bitsch et al., 2000).

Recent magnetic resonance imaging (MRI) studies could show that the degree of axonal damage and loss directly correlates with the degree of persistent disability in MS patients (De Stefano et al., 2005; De Stefano & Filippi, 2007; Kornek et al. 2000; Lovas et al. 2000; Hendriks et al. 2005). This emphasizes the importance of maintaining axonal and neuronal function in MS.

### 1.1.2 Histopathology of MS

The clinical heterogeneity of MS is also reflected in the diversity of lesion types. Lucchinetti et al. suggest a classification into four different lesion types:

1) The lesion has a clearly defined border and is characterized by macrophage and T cell infiltration as well as demyelination but only little oligodendrocyte loss. Subsequent remyelination takes place quickly.
2) Additionally to the characteristics of type 1 lesions, deposits of antibodies and complement factors occur.

3) Although dominated by macrophage and T cell infiltration like type 1 lesions, type 3 areas of inflammation have a diffuse border. Prominent apoptosis of oligodendrocytes is observed.

4) Type 4 lesions are defined by primary oligodendrocyte damage in the periplaque white matter, and have so far only been observed in autopsy cases.

1.2 Animal models of multiple sclerosis

For further investigation and basic research on CNS inflammation under defined and reproducible conditions animal models are indispensable.

Very first observations of accidentally induced encephalomyelitis resulting in paralytic disease go back to the end of the 19th century. Louis Pasteur used dried rabbit spinal cords as a vaccine against rabies, when in some cases patients developed paralyses (Balaguer, 1888). Being aware of this phenomenon, first experiments were done in rabbits using human spinal cord tissue (Koritschoner & Schweinburg, 1925). Thomas M. Rivers was the first who linked these findings to neurological complications in other diseases and established the most common model, experimental autoimmune encephalomyelitis (EAE) (Rivers et al. in 1933). Since then, different animal models of MS were established which represent different aspects of the disease. For instance, the cuprizone model is mainly used to analyze mechanisms of demyelination and remyelination (Blakemore, 1972).

Because it shares many immunological similarities with regard to histopathology, EAE became the most studied method. Many MS hypotheses as well as pharmaceutical drugs originate from EAE research.
1.2.1 Experimental autoimmune encephalomyelitis

Back in 1933 CNS tissue homogenate was injected subcutaneously in mammals to induce experimental inflammation of the CNS. Nowadays, purified myelin (or other CNS-related) proteins as well as short peptides are usually used. EAE has been successfully induced in many different mammals like dogs, cats, pigs, sheep, goats, monkeys, rabbits, hamsters, rats and mice (Gold et al., 2006). Depending on the species and the protein used for immunization, EAE shows different pathology and clinical course (Gold et al., 2006). However, until now there is no model mimicking all aspects of MS.

Because of the great variety of transgenic animals and advantages in breeding possibilities, the most common species used is mouse. Normally, animals are immunized with a small peptide carrying the sequence of MOG from amino acid position 35 to 55 (MOG\textsubscript{35-55}) emulsified in complete Freund’s adjuvant (CFA) (Mendel et al., 1995). This method is termed “active immunization”.

The disease that develops in C57 BL/6 mice is a monophasic, mostly self limited form where animals normally do not fully recover from their clinical symptoms. However, in contrast to MS, where also the brain and the neocortex could be affected, mainly the white matter of the spinal cord is affected in these animals (Engelhardt, 2006).

Active immunization of BL/6 mice with MOG\textsubscript{35-55} causes a rather destructive form of EAE with a high degree of axonal damage and subsequent axonal loss. Strong evidence has been collected that the disease is initiated by autoimmune, encephalitogenic CD4\textsuperscript{+} T cells with a Th1 phenotype (Halachmi et al., 1992). After immunization, the MOG peptide is presented by professional APCs, like dendritic cells and macrophages in peripheral lymph nodes. It is thought that T cells with a specific TCR proliferate and finally invade the CNS by recognizing MOG fragments loaded on major histocompatibility complex (MHC) class II molecules on perivascular APCs, e.g. macrophages. The following activation leads to a release of proinflammatory cytokines like IL-2, TNF-\alpha and IFN-\gamma, to an opening of the BBB, and to further recruiting of T cells and macrophages into the CNS. This inflammatory milieu causes damage to myelin and axons (Wekerle, 1991; Tsunoda & Fujinami, 1996; Engelhardt, 2008). In contrast to MS, in this model autoantibodies produced by B cells, complement factors and cytotoxic CD8\textsuperscript{+} T cells are considered to play no relevant role (Sospedra & Martin, 2005).

EAE is also inducible by transfer of myelin specific (e.g. MOG\textsubscript{35-55}-specific) CD4\textsuperscript{+} T cells (Driscoll et al., 1975). This method is termed “adoptive transfer” and it constitutes the
proof that encephalitogenic CD4\(^+\) T cells play the main role in the pathogenesis of EAE and therefore that EAE is caused by autoimmunity (Ben Nun et al., 1981). Here, the activation and proliferation of specific CD4\(^+\) T cells in the peripheral lymph nodes is completely circumvented.

By inducing EAE in different transgenic mouse strains many proteins and their roles during disease generation and maintenance have been analyzed to find potential targets for MS treatment. For instance, the successful and promising MS treatment by natalizumab (Sheremata et al., 1999) arose from early EAE experiments (Yednock et al., 1992). In this therapy an anti-\(\text{VLA-4}\) antibody is used to block the interaction of \(\text{VCAM-1}\) (on activated T cells and other leukocytes) and \(\text{VLA-4}\) (on activated high endothelia cells). This blockade impedes T cell diapedesis into the CNS and therefore induction of EAE by active immunization and adoptive transfer. Another example is the relative long established treatment of MS with glatiramer acetate, a synthetic, random polymer consisting of four different amino acids (Bornstein et al., 1987). This drug came also from promising results in EAE suppression (Teitelbaum et al., 1971).

### 1.3 Neurotrophins

Neurotrophins (NTs) are small, homodimeric proteins which belong to the family of growth factors (Lewin & Barde, 1996). Like all growth factors, neurotrophins have the ability to signal for cell survival, differentiation and / or growth upon binding to specific cell receptors (Thoenen, 1995).

Neurotrophins play a major role in the CNS and peripheral nervous system (PNS) during development with regard to cell connectivity and tissue plasticity. Neurons have been shown to compete for neurotrophins in developmental stages. Cells which do not bind enough of these molecules and therefore receive insufficient pro-survival signals undergo apoptosis (Thoenen, 1995). Neurotrophins are also capable of rescuing damaged or dying neurons, initiating sprouting and forming new axons to re-establish connections to a certain degree (Rabizadeh & Bredesen, 2003).

Neurotrophins originate from proneurotrophins that are cleaved by proteases to become functional. The truncated pro-part plays an important role in forming the correct tertiary structure of the neurotrophin (Lu, 2003).
Nerve growth factor (NGF) is the first discovered growth factor that enhances neuron growth and differentiation (Levi-Montalcini, 1966). Since then four neurotrophins have been classified (Thoenen, 1995):

1) **Nerve growth factor (NGF)**
   Without binding NGF neurites and neuronal cells degenerate during development. In the presence of NGF dendritic arborisation (Angeletti et al., 1971) and axonal branching is increased (Snider, 1988). Apoptotic neurons can be rescued by NGF (Williams et al., 1986).

2) **Brain derived neurotrophic factor (BDNF)**
   Because of its pro-survival signalling properties BDNF is required by many neuronal cell populations like midbrain dopaminergic neurons, cerebellar granule cells and hippocampal and cortical neurons during development (Snider, 1994; Hofer & Barde, 1988).

3) **Neurotrophin 3 (NT-3)**
   NT-3 is secreted by astrocytes to promote survival of OPCs and mature oligodendrocytes (Barres & Raff, 1994). Also, dorsal root ganglion cells (Hory-Lee et al., 1993) and trigeminal neurons (Ernfors et al., 1994) need NT-3 to survive.

4) **Neurotrophin 4/5 (NT-4/5)**
   NT-4/5 is expressed by skeletal muscle cells upon neuronal activation. It supports the generation of neuromuscular function in developmental stages (Funakoshin et al., 1995).

Although it was originally thought that neurotrophins only have “beneficial” effects on neurons, it became soon clear that the cellular response upon binding is defined by the involved receptor complexes and the underlying signalling pathways (Lu et al., 2005).
1.4 Neurotrophin receptors

There are two different neurotrophin receptors (NT-Rs):

1) The family of tropomyosin-related kinases (Trks) consisting of TrkA, TrkB and TrkC
2) The p75 low affinity neurotrophin receptor (p75\textsuperscript{NTR})

Trks bind neurotrophins with high and proneurotrophins with low affinity. TrkA mainly binds NGF, TrkB mainly BDNF and NT-4/5, and TrkC only NT-3 (Meakin & Shooter, 1992). Upon ligand binding single Trk receptors normally signal for cell survival (Fig. 1.3 d).

p75\textsuperscript{NTR} binds all neurotrophins with the same affinity. Compared to Trks p75\textsuperscript{NTR} has an overall lower affinity to neurotrophins, but a very high affinity to proneurotrophins (Kaplan & Miller, 1997). The ligand-receptor complex consisting of a proneurotrophin and a single p75\textsuperscript{NTR} normally leads to an apoptotic cell signal during development (Rabizadeh & Bredesen, 2003).

1.4.1 The p75 low affinity neurotrophin receptor

The p75\textsuperscript{NTR} is a 75 kDa protein consisting of one transmembrane domain, four extracellular immunoglobulin (Ig)-like domains at the N-terminus, which provide the ligand binding site, and six intracellular α-helices at the C-terminus (Fig. 1.2). p75\textsuperscript{NTR} also features a type II death domain which is able to induce apoptosis. It is posttranslationally modified at an N-linked and an O-linked glycosylation and a palmitoylation site.

The genomic region encoding the p75\textsuperscript{NTR} is located on chromosome 11 in \textit{mus musculus} and consists of almost 20,000 base pairs. It includes six exons. Exon three encodes three of the four neurotrophin binding Ig-like domains and is essential for a functional p75\textsuperscript{NTR} (Lee \textit{et al.}, 1992).

Because of its structural similarities, p75\textsuperscript{NTR} belongs to the TNF receptor superfamily (TNFRSF). It is also termed TNFRSF16, NGF receptor (NGF-R) and low affinity (L) NGFR.
1 Introduction

1.4 \( p75^{\text{NTR}} \)

\( p75^{\text{NTR}} \) is expressed by many different cells of the PNS and CNS as well as other cell populations not belonging to the nervous system, e.g. cells of the immune system (Labouyrie et al., 1997). Most of these cells express \( p75^{\text{NTR}} \) in developmental stages; however, it is often re-expressed under pathological conditions. During development \( p75^{\text{NTR}} \) plays a major role for the establishment of neural connections. It induces apoptosis in neurons which do not receive enough neurotrophins or too much proneurotrophins (Volosin et al., 2006).

For the following cell types \( p75^{\text{NTR}} \) expression has been shown in disease or under activated, disease-like conditions: neurons (e.g. after axotomy, stroke) (Dechant & Barde, 2002), reactive astrocytes (forming of glial scar) (Hanbury et al., 2002; Oderfeld-Nowak et al., 2003), oligodendrocytes and OPCs (after spinal cord injury, after cuprizone treatment, in MS lesions) (Cash et al., 2001; Beattie et al., 2002; Copray et al., 2005; Chang et al., 2000), rat microglia (Neumann et al., 1998) and monocytes (Dickensheets et al., 1997), activated B cells (Torcia et al., 1996) and dendritic cells (Jiang et al., 2007). Until now, \( p75^{\text{NTR}} \) was not detected on T cells (Torcia et al., 1996). Some specialized cell populations like Müller cells (Ding et al., 2001), CNS endothelial cells (Kim et al., 2004) and neural stem cells from the subventricular zone (SVZ) (Giuliani et al., 2004) express \( p75^{\text{NTR}} \) constitutively. The constant production of \( p75^{\text{NTR}} \) by CNS endothelial cells is upregulated in EAE (Copray et al., 2004; Küst et al., 2006).

1.4.2 \( p75^{\text{NTR}} \) mediated signal transduction

The cellular response to the binding of certain neurotrophins by \( p75^{\text{NTR}} \) is very diverse and strongly depends on associated coreceptors, intracellular adaptor proteins and neurotrophin concentrations (Fig. 1.3 and Fig. 1.4) (Lu et al., 2005).
Single p75 receptors not associated with any coreceptors induce apoptosis when no neurotrophin is bound (Fig 1.3 a). This happens via activation of the so called “stress kinase” c-Jun N-terminal kinases (JNKs), transcription factor p53-mediated production of the proapoptotic B cell lymphoma 2-associated X protein (BAX), subsequent release of cytochrome c from mitochondria and finally activation of caspases (Fig 1.3 a) (Casaccia-Bonnefil et al., 1996; Harrington et al., 2002; Huang & Reichardt, 2001). When neurotrophins are bound, p75NTR signalling leads to survival via activation of nuclear factor κB (NF-κB) and antiapoptotic B cell lymphoma-extra large (Bcl-xL) protein (Fig. 1.3 a and 1.5 a) (Hamanoue et al., 1999; Gentry et al., 2000; Rabizadeh & Bredesen, 2003).

If p75NTR is associated with Trks it depends on the binding neurotrophins, their concentration and the ratio of p75NTR to Trk molecules on the cell surface whether the response leads to prosurvival or proapoptotic signals (Fig. 1.3 b, c and Fig. 1.4 b) (Rabizadeh & Bredesen, 2003).

---

Fig. 1.3 | p75NTR signal variety.
Dependence of p75NTR signalling on ligands, ligand concentrations and receptor expression. (Rabizadeh & Bredesen, 2003)
In addition, \( p75^{\text{NTR}} \) is not only able to signal for cell death or survival but also for stabilization of the cytoskeleton by activation of the small guanosine triphosphatase (GTPase) Ras homologue gene family member A (RhoA) (Fig. 1.4 d). In this case \( p75^{\text{NTR}} \) is part of a receptor complex with neurite outgrowth inhibitor receptor (NogoR) and leucine rich repeat and Ig domain containing 1 (LINGO-1) (Wang et al., 2002; Mi et al., 2004). It binds myelin proteins like myelin associated glycoprotein (MAG) and is thought to mediate the inhibition of neurite outgrowth in neuronal processes approaching a myelin sheath.

\( p75^{\text{NTR}} \) can also act as a transcription factor via NGF-\( p75^{\text{NTR}} \) ligand-receptor complex internalization. In this case p75 is able to increase the expression level of the sodium (\( \text{Na}^+ \)) channel \( \text{Na}(v)1.8 \) (Damarjian et al., 2004). Enhanced numbers of \( \text{Na}^+ \) channels on axons are related to EAE pathology (Renganathan, 2003).
Fig. 1.5 | p75<sup>NTR</sup> signal transduction.
Mediated by p75<sup>NTR</sup> alone (a) and in combination with the coreceptor Trk (b). (Rabizadeh & Bredesen, 2003)
1.4.3 Processing and regulation of $p75^{\text{NTR}}$ by cleavage

It has been shown that after ectodomain shedding of the $p75^{\text{NTR}}$ and therefore removal of the extracellular NT binding site, the remaining intracellular C-terminal fragment acts as a substrate for $\gamma$-secretase, which also processes amyloid precursor protein (APP) to $\beta$-amyloid in a very similar way (Jung et al., 2003). This fragment is membrane-associated since the transmembrane domain is still functional (Fig 1.6). The latter carries the specific aminoacid target sequence for $\gamma$-secretase. After cleavage the soluble intracellular domain is released and immediately removed by proteasomal degradation. At the time, it is unknown if the released extracellular domain or the soluble intracellular fragment have got any further (signalling) functions. However, it has been suggested that the intracellular domain does not serve as a signalling molecule because of its immediate proteolysis. Nevertheless, the transmembrane cleavage of $p75^{\text{NTR}}$ by $\gamma$-secretase could function as an important regulatory mechanism of $p75^{\text{NTR}}$ expression and its association with Trks, thus determining the outcome of the cellular response.
In summary, p75\textsuperscript{NTR} is expressed by many different cell populations, like cells of the immune system and the CNS, under various conditions. It seems that p75\textsuperscript{NTR} plays major roles during damage and repair since it is re-expressed by many cell types. Besides cell death and survival, p75\textsuperscript{NTR} can induce a wide range of diverse cellular responses (e.g. inhibition of neurite outgrowth, support of myelination). These responses are mediated by distinct coreceptors as well as different adaptor proteins (e.g. Trk, LINGO-1, NogoR, Sortilin [Nykjaer \textit{et al.}, 2004], RhoA, TNF receptor associated factor [TRAF]-2, TRAF-6, neurotrophin receptor-interacting melanoma antigen gene [MAGE] homologue [NRAGE] [Salehi \textit{et al.}, 2000], p75\textsuperscript{NTR}-associated cell death executor [NADE] [Mukai \textit{et al.}, 2003]). Because p75\textsuperscript{NTR} itself does not have an intracellular kinase domain (Lu \textit{et al.}, 2005) and because of its ability to combine with other receptors, crosstalk between various signal transduction pathways might be possible.

### 1.5 Neurotrophin mediated interactions between the immune system and the CNS

Since widespread expression of neurotrophins (Kerschensteiner \textit{et al.}, 1999) and their receptors (Lomen-Hoerth & Shooter, 1995) has also been found on immune cells, the close connection between the immune system and the CNS under pathological conditions became clear. The increased production of neurotrophins and corresponding receptors by cells of the CNS and the immune system in disease led to the suggestion that neurotrophins might act as alert molecules able to prime tissue defence processes (Weskamp & Otten, 1987; Levi-Montalcini \textit{et al.}, 1996). For instance, neurotrophins are capable of modulating monocyte migration through the BBB (Flügel \textit{et al.}, 2001) via the p75\textsuperscript{NTR}. Neumann \textit{et al.} (1998) show that the MHC class II expression of cultured rat microglia is reduced by NGF - p75\textsuperscript{NTR} interaction. Furthermore, B cell proliferation, survival and Ig production is increased by NGF in an autocrine manner via p75\textsuperscript{NTR} (Kimata \textit{et al.}, 1991; Torcia \textit{et al.}, 1996).
1.6 \( p75^\text{NTR} \) suppression in EAE

Copray et al. (2004) showed increased clinical deficits and inflammatory infiltrations in spinal cords of \( p75^\text{NTR} \) deficient mice after MOG\textsubscript{35-55} EAE induction in comparison to wild type (wt) mice. The composition of the infiltrates regarding macrophages and T cells numbers however is comparable. They described \( p75^\text{NTR} \) expression on endothelial cells of blood vessels and perivascular astrocytes in the inflamed spinal cord by immunohistochemistry. Copray et al. interpret their findings as suggestive for a role of \( p75^\text{NTR} \) for BBB integrity.

In contrast, Soilu-Hänninen \textit{et al.} (2000) observed decreased incidence, clinical deficits, inflammation and demyelination in EAE mice in which \( p75^\text{NTR} \) was knocked down by specific antisense oligonucleotides. However, they also show \( p75^\text{NTR} \) expression on CNS endothelial cells.

1.7 Aims

The goal of the present work is to elucidate the role of the \( p75^\text{NTR} \) during experimental inflammation and demyelination of the CNS. Most likely, because of the widespread (re)expression, association with diverse coreceptors and initiation of complex signalling cascades, the role of \( p75^\text{NTR} \) in induction and repair of inflammatory demyelination is still a matter of debate.

In this study we examine the effects of a lack of \( p75^\text{NTR} \) signalling after active EAE induction and after adoptive transfer of encephalitogenic T cell clones by using a \( p75^\text{NTR} \) deficient mouse strain.

First, we compare responses of \( p75^\text{NTR} \) deficient and wt cells and tissues after various stimuli. Second, to further address the question if \( p75^\text{NTR} \) plays the major role within the CNS or in the infiltrating immune system we established a bone marrow chimera system in which the \( p75^\text{NTR} \) is exclusively expressed by cells of the immune system but not by other cell types of the body, namely CNS cells, and \textit{vice versa}. Third, we measure \( p75^\text{NTR} \) expression levels of diverse immune cell and CNS parenchymal cell populations under various experimental conditions \textit{in vitro}, \textit{ex vivo} and \textit{in vivo}.

We used a variety of techniques and readouts, such as cell culture systems, quantitative real time polymerase chain reaction (qRT-PCR), fluorescence activated cell
sorting (FACS), enzyme linked immunosorbent assays (ELISA), proliferations assays and immunohistochemistry.

The overall goal of this study is to check under which conditions which cell populations express p75NTR, what their contribution to mediating CNS inflammation, damage to axons and clinical outcome is, and possibly to apply new findings to MS treatment.
2 Materials and Methods

For further information on common procedures, reagents, buffers, solutions, chemicals, instruments, equipment, and manufacturers see appendix A.1, A.2, and A.3.

2.1 Animals

Young adult $p75^{NTR}$ -/- mice, first generated and described by Lee et al. (1992), aged 6-12 weeks were obtained from our own in-house breeding facility. They were mated heterozygously and, therefore, each mouse was genotyped. $p75^{NTR}$ -/- mice have a C57 BL/6-J background, and we used age-matched wild type C57 BL/6-J mice from Charles River (Sulzfeld, Germany) or our in-house breeding facility as controls.

The murine $p75^{NTR}$ gene is located on chromosome 11 and consists of six exons. In $p75^{NTR}$ -/- mice, exon 3 is knocked out. This exon encodes three of four extracellular, cysteine-rich, Ig-like domains, which are responsible for ligand binding (Welcher et al., 1991; Yan & Chao, 1991). The truncated version of $p75^{NTR}$ is still expressed in these knock-out mice; however, signalling is impeded (Yan & Chao, 1991).

The animals had free access to water and food ad libitum and a 12h/12h light/dark cycle. Experiments were carried out in accordance with the guidelines of the Central Department for Animal Experiments, University Medical Center, Göttingen, and the required permissions were obtained from the local authorities, then in Braunschweig, Germany.

2.1.1. Genotyping of animals

To genotype the offspring of the heterozygous mating, tissue was obtained during the earmarking procedure.

DNA extraction
- 350µl tail lysis buffer + 100µg proteinase K per biopsy
- 2-3h digestion at 56°C while shaking
Materials & Methods

2.1 Animals

- 5min centrifugation at 8000g
- collected supernatant + 350µl 70% isopropanol to precipitate DNA
- 5min centrifugation at 8000g
- discarded supernatant, washing pellet with 500µl 70% EtOH
- 5min centrifugation at 8000g
- discard supernatant, let pellet dry
- dissolved dry pellet in 100µl distilled H₂O

Polymerase chain reaction (PCR) kit (per sample)

- 2.5µl 5x buffer
- 0.5µl desoxyribonucleotides (dNTPs) (1:4) containing nucleotides of the bases adenine (dATP), cytosine (dCTP), guanine (dGTP), and thymine (dTTP), each 100mM
- 0.5µl each primer 1, primer 2, primer 3 (10µM each)
  primer sequence 1: GTGTTACGTTCCTCTGACGTTGTG
  primer sequence 2: TCTCATTCCGCGTCAGCCCAGGG
  primer sequence 3: GATTCGCAGCGCATCGCCTT
  (synthesized by MWG Biotech AG, Ebersberg, Germany)
- 0.25µl Taq polymerase
- 5.75µl H₂O
- 2µl sample DNA

PCR program

- 5min 95°C denaturation
- 1min 94°C denaturation
- 1min 59°C annealing
- 1min 72°C elongation
- 10min 72°C elongation
- forever 4°C storage

35 cycles

Agarose gel electrophoresis

- gel: 2% agarose in tris/borate/EDTA buffer (TBE) + 0.005% ethidium bromide
- electrophoresis in TBE buffer at 100V for 60min
- Visualization of PCR products by UV light
2.2 Generation of bone marrow chimeras

In a bone marrow chimeric animal the original immune system has been destroyed by irradiation and has been reconstituted by transfer of bone marrow from another mouse. Ionizing radiation causes damage of the DNA, resulting in cell apoptosis. Immune cells and hematopoietic stem cells are more susceptible to ionizing radiation than other cell populations of the body. After reconstitution with healthy bone marrow, the stem cells proliferate, differentiate, and repopulate the body compartments.

- p75<sup>NTR</sup> <s>-/-</s> and wt mice were whole body irradiated with 11.5 Gy from top and bottom each using a linear accelerator at the department of radiation therapy and radiooncology (Prof. Dr. Dr. Hess, Prof. Dr. Hille).
- after 1d irradiated mice were reconstituted with 6-8 x 10<sup>6</sup> bone marrow cells in 300µl PBS i.v. (see below).

To be able to distinguish between immune cells which survived the irradiation procedure and ones which repopulated the body after reconstitution, two different markers were used. For flow cytometric analysis a congenic mouse strain was used that expresses another variant of CD45, called CD45.1, on BL/6 background. p75<sup>NTR</sup> <s>-/-</s> mice have the natural CD45.2 gene on BL/6 background. For histological analysis a transgenic BL/6-J strain that expresses the green fluorescent protein (GFP) under the actin promoter was used as bone marrow donor. Thus, all cells express GFP. By means of specific antibodies against CD45.1, CD45.2, and GFP we were able to differentiate donor and acceptor cells.

- 8 weeks after the irradiation and cell transfer, the reconstitution efficacy was checked, and EAE was induced. During the repopulation phase, mice had low germ count acidic drinking water supplemented with neomycin (0.00016%) to prevent infections.
Preparation of bone marrow

- donor mice were deeply anesthetized and sacrificed by cervical dislocation
- bodies were disinfected with 70% EtOH
- hind legs were skinned and fleshed and the bones were rinsed in macrophage medium (DMEM + supplements, see appendix A.2)
- bones were cut at both ends under sterile conditions and rinsed from top and bottom with medium using a needle and syringe.
- washed with medium (cell washing see appendix A.1)
- passed through a 40µm cell strainer
- cell counting (see appendix A.1)
- 2x washed with PBS and resuspension of 6-8 x 10^6 cells in 300µl PBS per animal serum from the medium must not carried over
- *i.v.* injection (tail vein)
2.3 Induction of experimental autoimmune encephalomyelitis (EAE) by active immunization

Animals were immunized by subcutaneous injections of 200µl MOG\textsubscript{35-55} emulsion (2x 50µl \textit{peri-axillary}, 2x 50µl \textit{peri-inguinally}; left and right each). The MOG\textsubscript{35-55} emulsion contains 1µg/µl MOG\textsubscript{35-55} peptide and 5µg/µl killed \textit{mycobacterium tuberculosis} in a 1:1 ratio of sterile PBS and incomplete Freund’s adjuvant emulsified by sonication. During the immunization animals were anesthetized with diethyl ether \textit{per inhalationem}. Immediately and 48h after immunization 300ng pertussis toxin (PTX) in 300µl PBS were injected \textit{intraperitoneally} (i.p.) per mice to stimulate the immune response and permeabilize the BBB. Some EAE experiments were performed with Dr. med. Wolfgang Jäger and form a part of his medical doctor thesis, which was achieved also at the Institute of Neuropathology.

2.4 Induction of EAE by adoptive transfer

EAE was induced by \textit{i.p.} injection of an encephalitogenic MOG\textsubscript{35-55} specific T\textsubscript{H1} cell clone (clone 5.8, courtesy of Dr. med. Stefan Nessler). Before injection, T cells were re-stimulated in vitro.

2.4.1 Expansion and restimulation of T cell clones

T cell clones were stored in freezing medium (see appendix A.2), consisting of 40% fetal calf serum (FCS) and 20% dimethyl sulfoxide (DMSO) in Roswell Park Memorial Institute (RPMI) medium, in liquid nitrogen (N\textsubscript{2}). Aliquots of about $10^7$ T cells were thawed, disseminated, restimulated, and expanded in accordance with the following protocol. All steps were performed under sterile conditions. T cells were cultivated in a cell incubator at 37°C and 5% CO\textsubscript{2}.

\textit{Thawing and dissemination}
2 Materials & Methods

2.4 Adoptive transfer

- thawed frozen T cells at room temperature (RT)
- 2x washed with T cell medium (see appendix A.2) to get rid of the freezing medium (cell washing see appendix A.1)
- cell counting (see appendix A.1)
- dissemination of 750 000 cells/ml in 24-well plates + 2 ng/ml IL-2

First restimulation (after 3-4 d)
- collected and washed all cells
- cell counting
- dissemination of 750 000 cells/ml in 24-well plates
- added 6-8 x 10^6 irradiated splenocytes (see below) + 10µg/ml MOG_{35-55} for specific antigen presentation and restimulation of the T cell clone

Preparation of irradiated splenocytes
- naïve, female, 6-12 weeks aged mice were sacrificed by cervical dislocation while deeply anesthetized; spleens were extracted under sterile conditions
- washed with T cell medium and meshed through a 70nm cell strainer to obtain a single cell suspension
- washed and resuspended with 1% ammonium chloride in H_2O, incubation for 3 min to lyse the erythrocytes.
- 2x washed to get rid of the ammonium chloride
- transfer through a 40nm cell strainer to get rid of cell debris
- cell counting
- irradiation with 30Gy to inhibit proliferation of the splenocytes

Expansion of restimulated T cells
- 3-4d after the first restimulation, split the T cells by transfer of half of the resuspension in a new well every second day
- refilled the wells with medium + 2 ng/ml IL-2 to increase proliferation
2.4.2 Adoptive transfer

- second restimulation about 2 weeks after expansion according to the above protocol
- 2x washed and resuspended in sterile PBS 3-4d after the second restimulation
- cell counting (see appendix A.1)
- i.p. injection of ca. $2 \times 10^7$ T cells in 300µl sterile PBS per animal

2.5 Clinical evaluation of EAE

After EAE induction by immunization or adoptive transfer, animals were weighed and scored daily to measure disease severity. Scores are shown in table 2.1.

<table>
<thead>
<tr>
<th>Score</th>
<th>clinical symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>none</td>
</tr>
<tr>
<td>0.5</td>
<td>partial tail paresis</td>
</tr>
<tr>
<td>1</td>
<td>complete tail paralysis</td>
</tr>
<tr>
<td>1.5</td>
<td>slight hind limb paresis</td>
</tr>
<tr>
<td>2</td>
<td>distinct hind limb paresis, waddle gait</td>
</tr>
<tr>
<td>2.5</td>
<td>severe hind limb paresis, no normal gait on soles</td>
</tr>
<tr>
<td>3</td>
<td>complete hind limb paralysis</td>
</tr>
<tr>
<td>3.5</td>
<td>slight fore limb paresis</td>
</tr>
<tr>
<td>4</td>
<td>tetraparalysis</td>
</tr>
<tr>
<td>4.5</td>
<td>moribund</td>
</tr>
<tr>
<td>5</td>
<td>death</td>
</tr>
</tbody>
</table>

Tab 2.1 | Scores and clinical correlates.
Used EAE disease scores and their related clinical symptoms to assess disease severity.
2.6 Tissue preparation

At the end of the experiment mice were deeply anesthetized by i.p. injection of 300µl 14% chloral hydrate in H₂O. After checking the anesthesia by a pain stimulus on the leg and the eye lid reflex, animals were killed by cervical dislocation or transcardiac perfusion with PBS. No perfusion was done when collecting lymph nodes, spleens, bone marrow, and brains for primary microglia and astrocyte cultures. Animals were perfused when spinal cords and/or brains were dissected for histological, flow cytometric, and RNA analysis.

2.6.1 Transcardiac perfusion

The thoraces of deeply anesthetized animals were opened and the mice were anterogradely perfused with PBS through the left heart ventricle until all blood was washed out. For flow cytometric and RNA analysis, the native spinal cord and/or the brain was collected after PBS perfusion. For histological analysis PBS was followed by 4% paraformaldehyde (PFA) in PBS during the perfusion step to fix the tissue.

2.6.2 Tissue processing for histological analysis

After perfusion with PBS and 4% PFA skulls and spines were dissected and postfixed in 4% PFA over night at 4°C followed by a washing step with PBS over night at 4°C. Brains and spinal cords were dissected, cut into 3-4mm thick slices, dehydrated in an ascending sequence of alcohol solutions and embedded into paraffin (see chapter 2.7) over night using an automated tissue processor. Next day, tissues were embedded in paraffin blocks, cut into 1µm thin serial sections using a sliding microtome, and mounted on glass slides.
2.6.3 Tissue processing for RNA analysis

For RNA analysis, native spinal cords and/or brains were collected after perfusion with cold PBS and stored in RNAlater at -20°C until further processing.

2.7 Histochemical stainings

For the following histochemical stainings, tissue sections were deparaffinized and rehydrated. Stained sections were dehydrated after the staining procedures and mounted in DePex medium.

**Dehydration and embedding into paraffin**

\[
H_2O \rightarrow 50\% \text{ isopropanol (IPA)} \rightarrow 70\% \text{ IPA} \rightarrow 90\% \text{ IPA} \rightarrow 100\% \text{ IPA} \rightarrow \text{IPA/xylene (1:1)} \rightarrow 3x \text{ xylene} \rightarrow \text{paraffin}
\]

Deparaffinization and rehydration of the tissue sections were done according to the above protocol in reversed order. The duration for every step is 3 min.

2.7.1 Hematoxylin and eosin (HE) staining

HE staining provides a general overview of the tissue and facilitates the detection of inflammatory infiltrates. The inflammatory index was determined in HE stained spinal cord cross sections.

Hematoxylin stains cell nuclei blue by binding to basic nucleoproteins like histones. Eosin colours acidophilic and basic extra- and intracellular proteins pink and red (eosinophilic).

- deparaffinization and rehydration
- 8 min Mayer’s hemalum
- shortly washed with H2O
- differentiation by dipping 2-3x in 1% HCl alcohol
2.7 Histochemical stainings

- 10 min blued under flowing tap water
- 6 min 1% eosin solution
- shortly washed with H$_2$O
- dehydration
- mounted in DePeX medium

### 2.7.2 Luxol fast blue / periodic acid Schiff (LFB/PAS) staining

The LFB/PAS staining allows the assessment of demyelination in the white matter of spinal cord cross sections.

LFB stains myelin deep blue by binding to lipoproteins. PAS stains non-myelinated and demyelinated parenchyma pink.

- deparaffinization and rehydration to the 90% isopropanol step
- LFB solution over night, 60°C
- washed with 90% isopropanol
- differentiation in 0.05% lithium carbonate in H$_2$O to release LFB, 70% isopropanol to wash away released isopropanol, and H$_2$O to stop the washing step until only the myelin is stained deep blue
- 5 min 1% periodic acid in H$_2$O
- 5 min under flowing tap water
- washed with distilled H$_2$O
- 20 min Schiff’s solution
- 5 min under flowing tap water
- 2 min hemalum
- shortly washed with H$_2$O
- differentiated by dipping 2-3x in 1% HCl alcohol
- 10min blued under flowing tap water
- dehydration
- mounting in DePeX medium
2.7.3 Bielschowsky silver impregnation

Bielschowsky silver impregnation was used to enumerate axons in normal appearing white matter and in lesions and therefore to assess axonal loss.

Axons are argyrophilic (silver salt binding) structures. Insoluble silver nitrate binds to axons and is reduced to elementary silver leading to a black impregnation of axons. Parenchyma is stained yellow to brown.

- deparaffinization and rehydration
- 20 min 20% silver nitrate (AgNO$_3$) solution in distilled H$_2$O in the dark
- washed with distilled H$_2$O
- added 32% ammonium hydroxide in distilled H$_2$O drop by drop to the used silver nitrate solution until all precipitations have cleared up while stirring
- 15 min in this cleared silver nitrate/ammonium hydroxide solution in the dark
- washed with distilled water with some drops ammonium hydroxide in it
- added 10 drops of developer solution to the used silver nitrate/ammonium hydroxide solution
- developed in this solution until axons are stained black (5-6 min)
- washed with distilled H$_2$O
- 2 min fixation in 2% sodium thiosulfate in distilled H$_2$O solution
- dehydration
- mounted in DePeX medium

2.7.4 Immunohistochemical stainings

Immunohistochemical stainings were done to specifically detect cell marker proteins by antibodies. By means of these markers axonal damage, T cell infiltration, and other information can be gained.

For this technique, animals, e.g. rabbits, mice, or rats, were immunized with the target protein or peptide, e.g. mouse APP or CD3. The animals produce specific antibodies against certain epitopes. The antibodies are isolated, purified, and used to detect, e.g. APP in mouse tissue. The detection signal is then amplified and visualized by the use of biotin-conjugated
secondary antibodies, avidin-coupled peroxidase, and e.g., by precipitation of bound diaminobenzidine (DAB) (Fig. 2.2).

- deparaffinization and rehydration
- pretreatment of the tissue slices to unmask the epitopes:
  Boiled slices for 5x 3 min in 10 mM citric acid buffer using a microwave (800W).
- cooled down for 30 min at RT
- washed in H$_2$O followed by PBS (except for iNOS detection [see Tab. 2.2])
- 20 min 3% H$_2$O$_2$ in H$_2$O at 4°C to block endogenous peroxidase, which could produce a false positive signal when precipitating DAB during the developing step by H$_2$O$_2$.
- 3x washed with PBS
- 20 min blocked with 10% FCS in PBS at RT to prevent unspecific antibody binding (except for iNOS detection [see Tab. 2.2])
- incubation with the specific primary antibody diluted in 10% FCS in PBS over night at 4°C in a wet chamber (for further information on the primary antibodies applied see Tab 2.2).
- 3x washed with PBS
- 1h incubation with the secondary antibody diluted in 10% FCS in PBS at RT in a wet chamber to amplify the detection signal (for further information on the secondary antibodies, see Tab 2.3). The secondary antibody binds specifically to the Fc region of the primary antibody and is, e.g., conjugated with biotin.
- 3x washed with PBS
- 1h incubation with 0.1% streptavidin-horseradish peroxidase (POX) in 10% FCS in PBS at RT in a wet chamber. Streptavidin is isolated from bacterium streptomyces and binds biotin with high affinity bringing the coupled POX in close proximity to the specific antigen detection site.
- 3x washed with PBS
- development with DAB and H$_2$O$_2$ in PBS until a clear specific dark brown staining can be seen, preferentially without any background staining. DAB is oxidized by the bound POX using H$_2$O$_2$.
- 3x washed with PBS
- optional step: the DAB signal may be amplified by incubation with 2% copper sulphate (CuSO$_4$) in 0.9% NaCl solution for 10min.
- washed with H$_2$O
2. Materials & Methods

2.7 Histochemical stainings

- counter stain of nuclei with 20 s hemalum, shortly dipping in H₂O₂, and differentiation by 3-5 dips in HCl/EtOH.
- 10 min blued in flowing tap water
- dehydration
- mounted in DePeX medium

Fig. 2.2 | Scheme of detection in immunohistochemistry by using the avidin biotin method

The specific primary antibody binds to its antigen. Its Fc segment is detected by a biotinylated secondary anti-IgG specific antibody. (Strept)Avidin, coupled to peroxidase (POX), binds to biotin. POX oxidizes DAB by usage of H₂O₂. The precipitation of DAB reveals the location of the protein of interest. (From Dako pathology education guide “Immunohistochemical staining...”)
### 2.7 Histochemical stainings

<table>
<thead>
<tr>
<th>antigen</th>
<th>marker for</th>
<th>species</th>
<th>dilution</th>
<th>manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>early axonal damage</td>
<td>mouse</td>
<td>1:3000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>B220/CD45R</td>
<td>B cells</td>
<td>rat</td>
<td>1:200</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>CD3</td>
<td>T cells</td>
<td>rat</td>
<td>1:200</td>
<td>Serotec</td>
</tr>
<tr>
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<td>(Oertle et al., 2003)</td>
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**Tab. 2.2 | List of primary antibodies.**

Specificity, purpose, origin, dilution, and manufacturer of the used primary antibodies for immunohistochemical stainings.

* For the detection of iNOS not PBS but tris buffered saline (TBS) was used throughout the whole protocol. Also, for blocking not FCS but donkey serum was used.

<table>
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**Tab. 2.3 | List of secondary antibodies.**

Specificity, origin, dilution, and manufacturer of the used secondary antibodies for immunohistochemical stainings.
2.7.5 **Microscopic analysis of histochemically stained tissue slices**

In **HE stained sections** the mean number of inflammatory infiltrates per spinal cord cross section was determined at 40x magnification.

In the **LFB/PAS stained sections** the percentage of lesioned white matter was determined. This was done under 100x magnification by the computer program “cell^F”. The mean for every animal was calculated.

In **Bielschowsky stained sections** the axonal densities of normal appearing white matter and within lesions of spinal cord cross sections were determined by using an axon counting grid with 25 crossing points. At a magnification of 400x, the number of axons crossing one of these points was determined. The value obtained in the normal appearing white matter was set to 100%. The density of axons within lesions was expressed as the percentage of axonal density in the normal appearing white matter.

All **immunohistochemically stained sections** were analyzed at a magnification of 400x by using a 10x10 square cell counting grid. Immunoreactive (IR) cells or axons were counted within spinal cord lesions in a given area and given as IR structures/mm².

2.8 **RNA analysis**

Quantitative analysis of mRNA transcripts of selected inflammation related genes was done to examine differences in the quality of inflammation between p75\(^{NTR}\) \(-/-\) and wt mice during EAE. Furthermore, in combination with FACS sorting and primary cell cultures of microglia, bone marrow derived macrophages, and astrocytes we were able to measure p75\(^{NTR}\) expression by different cell subpopulations of the CNS and the immune system *ex vivo* and *in vitro*. We also stimulated these subpopulations *in vitro* to detect changes in the inflammatory response and p75\(^{NTR}\) expression.

For RNA analysis of spinal cords and brains, animals were perfused with cold PBS to wash out any remaining blood cells, and native tissues were put in RINAlater on ice to stabilize the mRNA. For RNA analysis of primary cell cultures and FACS sorted cell subpopulations, cell suspensions were centrifuged and directly resuspended in cell lysis buffer (see below).
2.8.1 RNA extraction

The Qiagen RNeasy Mini Kit was used for RNA extraction. All steps were done at RT according to the supplied protocol.

- homogenization of tissue with 500µl 1% β-mercaptoethanol in supplied RLT lysis buffer with an electric cell crusher;
  homogenization of cells from primary cell cultures or after FACS sorting by passing the lysate at least five times through a 20 gauge (0.9 mm diameter) needle or through a QiaShredder spin column in 500µl 1% β-mercaptoethanol in supplied RLT lysis buffer
- centrifugation for 3 min at 8000g to get rid of cellular debris
- collected supernatants and mixing it 1:1 with 70% EtOH to precipitate nucleic acids
- transferred the sample to an RNeasy spin column
- centrifugation for 1 min at 8000g. RNA binds to column. Discarded flow-through.
- Washed with 350µl supplied RW1 buffer. Centrifugation for 1 min at 8000g. Discarded flow-through.
- on-column DNA digestion to ensure that only RNA is bound by using the Qiagen RNase-free DNase set:
  According to the provided protocol, added ~27 Kunitz units DNase I in 80µl supplied RDD buffer per sample directly onto the column. DNA digestion for 15 min at RT.
- Washed with 350µl supplied RW1 buffer. Centrifugation for 1 min at 8000g. Discarded flow-through.
- Washed with 500µl supplied RPE buffer. Centrifugation for 1 min at 8000g. Discarded flow-through.
- Washed with 500µl supplied RPE buffer. Dried the column by centrifugation for 2 min at 8000g. Discarded flow-through avoiding carry-over of EtOH.
- Elution of bound RNA by 2x 30µl RNase-free H2O followed by centrifugation for 1 min at 8000g each.
- Storage at -80°C
2.8.2 cDNA reverse transcription

An Applied Biosystems High Capacity cDNA Reverse Transcription kit was used to reversely transcribe the mRNA to cDNA. All steps were done according to the supplied protocol.

- reverse transcription PCR (per sample): (All reagents supplied)
  8µl RT buffer
  3.2µl dNTPs (100mM)
  8µl random primers
  4µl reverse transcriptase (Multiscribe)
  16,8µl H₂O
  40µl sample RNA

- PCR program:
  10min 25°C annealing
  120min 37°C reverse transcription
  5s 85°C denaturation
  forever 4°C storage

- determination of cDNA concentration by using 1µl sample cDNA and a NanoDrop analyzer

2.8.3 TaqMan quantitative real time PCR (qRT-PCR)

In the TaqMan qRT-PCR technique not only forward and reverse primers complementary to the target gene are used, but also TaqMan probes, which anneal specifically to the target gene, too. These probes carry a fluorophore and a quencher in close proximity, inhibiting the detection of any fluorescence. If the single stranded DNA sequence with a bound primer is now specifically processed, the nucleotides of the TaqMan probes are removed one by one by the exonuclease activity of the Taq polymerase (Fig. 2.3). Therefore, fluorophore and quencher are released. Since the fluorophore is not in close proximity to the
quencher anymore, it is excitable and fluorescence can be detected. Accordingly, in the TaqMan qRT-PCR method, all measured fluorescence signals are specific.

![Scheme of the TaqMan qRT-PCR principle.](image)

Fig. 2.3 | Scheme of the TaqMan qRT-PCR principle.
Additional to primers, a specific TaqMan probe carrying a fluorophore and a quencher are used (1). In close proximity the quencher inhibits fluorescence detection. During the synthesis of the complementary strand, the probe is released by the exonuclease activity of the Taq polymerase (2). The distance of fluorophore and quencher is increasing (3), and fluorescence signals can be detected (4). (scheme by Ruhr-Universität Bochum)

TaqMan qRT-PCRs were prepared in 96-well-plates. All cDNA samples were also analyzed for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is constitutively expressed by all cell types.

**TaqMan qRT-PCR (per well)**
- 10µl TaqMan fast universal master mix
- 1µl gene expression assay mix (specific TaqMan probes)
- 8µl H₂O
- 1µl sample cDNA
2.9 Flow cytometry

Flow cytometric analysis was performed to check the frequency of immune cell subpopulations in lymph nodes and infiltrated spinal cords in p75NTR−/− and wt mice. Specific surface markers and intracellular stainings reveal not only different immune cell types, but also their maturation and activation status as well as their cytokine expression patterns. Therefore, flow cytometric analysis gives insights in both the quantity and quality of inflammation.

To see potential differences between p75NTR−/− and wt mice during peripheral priming, lymph nodes were analyzed from naïve mice as well as 3, 7, 10, and 14 days after active immunization. Also, spinal cords were examined with regard to infiltrating immune cell populations at peak of disease (score 3.0). Flow cytometric analysis was done by disseminating in vivo primed lymph node cells in culture and checking their activation upon in vitro stimuli.

To see if certain immune cell populations express p75NTR under specific conditions, lymph node cells were sorted and collected for further TaqMan qRT-PCR analysis.

Fluorescence signals were measured in real time by the PCR cycler. The instrument calculated cDNA concentrations (by using the GAPDH expression data) and CT and ΔCT values were computed. The cycle threshold (CT) value determines the numbers of cycles needed to get a fluorescent signal significantly above background noise. The less cycles are needed, the higher is the cDNA amount within the sample, the higher was the mRNA amount within the tissue / cells, and the higher was the expression of this gene of interest. To compare expression levels between individuals and groups, the ΔCT value is used. This value is the result of the CT value of the examined gene subtracted by the CT value of GAPDH. A ΔCT value difference of 1 means a 2-fold up- or downregulation of the gene; a difference of 2 cycles signifies a 4-fold regulation.

Data analysis was done using the StepOne Plus software from Applied Biosystems.
Furthermore, the reconstitution efficacy of bone marrow chimeras was measured by blood flow cytometry.

**FACS medium**

2% FCS in DMEM (high glucose)

**FACS buffer**

1% FCS in PBS

**Cell washing**

Filled up the tube with specified medium or buffer, centrifugation at 290g, 4°C, 6 min, discarding supernatant, and resuspension of the pellet in an appropriate amount of specified medium or buffer

### 2.9.1 Preparation of lymph nodes for flow cytometric analysis

Naïve mice or mice 3, 7, 10, or 14 days after active immunization were deeply anesthetized and sacrificed by cervical dislocation. Bodies were disinfected with 70% EtOH and 6 lymph nodes (4x peri-axillary, 2x peri-inguinally) were collected under sterile conditions.

- washed lymph nodes with FACS medium
- passed lymph nodes through a 70µm cell strainer and pipetted the suspension up and down to get a single cell suspension
- washed with FACS medium
- resuspension of the pellet in 500µl FACS buffer
- cell counting (see appendix A.1)
- splitting cells to have at least 50 000 in a FACS tube for each staining
- Staining of the cells (see chapter 2.9.4)
For *in vitro* stimulation of extracted lymph node cells followed by *flow cytometric analysis*, cells were disseminated in culture. The following additional steps were performed after preparing the single cell suspension according to the protocol above:

- washed with RPMI medium + supplements (same as T cell medium, see appendix A.2)
- resuspension of the pellet in RPMI medium + supplements
- cell counting and dissemination of $10^6$ cells per well in 24-well plates
- stimulation with 50 ng/ml phorbol 12-myristate 13-acetat (PMA) for 6h. Adding 4µl Golgi block reagent per every 6ml medium to stop protein transport for intracellular stainings. Golgi stop has to be added to every medium, buffer or solution from this point on to prevent protein release.
- Collected cells and washing with FACS buffer + Golgi stop to end the stimulation
- cell counting (see appendix A.1)
- splited the cells to have at least 50 000 in a FACS tube per staining
- surface protein staining of the cells (see chapter 2.9.4)
- fixation (2% PFA in PBS) over night at 4°C in the dark
- washed with FACS buffer
- intracellular staining with fluorochrome labelled antibodies diluted 1:100 in FACS buffer + Golgi stop in a volume of 100µl per FACS tube 30 min incubation at 4°C in the dark
- washed with FACS buffer
- resuspension of the pellet in 200µl 2% PFA in FACS buffer
- short-term storage at 4°C in the dark

### 2.9.2 Preparation of spinal cords for flow cytometric analysis

At the peak of disease (score 3.0) animals were deeply anesthetized with 300µl 14% chloral hydrate and perfused with cold PBS to wash out any remaining blood cells. Spinal cords were dissected, and collected in FACS medium on ice. Isolation of the immune cells was done by density centrifugation in a 3-phase Percoll gradient.
2 Materials & Methods
2.9 Flow cytometry

- passed the spinal cord tissue through a 70µm cell strainer and pipetted the suspension up and down to get a single cell suspension
- washed with FACS medium
- resuspension of the pellet in 2ml FACS medium + 20µl DNase solution (10µg/µl) to avoid DNA clots
  15 min incubation at 37°C
- washed with FACS medium
- resuspension of the pellet in 6ml 30% Percoll in FACS medium
- prepared the Percoll density gradient in a 15ml Falcon tube
  4ml 45% Percoll in FACS medium underlain by 2ml 70% Percoll in FACS medium
  Pipetted the cell suspension in 30% Percoll in FACS medium on top of the density gradient
- density centrifugation: 20 min at 1065g without acceleration and brake
- withdrawal of the uppermost layer containing the myelin by suction
- collecting the immune cells at the two interphases of the Percoll density gradient by using a long needle and a syringe
- passed through a 40µm cell strainer to get rid of cell debris
- washed with FACS medium
- resuspension of the pellet in 500µl FACS buffer
- cell counting (see appendix A.1)
- splitting cells to have at least 50 000 in a FACS tube per staining
- Staining of the cells (see chapter 2.9.4)

2.9.3 Preparation of blood for flow cytometric analysis

Animals were deeply anesthetized with diethyl ether per inhalationem. Blood was collected retrobulbarly by using a glass capillary and suspended in 1% 0.5M EDTA in FACS buffer. Staining of the blood leukocytes was performed according to the general flow cytometry staining protocol (see chapter 2.9.4). Additionally, erythrocytes were lysed after antibody incubation.
2.9.4 **Staining cells for flow cytometric analysis**

- washed with FACS buffer
- resuspension of the pellet in 50µl Fc receptor blocking solution (1:200 in FACS buffer) to inhibit unspecific binding of the Fc part of the antibodies to Fc receptors on immune cells
  10 min incubation at RT
- washed with FACS buffer
- staining with fluorochrome labelled antibodies diluted 1:300 in FACS buffer in a volume of 100µl per FACS tube (Tab. 2.4)
  15 min incubation at 4°C in the dark
- washed with FACS buffer
- erythrocyte lysis (only for blood flow cytometry):
  resuspension in 100µl OptiLyse. After incubation for 13 min at RT in the dark, addition of 1ml H₂O and incubation for 1-2h at RT in the dark
  washed with FACS buffer
- resuspension of the pellet in 200µl 2% PFA in FACS buffer
- short-term storage at 4°C in the dark

Flow cytometric analysis was done with a FACSCalibur. Raw data analysis was done with Cell Quest Pro, FlowJo V.7.6.1, and WinMDI 2.9 softwares.

For **TaqMan qRT-PCR analysis after cell sorting**, separated cell populations were processed according the mRNA analysis protocol in chapter 2.8.
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Tab 2.4 | List of antigen specific FACS antibodies.
Antigen specificity, host origin, immunoglobulin isotype, clone, commercial source, and coupled fluorochrome of the antibodies used for flow cytometry stainings. Detectable cell populations by the use of the markers are also indicated. Conjugated fluorophores were allophycocyanin (APC), fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP). Different fluorophores were used depending on the combination of antibodies applied. BD = Becton Dickinson Biosciences, BC = Beckman Coulter, eBio = eBioscience, IT = ImmunoTools
2.10 Enzyme-linked immunosorbent assay (ELISA)

ELISA analysis was done to specifically measure the amount of cyto- and chemokines released into the medium by cultured cells. ELISA plates are coated with a primary capture antibody, which is immobilized by binding to the plate surface. After applying the cell culture supernatants, the protein of interest is specifically captured by the antibody. The secondary biotinylated detection antibody binds to another epitope of the protein (“sandwich”). Streptavidin coupled to horseradish peroxidase (HRP) was used as detection system. HRP oxidizes the substrate tetramethyle benzidine (TMB) / peroxide, which then turns blue (Fig. 2.4).
This technique was used for *in vivo* primed lymph node cells (dpi10), which were restimulated *in vitro*, and primary cell cultures *in vitro*.

All pre-coated plates, solutions, buffers, antibodies, and the protein standard were supplied in the ELISA kits obtained from R+D Systems.

- transferred 100µl of the capture antibody solution per well in a 96-well ELISA plate
- incubation over night at 4°C
- 3x washed with 400µl wash buffer (0.05% Tween 20 in PBS) per well
- 1h blocking with blocking buffer at RT
- 3x washing with 400µl wash buffer per well
- added 100µl of the cell culture supernatant sample diluted 1:10 in the specific medium or protein standard per well
- 2h incubation at RT while shaking. The protein of interest binds specifically to the immobilized primary antibody.
- 3x washed with 400µl wash buffer per well
- added 100µl of the biotinylated secondary detection antibody per well
- 2h incubation at RT while shaking. The detection antibody binds specifically to another epitope of the protein.
- added 100µl streptavidin-HRP per well
- 20min incubation at RT in the dark. Streptavidin-HRP binds to the biotin of the detection antibody.
- 3x washed with 400µl wash buffer per well
- added 100µl substrate solution (TMB/peroxide) per well
- 20-30min incubation at RT in the dark. TMB/peroxide turns blue when modified by the HRP.
- added 50µl acidic stop solution (1M sulphuric acid) per well which turns the solution yellow
- photometric determination of the optical density by an ELISA reader at 540nm and 450nm as reference. Specific protein concentrations were calculated according to the supplied protein standards.
2.11 T cell clone analysis

2.11.1 Proliferation assay

Proliferation assays have been performed to check if impeded p75<sup>NTR</sup> signalling has effects on antigen specific T cell proliferation. Therefore, the incorporation of radioactive $^3$H-thymidine into newly synthesized DNA strands was measured. $^3$H-thymidine contains...
heavy hydrogen ($^3$H) in its methyl group, which is a weak beta emitter with a half-life of ~12.3 years. 3-4 days after T cell restimulation, radioactivity was measured as a marker for cell division frequency.

Two different approaches have been followed. First, we restimulated our T cell clone expressing functional p75$_{NTR}$ with p75$_{NTR}$-/- and wt APCs to dissect the effects of p75$_{NTR}$ deficiency on APCs. Second, we collected lymph node cells from immunized p75$_{NTR}$-/- and wt animals at dpi 10 and restimulated the MOG$_{35-55}$ specific T cells with wt APCs and the MOG peptide. This was done to look at possible differences in p75$_{NTR}$-/- and wt T cell proliferation.

- dissemination of 100 000 primed (dpi 10) lymph node cells (p75$_{NTR}$-/- or wt) or T cell clone cells in 50µl RPMI medium + supplements (T cell medium, see appendix A.2) per well in 96-well plates (see chapters 2.3.1 and 2.9.1 for dissemination of the T cell clone and preparation of primed lymph node cells).
- Added 100 000 irradiated APCs (p75$_{NTR}$-/- or wt) in 50µl medium per well to the T cells (see chapter 2.3.1 for preparation of irradiated APCs).
- Added 10µg/ml MOG$_{35-55}$ per well
- After 2d added $^3$H-thymidine with a radioactivity of 18.5 kBq in 25µl per well
- After 24h determination of cell proliferation by measurement of radioactivity of $^3$H-thymidine incorporated into the DNA by a cell harvester instrument and scintillation counter

**harvesting protocol**

Proliferating cells containing radioactive DNA are captured on a filter paper, whereas the radioactive medium is washed away.

- pre-wetted the filter paper by 3x washing (aspirating) with non-radioactive medium (cold)
- 1x aspirated radioactive cells onto the filter paper (hot)
- 3x washed by aspirating with medium to get rid of excess radioactivity within the cell medium (hot)
- 3x washed by aspirating with medium (cold)
- dried the filter paper at 60°C for 2h
- fused dry filter paper with a MultiLex film at 100°C
- measurement of radioactivity by a scintillation counter
2.11.2 mRNA analysis for p75<sup>NTR</sup> expression

To check if our T<sub>H1</sub> cell clone expresses p75<sup>NTR</sup> under various conditions, mRNA was isolated after different stimuli in vitro.

- dissemination of 750,000 cells/ml in T cell medium (see appendix A.2) in a 24-well plate (2ml/well)
- stimulation for the indicated time periods (3h, 6h, 24h):
  - PMA: 5 or 50 ng/ml (indicated)
  - α-CD3/α-CD28 antibodies: 0.1µl/ml from kit stock
  - agonistic α-CD3/α-CD28 antibodies provide costimulation
  - NGF: 100ng/ml
- washed with medium
- mRNA analysis (see chapter 2.8)

2.12 Bone marrow derived macrophages

Bone marrow derived macrophages (BMDM) were generated to have access to a pure, non-prestimulated, freshly differentiated primary macrophage culture. In contrast, common cell cultures obtained from peritoneal macrophages are likely prestimulated by thioglycolic acid or by the preparation procedure itself.

By addition of supernatants from L929 cell cultures to the medium, hematopoietic stem cells from the bone marrow differentiate into macrophages within 10 days. L929 is a mouse fibroblast culture, which produces a wide potpourri of cytokines, chemokines, and growth factors, e.g. granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF). L929 has been shown to drive hematopoietic stem cells into the macrophage niche (Boltz-Nitulescu <i>et al.</i>, 1987).
2.12 Bone marrow derived macrophages

2.12.1 Preparation and differentiation of BMDM

Cultivated BMDMs were stored in a cell incubator at 37°C with 10% CO₂.

- donor mice were deeply anesthetized and sacrificed by cervical dislocation
- bodies were disinfected with 70% EtOH
- hind legs were skinned and fleshed and the bones were washed in macrophage medium (DMEM + supplements, see appendix A.2)
- under sterile conditions bones were cut open at both ends and washed from top and bottom with medium using a needle and syringe.
- washed with medium (cell washing see appendix A.1)
- passed through a 40µm cell strainer
- cell counting (see appendix A.1)
- dissemination of $10^6$ cells/ml macrophage medium + 20% L929 medium in a Teflon coated pot
  A Teflon coated pot was used to easily harvest the adhering macrophages without risking a prestimulation, e.g. by trypsin or cell scraping.
- after 5d another 20% L929 was added
- after another 5d the cells were used for experiments
- washed with medium
- cell counting
- dissemination of $10^6$ cells/well in 500µl in a 24-well plate
- prestimulation (for 3d):
  fibrinogen: 10µM
  IFN-γ: 10 ng/ml
  lipopolysaccharide (LPS): 1 µg/ml
- washed with medium
- cell counting
- dissemination of $10^6$ cells/well in 500µl in a 24-well plate
- main stimulation (for indicated time periods)
  fibrinogen: 10µM
  IFN-γ: 10 ng/ml
  LPS: 1 µg/ml
  PMA: 50 ng/ml
Zymosan: 100µg/ml

- ELISA analysis (see chapter 2.10), NO analysis (see chapter 2.12.2), or ROS analysis (see chapter 2.12.3)

### 2.12.2 Measurement of nitric oxide (NO) production after stimulation of BMDM

To detect potential differences between p75\(^{\text{NTR}}\) \(^{-/-}\) and wt BMDM with regard to NO release, cells were stimulated with the TLR-4 activator lipopolysaccharide (LPS) and the TLR-2 activator Zymosan. The concentration of NO\(_2\), being a direct degradation product of NO, was measured by the Griess reaction protocol.

- filled each well of a 96-well plate with 50µl sample supernatant/protein standard and 50µl Griess reagents A + B (1:1)
- protein standard: NaNO\(_2\) (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.313 µM)
- shook
- photometrical measurement at 550 nm (reference 450 nm when using phenol red containing medium)

### 2.12.3 Measurement of reactive oxygen species (ROS) production after stimulation of BMDM

ROS, such as NO, have been shown to contribute to axonal damage. Therefore, ROS production by p75\(^{\text{NTR}}\) \(^{-/-}\) and wt BMDM after different (pre-) stimuli was measured.

After stimulation, intracellular ROS were directly bound by membrane permeable nitroblue tetrazolium (NBT), which leads to its precipitation and a blue staining. NBT binds both H\(_2\)O\(_2\) and O\(^2-\). After cell lysis and dissolving of NBT, ROS concentrations can be determined photometrically.
Before main stimulation:

- cell counting (see appendix A.1)
- dissemination of $10^6$ cells/well in 1 ml pure DMEM in a 24-well plate
- added 1 mg NBT/ml (in stock NBT is dissolved in DMSO)
- main stimulation for 6 h:
  - PMA: 50 ng/ml
  - Zymosan: 100 µg/ml
- fixation with 1 ml/well 100% methanol
- washed by aspiration with 70% methanol
- dried over night at RT under a hood
- lysed cells with 62.5 µl 2M potassium hydroxide (KOH) + 75 µl DMSO per well for ca. 5 min
- photometric determination of optical density at 650 nm

### 2.13 Astrocyte primary cell culture

To specifically check the role of p75\textsuperscript{NTR} \(-/-\) deficiency in astrocytes, primary astrocytes cultures were prepared.

- dissection of the brain from 0-2 d old mice
- removed meninges in Hank’s buffered salt solution (HBSS) in a petri dish on ice
- disintegrated the tissue with a razorblade and produced a single cell suspension by pipetting
- disseminating cells of 3-4 brains in astrocyte medium (BME + supplements, see appendix A.2) in a poly-L-lysine coated 75 cm\textsuperscript{2} flask
  - poly-L-lysine coating (0.1 mg/ml in distilled H\textsubscript{2}O) by incubation for 1 h at RT
- after 7 d changing medium by aspiration and added 200 µg/ml clodronate to deplete microglia cells
- after 2 d changing medium by aspiration and shook on a shaker at 37°C over night to get rid of oligodendrocytes
- washed by aspiration with medium
Materials & Methods

2.13 Astrocyte culture

- collected cells: 3-5 min treatment with 0.05% trypsin / EDTA at RT while shook to loosen astrocytes from the flask area
- washed with medium
- cell counting (see appendix A.1)
- dissemination of $10^6$ cells/well in 500µl in a 24-well plate
- prestimulation for 3d:
  IFN-γ: 10 ng/ml
  lipopolysaccharide (LPS): 1 µg/ml
- changed medium by aspiration
- main stimulation for indicated time periods or 20h
  IFN-γ: 5 ng/ml
  LPS: 10 or 100 µg/ml (indicated)
  PMA: 5 or 50 ng/ml (indicated)
  Zymosan: 100µg/ml
  NGF: 100ng/ml
- mRNA analysis (see chapter 2.8), ELISA analysis (see chapter 2.10), or ROS analysis (see chapter 2.13.1)

### 2.13.1 Measurement of ROS production after stimulation of astrocytes

ROS production was measured in p75NTR-/- and wt astrocytes.

Intracellular ROS were detected by the fluorescent dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyester (CM-H$_2$DCFDA). This molecule loses its membrane permeability upon ROS binding being trapped intracellularly.

Before main stimulation:
- collected cells: 3-5 min treatment with 0.05% trypsin/EDTA at RT while shook to loosen astrocytes
- cell counting (see appendix A.1)
- dissemination of $10^6$ cells/well in 1 ml pure HBSS in a 24-well plate
- added 10µM CM-H$_2$DCFDA (dissolved in DMSO)
- incubated for 30 min at 37°C and 5% CO$_2$
2.13 Astrocyte culture

- washed by aspiration with HBSS
- main stimulation:
  - PMA: 50 ng/ml
  - Zymosan: 100µg/ml
- measurement of arbitrary fluorescence units over time by a fluorescence reader
  - excitation wavelength: 490 nm
  - emission wavelength: 525 nm
3 Results

3.1 Induction of EAE by active immunization in $p75^{NTR}$-/- mice

3.1.1 More severe disease in $p75^{NTR}$-/- mice after active immunization

To address the question if $p75^{NTR}$ plays a role during experimental CNS inflammation, EAE was induced by active immunization with MOG$_{35-55}$ in CFA in $p75^{NTR}$-/- and wt mice. 13 days post immunization (dpi 13) the mice developed first clinical signs. From the beginning on until dpi 31, when the experiment was terminated, $p75^{NTR}$-/- mice suffered from a more severe disease course, as has been observed by Copray et al. (2004), (Fig 3.1).

![Disease course of $p75^{NTR}$-/- and wt mice after active immunization](image)

**Fig. 3.1 | Disease course of $p75^{NTR}$-/- and wt mice after active immunization.** Significantly higher scores for $p75^{NTR}$-/- mice during the entire experiment.
3.1.2 Increased demyelination in p75\textsuperscript{NTR} -/- mice after active immunization

The demyelinated white matter area was measured. In the chronic disease stage after active immunization the extent of demyelination within the spinal cord was higher in p75\textsuperscript{NTR} -/- mice (p75\textsuperscript{NTR} -/- 9.39 +/- 3.24%, wt 5.84 +/- 3.35%, p < 0.05) (Fig. 3.2), reflecting the more severe disease course of these mice. However, the density of mature, myelinating oligodendrocytes in the lesions (as indicated by NogoA immunohistochemistry) was similar (p75\textsuperscript{NTR} -/- 173 +/- 46 cells/mm\textsuperscript{2}, wt 157 +/- 56 cells/mm\textsuperscript{2}, p=0.6), giving no support for an enhanced vulnerability or impaired recruitment of oligodendrocytes in p75\textsuperscript{NTR} -/- mice (Fig. 3.3).

**Fig. 3.2 | Demyelination in the chronic disease stage after active immunization.**
LFB/PAS staining of spinal cord cross sections showed larger demyelinated areas within the white matter in p75\textsuperscript{NTR} -/- mice. The graph displays the percentage of demyelinated white matter. Scale bar = 200\textmu m

**Fig. 3.3 | Oligodendroglial damage in the chronic disease stage after active immunization.**
No difference in the density of mature NogoA\textsuperscript{+} oligodendrocytes was observed. Scale bar = 100\textmu m
3.1.3 Quantification of inflammation: Increased meningeal B cell numbers in \( \text{p75}^{\text{NTR}} \)\(^{-/-} \) mice after active immunization

To assess the quantity of inflammation, cross sections of spinal cords in the chronic disease stage (dpi 36) were stained immunohistochemically. Similar numbers of Mac-3\(^{+} \) macrophages/microglia (\( \text{p75}^{\text{NTR}} \)\(^{-/-} \) 952 +/- 130 cells/mm\(^2\), wt 935 +/- 165 cells/mm\(^2\), \( p = 0.91 \)) and CD3\(^{+} \) T cells (\( \text{p75}^{\text{NTR}} \)\(^{-/-} \) 192 +/- 54 cells/mm\(^2\), wt 242 +/- 129 cells/mm\(^2\), \( p = 0.37 \)), but increased numbers of B220/CD45R\(^{+} \) B cells within the meninges (\( \text{p75}^{\text{NTR}} \)\(^{-/-} \) 3.29 +/- 2 cells/mm meninges, wt 0.86 +/- 0.7 cells/mm meninges, \( p = 0.01 \)) were counted in \( \text{p75}^{\text{NTR}} \)\(^{-/-} \) mice (Fig. 3.4).

**Fig 3.4 | Inflammatory CNS infiltration in the chronic disease stage after active immunization.**
Immunohistochemical stainings of spinal cord cross sections revealed similar densities of Mac-3\(^{+} \) macrophages/microglia and CD3\(^{+} \) T cells, but increased numbers of meningeal B220/CD45R\(^{+} \) B cells in \( \text{p75}^{\text{NTR}} \)\(^{-/-} \) mice. Scale bar = 100µm
3.1.4 Neuronal damage and regeneration: Increased axonal loss in p75\textsuperscript{NTR} \(-/-\) mice after active immunization

The accumulation of axonally transported amyloid precursor protein (APP) was used as an indicator for early axonal damage. Healthy neurons express APP constitutively and deliver it to the presynapse via fast axonal transport. In acutely damaged axons this transport is impeded resulting in accumulation of APP and formation of axonal swellings. This event occurs in the acute disease stage and is thought to be only in part reversible. In the chronic disease stage acute axonal damage plays a minor role, but the degree of axonal damage is more precisely reflected by axonal loss. No differences were observed in the density of APP\textsuperscript{+} axonal spheroids within chronic lesions (p75\textsuperscript{NTR} \(-/-\) 198 \(\pm\) 54 spheroids/mm\textsuperscript{2}, wt 222 \(\pm\) 65 spheroids/mm\textsuperscript{2}, \(p = 0.48\)) (Fig. 3.5). However, axonal density was significantly lower within lesions in p75\textsuperscript{NTR} \(-/-\) mice compared to wt mice (p75\textsuperscript{NTR} \(-/-\) 22.5 \(\pm\) 1.6\%, wt 27.6 \(\pm\) 2.1\%, \(p = 0.0004\)). The extent of axonal loss was calculated in relation to the axon density in the normal appearing white matter (set at 100\%).

![Fig. 3.5](image)

**Fig. 3.5 | Axonal damage and loss in the chronic disease stage after active immunization.**

Immunohistochemical stainings of spinal cord cross sections showed similar densities of APP\textsuperscript{+} axonal spheroids in p75\textsuperscript{NTR} \(-/-\) and wt mice. Bielschowsky silver impregnation showed decreased axonal density in lesion areas of p75\textsuperscript{NTR} \(-/-\) mice and therefore increased axonal loss. Axonal density in the NAWM was set to 100\% Scale bar = 100\mu m
Neuronal damage was assessed by staining for hypophosphorylated heavy neurofilaments (NF-H), detected by the SMI35 antibody. After axonal transection, a neuronal reaction occurs leading to retrograde chromatolysis and accumulation of SMI35+ neurofilaments in the neuronal cytoplasm (Fig. 3.6). No significant difference was observed (\( p75^{NTR} /- 0.84 +/- 0.86 \) neurons/cross section, wt 0.52 +/- 0.73 neurons/cross section, \( p = 0.52 \)).

The capacity for neuroaxonal regeneration was analyzed by staining for growth associated protein 43 (Gap43). Gap43 is expressed by neuronal growth cones during axonal sprouting, but also in neuronal cell bodies after damage. No difference in the number of Gap43+ neurons was observed between wt (0.96 +/- 0.22) and \( p75^{NTR} /- \) mice (0.98 +/- 0.26) at dpi 31 (\( p=0.9 \)) (Fig 3.7).

To assess whether the difference in axonal loss was paralleled by a difference in the extent of inflammation, I examined the quantity and quality of the immune response during different disease phases (onset, peak, chronic), during peripheral immune priming at various time points (dpi 3, dpi 7, dpi 10) and under naïve conditions in \( p75^{NTR} /- \) and wt mice.
3.1.5 Quantity and quality of inflammation during peripheral priming and during disease after active immunization in p75\textsuperscript{NTR} -/- and wt mice

To examine whether any differences exist with regard to inflammation between wt and p75\textsuperscript{NTR} -/- mice after active EAE induction, I studied the effect of p75 deficiency on cellular and molecular level in various immune cell populations \textit{in vivo}, \textit{ex vivo}, and \textit{in vitro}.

3.1.5.1 Inflammatory infiltrates in spinal cords at the peak of disease after active immunization in p75\textsuperscript{NTR} -/- and wt mice

To confirm and extend our histological results and assess in more depth the populations of inflammatory cells infiltrating p75\textsuperscript{NTR} -/- and wt spinal cords at the peak of disease, spinal cords were collected at a score of 3.0 after active immunization with MOG\textsubscript{35-55}. p75\textsuperscript{NTR} -/- and wt animals were score-matched.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3-8.png}
\caption{Flow cytometric analysis of T cell populations in EAE spinal cords at the peak of disease. No differences between p75\textsuperscript{NTR} -/- and wt mice with regard to CD4\textsuperscript{+}, CD8\textsuperscript{+}, \(\gamma\delta\) T cell, and NK cell infiltration were observed.}
\end{figure}
The numbers of infiltrating CD4$^+$ T cells were about 10-fold higher than those of CD8$^+$ T cells (Fig. 3.8). Neither the CD4$^+$/CD8$^+$ ratio nor the numbers of γδ T cells and NK cells were significantly different between p75$^{NTR}$ -/- and wt mice.

CD11b is often used as a marker for infiltrating macrophages whereas GR1$^+$ cells are often referred to as granulocytes. In fact, both markers are expressed by subsets of both cell types as well as CNS residential microglia (Geissmann et al., 2008; Mildner et al., 2007). To distinguish between macrophages/microglia and granulocytes, CD11b$^+$ GR1$^+$ cell numbers were checked for MHC class II$^{hi}$ and MHC class II$^{lo}$ expression (Fig. 3.9 A, B). About 15% of the flow cytometric analyzed immune cells were GR1$^+$ inflammatory monocytes (CD11b$^+$ MHC class II$^{hi}$) and about 30% were granulocytes (GR1$^+$ CD11b$^+$ MHC class II$^{lo}$). The total number of all macrophages (CD11b$^+$ MHC class II$^{hi}$) was by far the largest population of immune cells (~83%). No differences between p75$^{NTR}$ -/- and wt mice were found. Activated macrophages and microglia can be distinguished by the expression level of the general leukocyte marker CD45. In both, p75$^{NTR}$ -/- and wt mice, about 30% of the immune cells were CD45$^{hi}$ CD11b$^+$ macrophages and another 30% were CD45$^{lo}$ CD11b$^+$ microglia (Fig. 3.9 C). By gating on these two populations, their degree of MHC class II expression was assessed (Fig. 3.9 D, E). Expression of MHC class II molecules indicated by a fluorescence intensity histogram was ~ 3-fold higher in macrophages compared to microglia. However, no differences between p75$^{NTR}$ -/- and wt were detected.
Fig. 3.9 | Flow cytometric analysis of CD11b\(^+\) cells in spinal cords at the peak of disease.

Similar cell counts for inflammatory monocytes (GR1\(^+\) CD11b\(^+\) MHC class II\(^{hi}\)) (A), granulocytes (GR1\(^+\) CD11b\(^+\) MHC class II\(^{lo}\)) (B), macrophages (CD11b\(^+\) CD45\(^{lo}\)), microglia (CD11b\(^+\) CD45\(^{hi}\)) (C), and MHC class II expression on macrophages (D) and microglia (E) were found in p75\(^{NTR}\)-/- and wt mice.
3.1.5.2 Regulation of inflammatory related genes after active immunization in \textit{p75}^{NTR} -/- and wt mice

To examine in more detail the quantity and quality of inflammation in \textit{p75}^{NTR} -/- and wt mice after active immunization, I studied the expression of inflammation related genes at different time points after peripheral priming and disease onset. Specific TaqMan probes were used to detect mRNA of various chemokines (CCL2, CCL5, CXCL10) and cytokines (IL-1, IL-6) to determine the quality of inflammation. As indicators for T cell and macrophage/microglia cell numbers, transcripts of cell lineage markers (CD4, CD68) were utilized. Also other molecules of interest, such as NGF, inducible nitric oxide synthase (iNOS), which produces NO, and \textit{p75}^{NTR} itself were analyzed. The aim of this analysis was to check which genes are regulated at which time points after EAE induction. Inflammation related gene regulations were also measured in the stage of peripheral priming, in which an immune response is generated in peripheral lymph nodes and in the spleen. The time point of this measurement was before disease onset at dpi 10 after immunization with MOG\textsubscript{35-55} in CFA or CFA alone (as negative control), respectively. During disease, time points were disease onset (at a score of 0.5), early disease (score of 1.5), peak of disease (score of 3.0), and the chronic disease stage at dpi 35 (score of 2.0). \textit{p75}^{NTR} -/- and wt mice were score-matched.

The $\Delta C_T$ value represents the number of cycles needed to detect a fluorescence signal significantly higher than the background signal in relation to the housekeeping gene GAPDH. The lower the $\Delta C_T$ value, the higher the mRNA amount within the sample, in this case the spinal cord.

![Fig. 3.10 | Similar expression of CD4 and CD68 in naïve and actively immunized \textit{p75}^{NTR} -/- and wt mice.](image)

Increasing CD4 and CD68 mRNA levels during disease evolution represent macrophage/microglia and T cell infiltration. In the chronic disease stage inflammatory cell numbers decrease. No differences between \textit{p75}^{NTR} -/- (red) and wt (black) were observed.
The amounts of CD4 and CD68 mRNA within the spinal cords increased with disease evolution until the peak of disease (score 3.0), representing the quantity of infiltration by T cells and macrophages/microglia (Fig. 3.10). In the chronic disease stage the higher ∆C_T values indicate that the numbers of T cells and macrophages have decreased. The mRNA levels were similar in p75NTR-/- and wt mice at all time points, showing that similar numbers of T cells and macrophages infiltrate the CNS in both mouse strains, when score-matched.

IL-1 and IL-6, which have proinflammatory properties in EAE, are strongly upregulated (~17-fold) during disease (Fig 3.11). The IL-1 and IL-6 levels showed no significant differences. NO production, indicated by higher expression of iNOS, is boosted during EAE (~12-fold increased). Similar expression of iNOS in p75NTR-/- mice and wt was
3 Results

3.1 Active EAE - Disease

seen (Fig. 3.11). The chemokines CCL2, CCL5 and CXCL10 have proinflammatory properties and are strongly upregulated during EAE (~ 18-fold) (Fig 3.11). No significant differences in chemokine expression were observed at any time point between p75$^{NTR}$ -/- and wt mice.

NGF is upregulated during EAE with the highest expression at peak of disease (Fig. 3.12). In naive lymph nodes, at dpi 10, during early disease evolution (score 1.5), and at the peak of disease (score 3.0) the mRNA levels of NGF are significantly lower in p75$^{NTR}$ -/- compared to wt mice (p=0.016). p75$^{NTR}$ itself is not regulated during EAE (p=0.33) (Fig. 3.12). Since an exon 3 specific p75$^{NTR}$ TaqMan probe was used, no mRNA could be detected in the p75$^{NTR}$ exon 3 deficient mice, validating the genotype on the mRNA level.
3.1.5.3 Leukocyte subpopulations in naïve and immunized lymph nodes of \( p75^{\text{NTR}} \)−/− and wt mice

I performed a detailed four colour flow cytometry study of leukocyte subpopulations in the inguinal and axillary lymph nodes of \( p75^{\text{NTR}} \) deficient animals and wild type controls before and after immunization with MOG\(_{35-55}\). Specifically, I wanted to clarify, if differences in lymphocyte subpopulations were responsible for the earlier disease onset and more aggressive EAE disease course in \( p75^{\text{NTR}} \) deficient animals. Major leukocyte subpopulations were differentiated by their properties in forward (FSC) and side scatter (SSC) light, which reflect their size and granularity/internal complexity, respectively. The lymphocyte subpopulation is encircled in a correlated measurement of FSC and SSC in Fig. 3.13. Fluorescent antibodies against characteristic surface molecules were applied to further differentiate lymphocyte subsets.

Representative flow cytometry dot plots for CD4 and CD8 T cells (Fig. 3.14), γδ T cells (Fig. 3.15), NK and NK T cells (Fig. 3.16), regulatory T cells (T\(_{\text{reg}}\)) (Fig. 3.17), and B cells (Fig. 3.18) are depicted.

Since CD4 T cells are the key players in the pathogenesis of EAE, we started the analysis with this lymphocyte subset. We could not detect any difference in the ratio of T helper cells (CD4\(^+\)) to cytotoxic T cells (CD8\(^+\)) in naïve (Fig 3.14B: \( p75^{\text{NTR}} \)−/− 1.45 +/- 0.05, wt 1.3 +/- 0.19) or immunized animals (Fig. 3.14C: \( p75^{\text{NTR}} \)−/− 1.36 +/- 0.16, wt 1.27 +/- 0.1).

γδ T cells have been shown to enhance autoimmunity by restraining T\(_{\text{reg}}\) responses (Petermann et al., 2010). We therefore analyzed the γδ T cell subpopulation (Fig. 3.15 A, B) with similar frequencies of γδ T cells in naïve (\( p75^{\text{NTR}} \)−/− 1.03 +/- 0.1, wt 0.74 +/- 0.18) (Fig. 3.15 C) and immunized animals (\( p75^{\text{NTR}} \)−/− 0.87 +/- 0.23, wt 1.15 +/- 0.2) (Fig. 3.15 D) in both genotypes.
3. Results

3.1 Active EAE - Priming

**Fig. 3.14 | Flow cytometric analysis of T helper and cytotoxic T cell populations in naïve and immunized lymph nodes.**

A representative CD4/CD8 flow cytometry dot plot of lymph node cells is depicted in A. The cells were previously gated on the lymphocyte subpopulation by their FSC/SSC properties. The CD4/CD8 ratio did not differ between the genotypes in naïve lymph nodes (B) nor after immunization with MOG\textsubscript{35-55} (C). Data are presented as mean +/- SD.

**Fig. 3.15 | Flow cytometric analysis of the γδ T cell population in naïve and immunized lymph nodes.**

A representative γδ TCR/CD3 flow cytometry dot plot of lymph node cells is depicted in A. The isotype control staining is shown in B. γδ T cells are expressed as percentage of total lymphocytes and did not differ between the genotypes in naïve lymph nodes (C) nor after immunization with MOG\textsubscript{35-55} (D).
The role of natural killer (NK) and NK T cells in autoimmunity has been studied in a number of animal models, among them EAE. Depletion of NK cells previous to EAE induction resulted in clinically more severe EAE (Zhang et al., 1997) and beneficial effects have been proposed for NK T cell activation at the time of MOG<sub>35-55</sub> immunization (Singh et al., 2001). A typical staining for NK cells (NK1.1<sup>hi</sup>, CD3<sup>8</sup>) and NK T cells (NK1.1<sup>intermediate</sup>, CD3<sup>intermediate</sup>) is demonstrated in Fig. 3.16 A.

![Flow cytometric analysis of NK cell and NK T cell populations in naïve and immunized lymph nodes.](Image)

A representative NK1.1/αβ TCR flow cytometry dot plot of lymph node cells is depicted in A, the isotype control panel is shown in B. NK (C, D) and NK T cells (E, F) are expressed as percentage of total lymphocytes and did not differ between the genotypes in naïve lymph nodes (C, E) nor after immunization with MOG<sub>35-55</sub> (D, F).

The relative numbers of NK cells (NK1.1<sup>hi</sup> αβ TCR<sup>+</sup>) were similar in p75<sup>NTR</sup><sup>-/-</sup> and wt mice in naïve and immunized animals. (Fig. 3.16 C, D). Whereas the percentage of NK cells dropped after immunization (Fig 3.16 C, D), the frequency of NK T cells remained stable (Fig. 3.16 E, F).
p75$^{\text{NTR}}$ -/- and wt mice had comparable frequencies of NK T cells (NK1.1$^{\text{int}}$ $\alpha\beta$TCR$^{\text{int}}$) in lymph nodes before and after immunization (Fig. 3.16 E, F).

Regulatory T cells (T$^{\text{reg}}$s) were assessed due to their prominent function in regulating the proliferation and cytokine secretion of effector T cells. FoxP3, a forkhead/winged helix transcription factor is essential for the development and control of T$^{\text{reg}}$s and can be detected with an intracellular staining as shown in Fig. 3.17.

p75$^{\text{NTR}}$ deficient mice had less FoxP3$^{+}$ T regulatory cells in naive lymph nodes compared to wild type controls (p75$^{\text{NTR}}$ -/- 11.03 +/- 1.09, wt 15.94 +/- 4.18; p = 0.04) (Fig. 3.17 C) but this difference did not persist after immunization with MOG$_{35-55}$ (p75$^{\text{NTR}}$ -/- 12.31 +/- 1.85, wt 11.54 +/- 1.37; p = 0.4) (Fig. 3.17 D).
Finally, we analyzed the number and frequency of B cells in p75\textsuperscript{NTR} deficient mice by using an antibody against CD19.

The ratio of T cells to B cells in naïve (p75\textsuperscript{NTR} \(-/-\) 2.58 +/- 0.03, wt 2.91 +/- 0.51) and immunized lymph nodes (p75\textsuperscript{NTR} \(-/-\) 0.72 +/- 0.16, wt 0.82 +/- 0.22) was similar in p75\textsuperscript{NTR} \(-/-\) and wt animals (Fig. 3.18). Surprisingly, after immunization with MOG\textsubscript{35-55} the CD3/CD19 ratio decreased considerably.
3.1.5.4 Immunization response of lymph node cells to MOG$_{35-55}$

3.1.5.4.1 No difference in the expression of T cell activation and maturation markers in naïve and immunized lymph nodes of p75$^{NTR}$ -/- and wt mice

Next, we analyzed the activation status of CD4 T cells before and after immunization with MOG$_{35-55}$ in the respective genotypes. We differentiated naïve T cells (CD44$^{lo}$, CD62L$^{hi}$) from effector T cells (CD44$^{hi}$, CD62L$^{lo}$) with antibodies directed against CD44 and CD62L. A typical dot plot is depicted in Fig. 3.19. The percentage of CD4 T cells with an effector phenotype increased from around 4% to 8-9% at day 7 post immunization in both genotypes comparably (Fig 3.19).

![Fig. 3.19 | Flow cytometric analysis of the T cell activation markers CD62L and CD44 in naïve and immunized lymph nodes. CD4 T cells were identified (A) and their CD62L and CD44 expression was analyzed (B). The percentage of CD4 T cells with an effector phenotype was significantly higher at d7 after immunization compared to naïve mice (n=2), but both genotypes generated comparable CD4 effector phenotype frequencies after MOG$_{35-55}$ immunization.](image-url)
In naïve animals around 5% of all CD4 T cells were positive for CD69, an early T cell activation marker. The percentage of CD69 positive CD4 T cells increased to 20.1% in wild type animals and 17.1% in p75NTR deficient animals following immunization with MOG35-55 (Fig. 3.20).

CD69 is expressed as one of the earliest activation markers by T cells. In non-inflamed lymph nodes the expression was moderate (~5.5%) (Fig. 3.20). After MOG35-55 immunization the number of CD69+ T cells increased (~15% at dpi 3, ~18.5% at dpi 7). The frequencies did not differ between p75NTR-/- and wt mice in naïve nor in immunized lymph nodes.
### 3.1.5.4.2 T cell adhesion molecules naïve and immunized lymph nodes of p75<sup>NTR</sup> -/- and wt mice

We analyzed adhesion molecules on CD4 T cells, which play a role in the T cell recruitment to the CNS. These included LFA-1 (CD11a/CD18), which is relevant for the transendothelial migration of encephalitogenic T cells to the CNS (Laschinger et al., 2002) and VLA-4 (CD29/CD49d) (Yednock TA et al. Nature 1992), which is the target of natalizumab, an approved monoclonal antibody therapy for active relapsing multiple sclerosis. Whereas CD11a was constitutively expressed on all lymph node T cells (Fig. 3.21), a comparable upregulation of CD29 after MOG<sub>35-55</sub> immunization could be observed in both genotypes and is depicted in Fig. 3.22.

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**Fig. 3.21 | Flow cytometric analysis of the T cell adhesion molecule CD11a**

A representative CD3/CD11a flow cytometry dot plot of lymph node cells is depicted in A. The cells were previously gated on CD3 T cells. B shows the isotype control. CD11a was constitutively expressed already in naïve animals and no further upregulation could be documented after immunization. n=2
3.1.5.4.3 Cytokine profile of MOG\textsubscript{55-55} immunized CD4\textsuperscript{+} T cells in p75\textsuperscript{NTR} \textasciitilde/- and wt mice

Encephalitogenic CD4 T cells secrete T\textsubscript{H}1 or T\textsubscript{H}17 cytokines. We therefore determined the prototypic T\textsubscript{H}1 cytokine IFN\textsubscript{\gamma} and the prototypic T\textsubscript{H}17 cytokine IL-17 after immunization with MOG\textsubscript{55-55} on a single cell level by intracellular cytokine stainings. Regional lymph nodes were collected at dpi 3 and dpi 7 after immunization and the single cell suspensions were stimulated with PMA/Ionomycin at a concentration of 50ng/ml in the presence of Golgi block for 6h. Unstimulated cells were kept on Golgi Block only. A representative flow cytometry dot plot is presented in Fig. 3.23.
The frequency of IFNγ+ CD4+ T cells as well as IL17+ CD4+ T cells was in the range of 1-2% after immunization with MOG35-55. Most CD4+ T cells produced either IFNγ or IL17, cells producing both cytokines were extremely rare (data not shown). Whereas IFNγ+ CD4+ T cells can be detected as early as dpi 3 (Fig 3.24 E, F), there was an increase in the frequency of IL-17 producing T cells from dpi 3 to dpi 7 (Fig. 3.24 G, H). MOG35-55 immunization resulted in comparable frequencies of T_H1 and T_H17 cells in both genotypes.
3. Results

3.1 Active EAE - Priming

IL-17⁺ CD4⁺ T cell frequencies did not increase over time at dpi 3 and dpi 7 in MOG₃₅₅₅ immunized lymph nodes in both mouse strains (Fig 3.24 E, F). After stimulation of the T cells, IL-17 production was about 2-fold higher compared to unstimulated T cells (Fig. 3.24 G, H). T cells from time point dpi 7 (Fig. 3.24 F, H) responded more to stimulation than T cells from dpi 3 (Fig. 3.24 E, G). This shows that the cells are more susceptible to exogenous stimuli and have a more activated phenotype in comparison to dpi 3. Again, no significant differences between the two mouse strains analyzed were seen.
3.1.5.4.4. Similar numbers of IFNγ and IL-17 producing γδ T cells in lymph nodes of MOG\textsubscript{35-55} immunized p75\textsuperscript{NTR} \textsuperscript{-/-} and wt mice

γδ T cells have come into focus due to their potential to restrain T\textsubscript{reg} responses. We therefore analyzed IFNγ and IL-17 production in the γδ T cell subpopulation at dpi 3 and dpi 7 with and without PMA stimulation (Fig. 3.25).

The numbers of IFNγ\textsuperscript{+} γδ T cells were very low and did not significantly change from dpi 3 to dpi 7 (Fig. 3.26 A, B). After PMA treatment IFNγ expression was about 15-fold increased at dpi 3 and dpi 7 (Fig. 3.26 E, F). The frequency of IL-17\textsuperscript{+} γδ T cells increased from ~1% (dpi 3) to ~4% (dpi 7) (Fig. 3.26 C, D). After PMA stimulation, IL-17\textsuperscript{+} γδ T cell numbers were ~10-fold higher at dpi 3 and dpi 7 (Fig. 3.26 G, H). The frequencies of γδ T cells secreting the given cytokines were comparable between the genotypes.
3.1.5.4.5 No difference in numbers of macrophages expressing MHC class II and T cell costimulatory molecule CD80 in lymph nodes of naïve and immunized p75NTR-/- and wt animals

I furthermore analyzed the population of antigen presenting macrophages (CD45<sup>+</sup> CD11b<sup>+</sup> MHC class II<sup>+</sup>) and their expression of the co-stimulatory molecule CD80 (Fig. 3.27).
Results

3.1 Active EAE - Priming

Flow cytometric data revealed similar numbers of CD11b+ CD80+ MHC class II+ macrophages within the lymph nodes of immunized p75NTR-/- and wt mice (Fig. 3.27). We further distinguished dendritic cells within the CD11b+ population by antibodies against CD11c and found similar percentages and cell numbers expressing MHC-II and CD80 between the genotypes (data not shown).

The percentage of MHC class II+ cells within the CD11b+ compartment was already high in naïve lymph nodes (96%) and did not change after immunization with MOG35-55 (93%) (Fig. 3.28 A, C). However, the median fluorescence intensity for MHC class II increased significantly, suggesting that MHC class II expression was upregulated by immunization. In naïve lymph nodes, a very small fraction of CD11b+ cells expressed CD80+ (Fig. 3.28 B) and this fraction increased to around 40% after MOG35-55 immunization.

Fig. 3.27 | Flow cytometric analysis of antigen presenting macrophages in immunized lymph nodes.

A representative CD80/MHC class II flow cytometry dot plot of lymph node cells is depicted in A. The cells were previously gated on CD45+ CD11b+ cells. B shows the isotype control. The number of CD80+ MHC class II+ macrophages did not differ between the genotypes in MOG35-55 immunized lymph nodes (C).
(Fig. 3.28 D). No differences between p75<sup>NTR</sup> <sup>-/-</sup> and wt animals with respect to antigen presenting cells were found.

**Fig. 3.28 | Flow cytometric histograms for MHC class II and CD80 expression by CD11b<sup>+</sup> cells in naïve and immunized lymph nodes.**

Representative MHC class II (A, C) and CD80 (B, D) flow cytometry histograms of naïve (A, B) and immunized lymph node cells (C, D) are shown. The cells were previously gated on CD45<sup>+</sup> CD11b<sup>+</sup> cells. The percentage of MHC class II<sup>+</sup> cells within the CD11b<sup>+</sup> compartment did not change after MOG<sub>35-55</sub> immunization (~95%) (A, C). However, there is a clear increase in median fluorescence intensity of MHC class II after immunization, suggesting upregulation of this molecule. In naïve lymph nodes, only a minimal fraction of CD11b<sup>+</sup> cells was positive for CD80 (B). After immunization, this fraction increased to around 40% (D). The red line represents the isotype control. n=5
3.1.5.4.6 No difference in the cytokine/chemokine production of lymph node cells of MOG\textsubscript{35-55} immunized p75\textsuperscript{NTR} +/- and wt mice

Cytokine and chemokine concentrations were also measured by ELISA in the supernatant of restimulated lymph node cells taken from animals 10 days after MOG\textsubscript{35-55} immunization (Fig. 3.29). Comparable cytokine concentrations were found.

\begin{figure}
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\includegraphics[width=\textwidth]{fig329}
\caption{ELISA analysis of lymph node cells (dpi 10) after MOG\textsubscript{35-55} restimulation.}
\end{figure}

In summary, an extensive immunological workup failed to detect differences between p75\textsuperscript{NTR} deficient animals and wild type controls.
3.2 \( \text{p75}^{\text{NTR}} \) deficiency has no effect on T cell proliferation and antigen presentation to T cells.

The role of \( \text{p75}^{\text{NTR}} \) deficiency for T cell proliferation was determined by proliferation assays. At dpi 10 lymph node cells with and without a functional \( \text{p75}^{\text{NTR}} \) gene were restimulated \textit{in vitro} with MOG\textsubscript{35-55}. The medium was supplemented with radioactive \( ^3\text{H} \) thymidine. After 3d, counts per minute were measured showing how much \( ^3\text{H} \) thymidine was incorporated into the DNA by \textit{de novo} synthesis. Since only antigen specific T cells respond to MOG\textsubscript{35-55} restimulation, most of the proliferation activity observed can be ascribed to T cells. \( \text{p75}^{\text{NTR}} \) deficiency in CD4\textsuperscript{+} T cells had no effect on antigen specific T cell proliferation (Fig. 3.30 A).

To test whether APCs derived from \( \text{p75}^{\text{NTR}} \text{-/-} \) and wt mice differ in their respective capacity to stimulate MOG\textsubscript{35-55} specific wt T-cells, T cell clone cells were restimulated using APCs from \( \text{p75}^{\text{NTR}} \text{-/-} \) and wt spleens. T cells showed similar proliferation activity. Therefore, \( \text{p75}^{\text{NTR}} \) deficiency plays no role in antigen presentation by APCs to T cells (Fig. 3.30 B).

\begin{figure}[h]
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\includegraphics[width=	extwidth]{Fig_3.30.png}
\caption{\textbf{T cells proliferation after MOG\textsubscript{35-55} restimulation.}\label{fig:3.30}
Lymph nodes were collected at dpi 10 from actively immunized \( \text{p75}^{\text{NTR}} \text{-/-} \) and wt mice and \textit{in vitro} restimulated with MOG\textsubscript{35-55} (A). \( \text{p75}^{\text{NTR}} \) deficiency on T cells had no effect on T cell proliferation. MOG\textsubscript{35-55} specific wt T cells were also restimulated using APCs derived from \( \text{p75}^{\text{NTR}} \) and wt mice (B). \( \text{p75}^{\text{NTR}} \) deficiency in APCs had no effect on T cell proliferation upon restimulation.}
\end{figure}
3.2.1 No p75<sup>NTR</sup> expression by T<sub>H1</sub> cells

To test whether p75<sup>NTR</sup> is expressed by the MOG<sub>35-55</sub> specific T<sub>H1</sub> cell clone used in this study, p75<sup>NTR</sup> expression was measured after LPS, PMA (two concentrations), and CD3/CD28 stimulation at different time points by TaqMan qRT-PCR analysis (Tab. 3.1). No p75<sup>NTR</sup> gene transcripts were detected. Also, p75<sup>NTR</sup> expression was not modulated by the presence of NGF. The high levels of CCL5 produced by our T cell clone after CD3/CD28 as well as PMA stimulation did not change when NGF was applied (three different concentrations) (data not shown). Therefore, it could be ruled out that p75<sup>NTR</sup> deficiency has a direct effect in T cells. However, since T cell responses are dependent on the stimulation by other cell populations (e.g., DCs, B cells, endothelia, pericytes), they could be affected indirectly by p75<sup>NTR</sup> deficiency.

3.3 p75<sup>NTR</sup> deficiency has no effect on NO, ROS, and cytokine/chemokine production in bone marrow derived macrophages

To obtain a pure, non-prestimulated macrophage population, hematopoietic stem cells from the bone marrow were differentiated to macrophages by incubation with L929 medium. The mature immune cells had a distinct macrophage, but not granulocyte phenotype (Fig. 3.31 and Fig. 3.32).
3.3 Bone marrow derived macrophages

Fig. 3.32 | Flow cytometric analysis of BMDM showed a distinct macrophage phenotype.
BMDMs were CD45+ CD11b+ and only a small percentage expressed MHC class II. Only very few cells carried the granulocyte marker GR1.

3.3.1 No effect of p75\textsuperscript{NTR} deficiency on NO release by BMDMs

BMDMs were stimulated by the TLR-4 ligand LPS and the TLR-2 ligand Zymosan. NO release was indirectly detected by its reduction products nitrite and nitrate using the Griess reaction. No differences between p75\textsuperscript{NTR}-/- and wt BMDMs could be found (Fig. 3.33).

Fig. 3.33 | NO production by p75\textsuperscript{NTR}-/- and wt BMDMs after LPS (A) and Zymosan (B) stimulation.
No differences in NO release after TLR4 activation by LPS (A) and after TLR2 activation by Zymosan (B) between p75\textsuperscript{NTR}-/- and wt BMDMs were found.
3.3.2 ROS production of p75\textsuperscript{NTR} \textasciitilde/- and wt BMDMs

To examine if p75\textsuperscript{NTR} deficiency leads to altered ROS production by BMDMs, cells were prestimulated under various conditions. After stimulation with and without PMA and Zymosan, intracellular ROS were directly bound by nitroblue tetrazolium (NBT) which binds both H\textsubscript{2}O\textsubscript{2} and O\textsuperscript{2-}.

Highest ROS levels were measured after Zymosan stimulation. ROS production of unstimulated, PMA, and Zymosan stimulated BMDM did not differ significantly between p75\textsuperscript{NTR} \textasciitilde/- and wt cells (Fig 3.34). ROS production was always increased when the cells were prestimulated with LPS, IFN\textgamma, or fibrinogen.

3.3.3 Cytokine/chemokine production of p75\textsuperscript{NTR} \textasciitilde/- and wt BMDMs

Secretion of TNF\alpha, IL-6, and CCL5 was measured by ELISA after IFN\gamma, LPS, and fibrinogen stimulation (Fig. 3.35). No differences in the amount of TNF\alpha, IL-6 and CCL5 production could be found. In general, LPS stimulation had the greatest effect on BMDM activation, whereas IFN\gamma and fibrinogen induced production of only small amounts of cytokines and chemokines.
3.4 \textit{p75}^{\text{NTR}} expression in naïve lymph nodes and after priming with MOG\textsubscript{35-55}

To study which immune cell types of the lymph node express \textit{p75}^{\text{NTR}} under naïve conditions and after active immunization, cell populations were separated by FACS sorting and the amount of mRNA transcripts for \textit{p75}^{\text{NTR}} was determined by TaqMan qRT-PCR. Time points were naïve, dpi 3, dpi 6, and dpi 14 after immunization with MOG\textsubscript{35-55} peptide.

Only in B cells a constitutive expression of \textit{p75}^{\text{NTR}} was found under naïve as well as priming conditions (Tab. 3.2). In the conditions examined, \textit{p75}^{\text{NTR}} was never expressed by any of the examined T cell types (CD\textsuperscript{4+}, CD\textsuperscript{4+} effector, CD\textsuperscript{8+}, γδ, NK T), NK cells, CD11\textsuperscript{b+} CD11c\textsuperscript{+} (macrophages, neutrophils), and CD11\textsuperscript{b−} CD11c\textsuperscript{+} (DCs, neutrophils) cells. These findings are in accordance with our data revealing no \textit{p75}^{\text{NTR}} expression by a CD4\textsuperscript{+} T\textsubscript{H}1 T cell clone and by microglia after various stimuli \textit{in vitro} (Tab. 3.1, Tab. 3.3). Constitutive expression of \textit{p75}^{\text{NTR}} by B cells and no expression by T cells were shown before by Torcia \textit{et al.}, 1996.
3. Results

3.4 p75<sup>NTR</sup> expression by lymph node cells

<table>
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<th>ΔCT values</th>
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<th>dpi 6</th>
<th>dpi 14</th>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt; Effector T cells</td>
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<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
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Tab. 3.2 | p75<sup>NTR</sup> expression in lymph nodes after priming with MOG<sub>35-55</sub>

Constitutive expression of p75<sup>NTR</sup> by B cells in naive and primed lymph nodes. No expression by CD4<sup>+</sup>, CD4<sup>+</sup> effector, CD8<sup>+</sup>, γδ, NK T, NK, CD11b<sup>+</sup> CD11c<sup>+</sup>, and CD11b<sup>-</sup> CD11c<sup>+</sup> cells. bdl = below detection limit; nd = not determined, n=2
3.5 Active EAE in bone marrow chimeras

To further elucidate whether the observed increase in axonal damage after active immunization is derived from a (subtle) immune phenotype or an inherent enhanced vulnerability of the CNS, a bone marrow chimera system was established (Fig. 2.1). p75<sup>NTR</sup>/- and wt mice were whole-body irradiated with 11.5 gray. This results in a complete destruction of the immune system, except for non-dividing memory T and B cells. Irradiated p75<sup>NTR</sup>/- mice were reconstituted with wt bone marrow and vice versa. This results in chimeras that have a complete, functioning p75<sup>NTR</sup> gene in all cells of the body except immune cells (irradiated wt mice reconstituted with p75<sup>NTR</sup>/- bone marrow), and chimeras in which only immune cells express a functioning p75<sup>NTR</sup> but no other cells (irradiated p75<sup>NTR</sup>/- mice reconstituted with wt bone marrow). By using this system we were able to separate effects of local p75<sup>NTR</sup> deficiency in the CNS (wt → p75<sup>NTR</sup>/-) and in the immune system (p75<sup>NTR</sup>/- → wt). Control groups were p75<sup>NTR</sup>/- → p75<sup>NTR</sup>/- and wt → wt.

3.5.1 Effects of irradiation on immune cell infiltration and cytokine/chemokine production in the CNS

To first assess the effects of the irradiation procedure on immune cell infiltration and cytokine production in the spinal cord and brain, irradiated and reconstituted animals (wt → wt and p75<sup>NTR</sup>/- → p75<sup>NTR</sup>/-) were compared at d16 after irradiation to age-matched, naïve mice. Spinal cords (Fig. 3.36) and brains (Fig. 3.37) were prepared for TaqMan qRT-PCR analysis of cell lineage markers and cytokine/chemokine transcripts. Measurements of naïve and irradiated and p75<sup>NTR</sup>/- and wt CNS tissues were performed simultaneously. The ∆CT value shows the number of cycles required to detect the first signal significantly above the background noise. The lower the ∆CT value, the higher the specific mRNA amounts.

The expression of the macrophage/microglia surface marker CD68 and the T cell marker CD4 was not elevated in irradiated mice. TNFα and the chemokines CCL2 and CCL5 were slightly (2-fold) upregulated. CXCL10 was the only gene examined that was significantly modulated. There, fluorescence intensities of specific transcripts were 2-3 cycles earlier above background noise in irradiated CNS tissue than in naïve CNS tissues, which
equates a 4-6-fold upregulation. Neither p75\textsuperscript{NTR} itself nor Troy, another apoptosis inducing receptor of the same family, were regulated. No differences between p75\textsuperscript{NTR} -/- and wt mice were observed.

**Fig. 3.36 | Expression of inflammation related genes in naïve and irradiated spinal cords.**
No infiltration or proliferation/activation of macrophages/microglia (CD68) or T cells (CD4) was observed after irradiation. CXCL10 transcript levels were increased 4-6-fold (2-3 cycles). TNF\textalpha, CCL2 and CCL5 were only slightly upregulated (2-fold, equates 1 cycle). p75\textsuperscript{NTR} and Troy were not regulated. No differences between p75\textsuperscript{NTR} -/- (red) and wt (black) mice were observed. The lower the \( \Delta CT \) value, the higher the mRNA amount.
Very similar values for the expression of these genes were found in the brains of the same naïve or irradiated animals.

**Fig. 3.37 | Expression of inflammation related genes in naïve and irradiated brains.**
No infiltration or proliferation of macrophages/microglia (CD68) or T cells (CD4) took place after irradiation. CXCL10 transcription was increased 4-6-fold (2-3 cycles). TNFα, CCL2 and CCL5 were only slightly upregulated (2-fold, equates 1 cycle). p75\textsuperscript{NTR} and Troy were not regulated. No differences between p75\textsuperscript{NTR} -/- (red) and wt (black) mice were found. The lower the ΔCT value, the higher the mRNA amount.
3.5.2 Effects of irradiation on the infiltration of macrophages and T cells into the CNS

Similarly, on the histological level no cell infiltrations were found within the irradiated CNS (data not shown). Numbers of Mac-3$^+$ macrophages/microglia were similar in naïve and irradiated spinal cords. Only very few CD4$^+$ T cells were observed, but no differences between naïve and irradiated tissue and between p75$^{NTR}$ +/- and wt mice were found. To specifically detect immune cells infiltrating from the periphery, mice were reconstituted with transgenic actin-GFP expressing bone marrow cells. Overall numbers of GFP$^+$ immune cells were similar in naïve and irradiated mice as well as in p75$^{NTR}$ +/- and wt mice (data not shown).

Thus, we could exclude any significant microglia activation and cyto-/chemokine production in the brain and spinal cord of p75$^{NTR}$ +/- and wt mice after irradiation.

3.5.3 Reconstitution efficiency of bone marrow chimeras

To test to which degree the irradiation had destroyed the hematopoietic stem and immune cells, and to which degree a repopulation of the immune system by the transplanted cells had occurred, flow cytometric and immunohistochemical analyses were performed. For flow cytometric analysis a congenic mouse strain was used that expresses CD45.1.

When p75$^{NTR}$ +/- mice (CD45.2$^+$) receive bone marrow from wts (CD45.1$^+$) (wt $\rightarrow$ p75$^{NTR}$ +/-), immune cells should be CD45.1$^+$ 8 weeks after reconstitution. Vice versa, when wt mice (CD45.1$^+$) are reconstituted with p75$^{NTR}$ +/- bone marrow (CD45.2$^+$) (p75$^{NTR}$ +/- $\rightarrow$ wt), the majority of the immune cells should express CD45.2. Fig. 3.38 A shows that almost all leukocytes in the peripheral blood of p75$^{NTR}$ +/- mice expressed CD45.1 after reconstitution with wt (CD45.1$^+$) bone marrow. Similarly, almost all immune cells in wt (CD45.1$^+$) mice expressed CD45.2 after reconstitution with p75$^{NTR}$ +/- (CD45.2$^+$) bone marrow (Fig. 3.38 B). The reconstitution efficiency in our system was between 90% and 98% in all the experiments.
3. Results

3.5 Active EAE in bone marrow chimeras

Fig. 3.38 | Flow cytometric analysis of CD45+ immune cells in the peripheral blood in bone marrow chimeras 8 weeks after reconstitution.

Wt CD45.1+ bone marrow was received by CD45.2+ p75NTR-/- mice (wt → p75NTR-/-) (A). p75NTR-/- CD45.2+ bone marrow was received by CD45.1+ wt mice (p75NTR-/- → wt) (B). The reconstitution efficiency was above 90% in both groups.

Fig. 3.39 | Flow cytometric analysis for blood T and B cells in bone marrow chimeras.

Reconstitution efficiency of T cell population was 93% (A). It is very likely that the rest of the T cells (6.5%) are non-dividing memory T cells. A control group (p75NTR-/- → p75NTR-/-) shows specific binding of the antibodies and successful chimera generation (B).
To determine the reconstitution efficiency of immune cell subsets, T and B cells populations were also analyzed separately (Fig. 3.39 A). For that purpose, all lymphocytes were gated and separated in CD3⁺ T cells and CD3⁺ B cells. In wt \( \rightarrow \) p75NTR⁻/⁻ chimeras 6.5% of all T cells were CD45.2⁺. This population represents a non-dividing memory T cell subset that survived irradiation. The majority of T cells, however, (93%) were derived from the reconstituted bone marrow (CD45.1⁺). In a control group (p75NTR⁻/⁻ \( \rightarrow \) p75NTR⁻/⁻) ~ 99% of the T and B cells were CD45.2⁺ (Fig. 3.39 B).

![Image of chimerism analysis](image_url)

**Fig. 3.40 | Visualization of chimerism: Immunohistochemical analysis of spinal cord sections at the peak of disease after active immunization.**

Immunohistochemical staining of GFP (brown) detects only infiltrating immune cells in wt \( \rightarrow \) p75NTR⁻/⁻ (A), all CNS residential cells but no infiltrating cells in p75NTR⁻/⁻ \( \rightarrow \) wt (B), no cells at all in the control group p75NTR⁻/⁻ \( \rightarrow \) p75NTR⁻/⁻ (C) and all cells in GFP \( \rightarrow \) GFP wt mice (D). Scale bars = 50µm (A, B, D), 200µm (C)
The bone marrow chimerism can be visualized by immunohistochemistry for GFP in mouse spinal cord sections after EAE induction. For these experiments, wt mice were not carrying the congenic CD45.1 marker but expressed GFP under the actin promoter. After active immunization at peak of disease the infiltrating GFP+ immune cells are clearly distinguishable from the residential GFP− CNS cells in wt → p75NTR−/- chimeras (Fig. 3.40 A). *Vice versa*, in p75NTR−/- → wt chimeras the infiltrating immune cells are GFP− and all other cells are GFP+ (Fig. 3.40 B). The control groups show that no cell is GFP+ (p75NTR−/- → p75NTR−/-) (Fig. 3.40 C) or all cells are GFP+ (wt → wt), respectively (Fig. 3.40 D).

### 3.5.4 Significantly reduced incidence and less severe disease course in wt → wt chimeras in the acute stage after active immunization

The active immunization of the bone marrow chimeras was performed to examine the question, whether the lack of p75NTR on immune or CNS resident cells is responsible for the more severe disease observed in p75NTR−/- after active immunization.

When active EAE was induced in bone marrow chimeras by immunization with MOG35-55, wt → wt chimeras suffered from a significantly milder disease course (Fig. 3.41 A). From the beginning on, p75NTR−/- → p75NTR−/- (p < 0.01) and p75NTR−/- → wt (p < 0.05) chimeras had higher scores. Compared to these two groups, wt → p75NTR−/- chimeras showed a trend towards a milder disease course. However, no statistically significant difference between the groups wt → p75NTR−/- and wt → wt were found. The onset was also delayed in wt → wt. A trend towards higher cumulative scores in p75NTR−/- → p75NTR−/- chimeras was found (p = 0.06) (Fig. 3.41 B) (p75NTR−/- → p75NTR−/- 17.2 +/- 5.6, wt → p75NTR−/- 14.5 +/- 6.3, p75NTR−/- → wt 13.7 +/- 7.2, wt → wt 9 +/- 3.9).
3 Results

3.5 Active EAE in bone marrow chimeras

100% of all p75<sup>NTR</sup> /^-^ → p75<sup>NTR</sup> /^-^ and p75<sup>NTR</sup> /^-^ → wt chimeras developed EAE. This is a significant difference in incidence when compared to 83.3% in wt ^-^ → p75<sup>NTR</sup> /^-^ and 42.86% in wt → wt (Fig. 3.42) (p < 0.05).
In summary, mice with a p75<sup>NTR</sup> -/- immune system display more severe EAE; however, also mice with p75<sup>NTR</sup> deficiency in the CNS develop severe EAE. These data suggest that p75<sup>NTR</sup> plays a role in both, the immune system as well as the CNS.

### 3.5.5 Histopathological disease correlates in bone marrow chimeric mice

Wt → wt chimeras had less inflammatory infiltrates per spinal cord cross section (inflammatory index) (9.2 +/- 3.2 infiltrates/cross section) than p75<sup>NTR</sup> +/- → p75<sup>NTR</sup> +/- (12.5 +/- 2.1 infiltrates/cross section) (wt → p75<sup>NTR</sup> +/- 10.5 +/- 1.2 infiltrates/cross section, p75<sup>NTR</sup> +/- → wt 7.6 +/- 1.4 infiltrates/cross section) (Fig. 3.43). However, no significant difference was found (p > 0.05).

**Fig. 3.43 | Inflammatory index in the acute stage after active immunization of bone marrow chimeric mice.**

Slightly lower mean number of infiltrations per spinal cord cross section in wt → wt chimeras compared to p75<sup>NTR</sup> +/- → p75<sup>NTR</sup> +/-.
In this very acute disease stage demyelinated areas in the white matter of the spinal cord mainly reflect the dense infiltrations of immune cells, which displace the myelin. No difference in the percentage of demyelinated white matter was observed between the groups (p75NTR−/− → p75NTR−/− 9.02 +/- 1.75%, wt → p75NTR−/− 8.16 +/- 1.27%, p75NTR−/− → wt 7.62 +/- 3.48%, wt → wt 9.6 +/- 3.24%) (Fig. 3.44).

Axons in p75NTR deficient animals suffered from more pronounced early axonal damage indicated by APP accumulation in the acute disease stage (Fig. 3.45). Wt → wt (501 +/- 212 spheroids/mm²) and p75NTR−/− → wt (513 +/- 273 spheroids/mm²) chimeras had the lowest numbers of APP+ spheroids per mm². No significant difference between these two groups was found (p = 0.95). p75NTR−/− → p75NTR−/− (978 +/- 214 spheroids/mm²) and wt → p75NTR−/− (1031 +/- 268 spheroids/mm²) chimeras attained the highest numbers. Again, no difference between the latter two groups was observed (p = 0.74). However, both chimera groups with wt CNS show significantly lower early axonal damage when compared to the two

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**Fig. 3.44 | Demyelinated area in spinal cords of chimeras in the acute disease stage after active immunization.**

No difference in white matter demyelination in the spinal cord was found. The graph displays the percentage of demyelinated white matter.
other chimera groups with p75NTR deficiency within the CNS (p < 0.03). p75NTR -/- → wt chimeras had relatively high disease scores while relatively few axonal damage; wt → p75NTR -/- chimeras had also relatively high scores but more damaged axons. These findings indicate an increased vulnerability of p75NTR deficient CNS axons under inflammatory conditions.

In combination with the data obtained from the active immunization experiments, these results suggest that p75NTR deficiency plays a role in both, the CNS and the immune system to aggravate the inflammatory disease phenotype. Without functional p75NTR, immune cells could generate a more aggressive inflammatory response, as suggested by the severe early disease course in p75NTR -/- mice. On the other hand, p75NTR deficiency within the CNS (in neurons, oligodendrocytes, microglia, or astrocytes) results in increased axonal damage.

Fig. 3.45 Early axonal damage in bone marrow chimeric mice in the acute disease stage after active immunization.

p75NTR -/- → p75NTR -/- and wt → p75NTR -/- chimeras suffer from significantly more early axonal damage than p75NTR -/- → wt and wt → wt chimeras.
3.6 Induction of EAE by adoptive transfer of encephalitogenic T cells in p75<sup>NTR</sup> -/- and wt mice

To address the question if the increased disease severity in p75<sup>NTR</sup> -/- mice results from events during priming in peripheral lymph nodes or the spleen before disease onset, EAE was induced by adoptive transfer of an encephalitogenic MOG<sub>35-55</sub> specific CD4<sup>+</sup> T cell clone. In contrast to active EAE induction by immunization, adoptive transfer completely circumvents de novo stimulation and proliferation of antigen-specific T cells. The T-cell clone expresses wt p75<sup>NTR</sup>.

3.6.1 Similar disease onset and severity in the acute disease stage after adoptive transfer of MOG<sub>35-55</sub> specific CD4<sup>+</sup> T cells

Six days after adoptive transfer of T cells both mouse strains showed first clinical symptoms. No difference between p75<sup>NTR</sup> -/- and wt mice with regard to incidence (100% each) (data not shown), disease onset, and severity (Fig. 3.46) were observed at any time point (p = 0.38). The mice were sacrificed at the peak of disease (dpi 11) for histological analysis.

![Fig. 3.46 | Disease course in the acute disease phase after the transfer of MOG<sub>35-55</sub> specific CD4<sup>+</sup> T cells in p75<sup>NTR</sup> -/- and wt animals. Similar disease onset and severity during the acute stage in p75<sup>NTR</sup> -/- and wt mice. The mice were sacrificed at the peak of disease (dpi 11) for histological analysis.](chart.png)
3.6.1.1 Similar quantity and quality of inflammation in the acute disease stage after adoptive transfer of a CD4$^+$ T cell clone

To determine whether the similar disease course is also reflected on the histological level with regard to immune cell infiltration and tissue damage, immunohistochemical stainings of spinal cord sections were analyzed. No differences in the mean number of inflammatory infiltrates per spinal cord cross section were found at the peak of disease in $p75^{NTR}$ /- and wt mice (Fig 3.47 A) ($p75^{NTR}$ /- 2.7 +/- 1 infiltrates/spinal cord cross section, wt 2.7 +/- 1 infiltrates/spinal cord cross section, p = 0.97).

HE (A) and immunohistochemical stainings (B-D) of spinal cord cross sections show similar numbers of inflammatory infiltrates (A), Mac-3$^+$ macrophages/microglia (B), infiltrating CD3$^+$ T cells (C) and B220/CD45R$^+$ B cells (D). Scale bars = 100µm.
Also, no differences in the cellular composition of the infiltrates were observed with respect to macrophage/microglia numbers (Fig. 3.47 B) \((p75^{\text{NTR}}-/- 2396 +/- 165 \text{ cells/mm}^2, \text{wt } 2472 +/- 298 \text{ cells/mm}^2, p = 0.57)\), infiltration of T cells (Fig. 3.47 C) \((p75^{\text{NTR}}-/- 688 +/- 82 \text{ cells/mm}^2, \text{wt } 720 +/- 73 \text{ cells/mm}^2, p = 0.47)\) and B cells (Fig. 3.47 D) \((p75^{\text{NTR}}-/- 8.29 +/- 3.77 \text{ cells/mm meninges}, \text{wt } 10 +/- 3.11 \text{ cells/mm meninges}, p = 0.38)\). These similarities in the extent of inflammation are in accordance with the comparable disease course in both mouse strains.

To further also assess the quality of inflammation on the histological level, spinal cord slices were immunostained for S100A9, which reveals early activated macrophages, and iNOS to show NO producing cells. Spinal cord lesions in \(p75^{\text{NTR}}-/-\) mice showed similar numbers of S100A9\(^+\) \((p75^{\text{NTR}}-/- 491 +/- 108 \text{ cells/mm}^2, \text{wt } 399 +/- 99 \text{ cells/mm}^2, p = 0.12)\) and iNOS\(^+\) \((p75^{\text{NTR}}-/- 1861 +/- 941 \text{ cells/mm}^2, \text{wt } 2282 +/- 405 \text{ cells/mm}^2, p = 0.31)\) macrophages/microglia as in wt mice (Fig. 3.48).

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**Fig. 3.48** Early activated and NO producing macrophages/microglia at the peak of disease after adoptive transfer.

Immunohistochemical stainings of S100A9\(^+\) early activated macrophages and iNOS\(^+\) NO producing macrophages/microglia within lesions of the spinal cord showed no differences in lesional densities.
3.6.1.2 Increased axonal damage and loss in p75\textsuperscript{NTR} \textasciitilde/- mice in the acute disease stage after adoptive transfer

Axonal damage was assessed by APP immunostaining, and axonal loss by Bielschowsky silver impregnation. APP\textsuperscript{+} axonal spheroids indicative of disturbed axonal transport occurred more often in spinal cord lesions of p75\textsuperscript{NTR} \textasciitilde/- mice (Fig. 3.49) (p75\textsuperscript{NTR} \textasciitilde/- 873 +/- 196 spheroids/mm\textsuperscript{2}, wt 462 +/- 107 spheroids/mm\textsuperscript{2}, p = 0.0004). Axonal densities were decreased in p75\textsuperscript{NTR} \textasciitilde/- mice (p75\textsuperscript{NTR} \textasciitilde/- 27.6 +/- 2.2 %, wt 33 +/- 1.5 %, p = 0.0003).

![Fig. 3.49 | Axonal damage and loss in the acute disease stage after adoptive transfer.](image)

Immunohistochemical staining for APP\textsuperscript{+} axonal spheroids shows increased axonal damage in p75\textsuperscript{NTR} \textasciitilde/- mice. Bielschowsky silver impregnation reveals decreased axonal density within lesions of the spinal cord in p75\textsuperscript{NTR} \textasciitilde/- mice. Axonal densities are given as percentages of axonal density in the NAWM (set to 100%). Scale bars = 50\textmu m

The increased axonal damage and loss while very similar disease onset, severity, quantity, and quality of inflammation in p75\textsuperscript{NTR} \textasciitilde/- mice suggests that p75\textsuperscript{NTR} on CNS resident cells (neurons, astrocytes, microglia, oligodendrocytes) is neuroprotective in CNS inflammation. However, an effect of p75\textsuperscript{NTR} on infiltrating immune cells could not be fully excluded by the immunohistological analysis (i.e. a more damaging immune reaction). Therefore, the quality of inflammation was further analyzed on the mRNA level by qRT-PCR.
3.6.1.3 No difference in the expression of inflammation related genes in the acute disease stage after adoptive transfer between \( \text{p75}^{\text{NTR}} \)\(-/-\) and wt mice

After EAE induction by adoptive transfer of MOG\(_{35-55}\) specific encephalitogenic T cells, spinal cords were collected at the peak of disease and total mRNA of inflammation related genes was determined by TaqMan qRT-PCR. Both, naïve and inflammed \( \text{p75}^{\text{NTR}} \)\(-/-\) and wt spinal cords were analyzed simultaneously and in direct comparison. The ∆CT value represents the number of cycles needed to measure the first signal significantly above background. Therefore, the lower the ∆CT value the higher the amount of specific mRNA within the sample. As expected, several genes were strongly upregulated in EAE at the peak of disease, such as IFN\(_{\gamma}\), TNF\(\alpha\), IL-1, IL-6, iNOS, CCL2, CCL5, and CXCL10, reflecting an intense proinflammatory milieu (Fig. 3.50). Increased mRNA levels of CD4 and MHC class II reflect T cell and macrophage infiltration and activation. Other molecules, such as IL-17, macrophage colony stimulating factor (M-CSF), S100A8 (an marker for early activated macrophages and granulocytes), and MMP9 do not seem to play important roles in this phase of adoptive transfer EAE. Expression of the anti-inflammatory IL-10 was also increased at the peak of disease. The potential ligands for \( \text{p75}^{\text{NTR}} \), BDNF and NGF, were regulated only very little.

Expression levels of all analyzed genes were similar in \( \text{p75}^{\text{NTR}} \)\(-/-\) and wt mice, under naïve conditions as well as at the peak of disease. Consequently, to this point, the increased axonal damage in \( \text{p75}^{\text{NTR}} \)\(-/-\) mice cannot be explained by differences in the quantity or the quality of inflammation.
3 Results

3.6 Adoptive transfer EAE

Fig 3.50 | mRNA expression levels of inflammation related genes in naive mice and after adoptive transfer of encephalitogenic T cells.

While some cytokines and chemokines were strongly upregulated at the peak of disease, other genes, such as NGF and BDNF are barely regulated. No difference between p75NTR -/- (red) and wt (black) mice was observed. Shown are the cycle threshold (CT) values in relation to the housekeeping gene GAPDH (ΔCT). The lower the ΔCT value the higher the mRNA amount.
3.6.2 More severe disease course in p75\textsuperscript{NTR} \textasciitilde/- mice in the chronic disease stage after adoptive transfer of encephalitogenic T cells

A “late chronic” EAE experiment (>50 days of disease) was undertaken to examine the question whether the increased early axonal damage in p75\textsuperscript{NTR} \textasciitilde/- mice has effects on the disease severity and axonal density in the chronic stage. Therefore, EAE was induced by adoptive transfer of a CD4\textsuperscript{+} T cell clone and the mice were sacrificed at dpi 65. Again, disease onset and peak were very similar in p75\textsuperscript{NTR} \textasciitilde/- and wt mice (Fig. 3.51). However, from dpi 50 on daily clinical scores were significantly higher in p75\textsuperscript{NTR} \textasciitilde/- mice (p < 0.0001).

3.6.2.1 Less T cell infiltration in p75\textsuperscript{NTR} \textasciitilde/- mice in the chronic disease stage after adoptive transfer

At dpi 65 after adoptive transfer the inflammatory spinal cord lesions were analyzed with regard to macrophage, T cell and B cell infiltration (Fig. 3.53). In the chronic disease stage fewer inflammatory cells were observed than at the peak of disease. No differences in the density of Mac-3\textsuperscript{+} macrophages/microglia (Fig. 3.52 A) (p75\textsuperscript{NTR} \textasciitilde/- 406 +/- 50 cells/mm\textsuperscript{2}, wt 398 +/- 53 cells/mm\textsuperscript{2}, p = 0.76) and meningeal B220/CD45R\textsuperscript{+} B cells (Fig. 3.52 C)
(p75<sup>NTR</sup> +/- 1.05 +/- 0.42 cells/mm meninges, wt 0.81 +/- 0.44 cells/mm meninges, p = 0.32) were found between p75<sup>NTR</sup> +/- and wt mice. Surprisingly, despite the fact that the disease course was more severe, significantly less CD3<sup>+</sup> T cells were counted in p75<sup>NTR</sup> +/- compared to wt mice at this time point (Fig. 3.52 B) (p75<sup>NTR</sup> +/- 58.8 +/- 25.7 cells/mm<sup>2</sup>, wt 141.5 +/- 32.7 cells/mm<sup>2</sup>, p = 0.0002).

**Fig. 3.52 | Inflammation in the chronic disease stage after adoptive transfer.**

Immunohistochemical stainings for Mac-3 (A), CD3 (B) and B220/CD45R (C) reveal similar numbers of macrophages/microglia and B cells. Numbers of T cells were increased in wt mice although less severe disease course was observed. Scale bars = 100µm (A, B), 50µm (C)
3.6.2.2 Similar extent of demyelination and oligodendroglial damage in \( \text{p75}^{\text{NTR}}^-/- \) and \text{wt} mice in the chronic disease stage after adoptive transfer of encephalitogenic T cells

In this late chronic disease stage, the assessment of small perivascular, subpial or intraparenchymal inflammatory infiltrates (as performed in animals examined at the peak of disease) does not reflect the severity of tissue damage and disease. Instead, the extent of demyelinated lesions was assessed and given as percentage of the total white matter (Fig. 3.53 A) (\( \text{p75}^{\text{NTR}}^-/- 1.4 +/- 0.74 \% \) demyelination of WM, \text{wt} 1.41 +/- 0.65 \% demyelination of WM, \( p = 0.99 \)). Oligodendroglial damage was assessed by the density of NogoA\(^+\) mature oligodendrocytes within lesions (Fig. 3.53 B) (\( \text{p75}^{\text{NTR}}^-/- 466 +/- 123 \text{ cells/mm}^2, \text{wt} 330 +/- 112 \text{ cells/mm}^2, p = 0.11 \)). No differences were found between \( \text{p75}^{\text{NTR}}^-/- \) and \text{wt} mice.

![Image](image.png)

**Fig. 3.53 | Demyelination in the chronic disease stage after adoptive transfer.**
The percentage of the demyelinated area of the total white matter in spinal cord cross sections was calculated in LFB/PAS stained sections (A). Oligodendroglial damage was assessed by detection of mature oligodendrocytes expressing NogoA (B). No differences were observed between \( \text{p75}^{\text{NTR}}^-/- \) and \text{wt} mice. Scale bars = 200\( \mu \text{m} \) (A), 100\( \mu \text{m} \) (B)
3.6.2.3 Increased axonal damage and loss in p75$^{NTR}$/-/- mice in the chronic disease stage after adoptive transfer

The capacity of neuroaxonal regeneration was assessed by immunostaining for Gap43$^+$ neurons (Fig. 3.54). Only single GAP43$^+$ neurons were observed. No differences between p75$^{NTR}$/-/- and wt mice were found (p75$^{NTR}$/-/- 0.8 +/- 0.15 cells/mm², wt 0.89 +/- 0.22 cells/mm², p = 0.35).

![Graph showing axonal damage and loss in p75$^{NTR}$/-/-](image1)

**Fig. 3.54 | GAP43$^+$ neurons in the chronic disease stage after adoptive transfer.**
No differences between p75$^{NTR}$/-/- and wt mice were observed with regard to numbers of immunohistochemically detected Gap43$^+$ neurons.

![Images showing immunostaining for APP and Bleichowsky](image2)

**Fig. 3.55 | Axonal damage and loss in the chronic disease stage after adoptive transfer.**
Immunohistochemical staining for APP revealed an increased degree of axonal damage in spinal cord lesions of p75$^{NTR}$/-/- mice. The relative axonal density in lesions was significantly lower in p75$^{NTR}$ deficient mice. Axonal densities are shown as percentages of axonal density in the NAWM (set to 100%). Scale bars = 50µm.
The degree of axonal damage was determined by the density of APP\(^+\) axonal spheroids within lesions; the degree of axonal loss by Bielschowsky silver impregnation (Fig. 3.55). Axonal damage (p75\(^{NTR}\)\(^{-/-}\) 205 +/- 20 spheroids/mm\(^2\), wt 115 +/- 25 spheroids/mm\(^2\), p=0.000009) and loss (p75\(^{NTR}\)\(^{-/-}\) 29 +/- 1.8 %, wt 36 +/- 2.2 %, p=0.0001) were significantly increased in spinal cord lesions of p75\(^{NTR}\)\(^{-/-}\) mice.

Having shown the similar quantity and quality of inflammation in the acute and chronic disease stage after adoptive transfer, the more severe axonal damage and loss at both time points in p75\(^{NTR}\)\(^{-/-}\) mice suggested an important neuroprotective role for p75\(^{NTR}\) within the CNS, i.e. in neurons, astroglia, microglia, and oligodendroglia. Therefore, primary cell cultures of microglia and astrocytes were analyzed with regard to p75\(^{NTR}\) and cytokine/chemokine expression, ROS production, and modulation thereof by NGF.

3.7 No p75\(^{NTR}\) expression in primary microglia cultures

To analyze the role of p75\(^{NTR}\) deficiency in the CNS in more detail, primary microglia cultures from wt mice were used (kindly provided by Prof. Dr. Hanisch). No p75\(^{NTR}\) expression was detected after LPS and PMA stimulation at different time points by TaqMan qRT-PCR (Tab. 3.3). Also, different NGF concentrations had no effect on p75\(^{NTR}\) expression and did not modulate the amount of CCL5 produced after LPS stimulation (data not shown).

Therefore, a direct modulation of microglia via p75\(^{NTR}\) during EAE can be ruled out.

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Tab. 3.3 | No p75\(^{NTR}\) expression in primary microglia cultures. No p75\(^{NTR}\) expression by microglia upon various stimuli at different time points.
3.8 Constitutive \( p75^{NTR} \) expression in primary astrocytes cultures

To obtain a primary culture of astrocytes, after removing the meninges, single cell suspensions of the whole brain from \( p0 \) mice were disseminated in dishes. Oligodendrocytes were shaken off and microglia were depleted by clodronate. \( p75^{NTR} \) expression, assessed by TaqMan qRT-PCR, was found in unstimulated and PMA stimulated conditions at all time points (Tab. 3.4). This constitutive \( p75^{NTR} \) expression was not regulated by NGF.

Therefore, the effect of \( p75^{NTR} \) deficiency on astrocytes was analyzed in more detail with regard to ROS and cytokine/chemokine production and its modulation by NGF.

3.8.1 No difference in the ROS production by primary \( p75^{NTR} \) deficient and wt astrocytes

ROS production by primary \( p75^{NTR} /-/- \) and wt astrocytes cultures was measured in vitro after several pretreatments (LPS, IFN\( \gamma \)) and stimuli (PMA, Zymosan). Intracellular ROS were detected by the fluorescent dye CM-H\( _2 \)DCFDA. This molecule loses its membrane permeability upon ROS binding and is then trapped intracellularly. Arbitrary units for fluorescence intensity were measured over time (Fig. 3.56). Under none of the conditions examined differences between \( p75^{NTR} \) deficient and wt astrocytes could be found. Besides, pretreatment with LPS or IFN\( \gamma \) as well as stimulation with PMA or Zymosan did not lead to an increase in ROS production compared to untreated unstimulated controls.

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3.8.2 No difference in the cytokine/chemokine production of p75<sup>NTR</sup> −/− and wt astrocytes

TNFα, IL-6, IL-10, and CCL5 levels in supernatants of p75<sup>NTR</sup> −/− and wt astrocytes cultures were checked by ELISA after IFNγ and LPS stimulation (Fig. 3.57). IL-10 levels of astrocytes were low after LPS stimulation, whereas TNFα levels were high. IFNγ is no potent activator for cytokine / chemokine production. No differences between p75<sup>NTR</sup> −/− and wt astrocytes were detected.

Fig. 3.56 | ROS production by p75<sup>NTR</sup> −/− and wt astrocytes in vitro.
ROS detection by CM-H2DCFDA over time. Similar curves for p75<sup>NTR</sup> deficient and wt astrocytes regardless of pretreatment (none, IFNg, LPS) and stimulus (none, PMA, Zymosan).
Fig. 3.57  | Cytokine / chemokine release of p75NTR -/- and wt astrocytes in vitro. IFNγ did not activate astrocytes. No significant differences between p75NTR -/- and wt astrocytes were observed.
5 Discussion

5.3 The role of p75NTR in experimental CNS inflammation

In the present study I examined the role of p75NTR during EAE. EAE induction by active immunization with the MOG35-55 peptide in C57 BL/6 mice is the most common animal model for MS, because it mimics many pathological hallmarks of the disease, such as inflammation, demyelination, and axonal damage and loss. Copray et al. (2004) had shown before that p75NTR deficient mice suffer from a more severe disease course than wild type mice. However, the underlying mechanisms remained unclear.

We induced EAE not only by active immunization with the MOG35-55 peptide, but also by adoptive transfer of a MOG35-55 specific T cell clone. Thereby, the generation of a peripheral immune response within the lymph nodes was completely circumvented. Using this technique helped us to dissect the role of p75NTR during preclinical disease generation.

Furthermore, a bone marrow chimera system was used to separate the effect of p75NTR deficiency in the immune system and in the CNS.

In this work I address the question why p75NTR -/- mice get sicker, which cellular subsets express p75NTR under pathological conditions and what inflammatory or CNS-autonomous mechanisms are regulated by p75NTR after active immunization or transfer of encephalitogenic T cells.

4.1.1 More severe disease course in p75NTR -/- mice after active immunization with MOG35-55

As the starting point of this project, I found that p75NTR -/- mice suffer from a more severe disease course after active immunization with MOG35-55 compared to wt mice (Fig. 3.1). This is true from the very beginning on and extends into the chronic disease stage (dpi 31). Accordingly, in the chronic disease stage p75NTR deficient mice showed increased demyelination and axonal loss. However, the degree of inflammation, with regard to numbers of macrophages/microglia and T cells within the lesions, was similar in p75NTR -/- and wt...
mice. Furthermore, the number of acutely damaged axons, indicated by APP accumulation and hypophosphorylation of neurofilaments (SMI35+) did not differ between the genotypes, while axonal loss, assessed by Bielschowsky silver impregnation, was significantly increased in p75NTR-/- mice. These findings are in accordance with Copray et al. (2004) who also found a more severe disease course in p75NTR-/- mice while similar numbers of macrophages/microglia and T cells within the lesions compared to wt animals.

No differences in numbers of mature oligodendrocytes (NogoA+) (Fig. 3.3) were found in chronic lesions. This indicates that p75NTR-/- mice did not suffer from a more severe disease course because of increased oligodendroglial damage.

In contrast to Copray et al. (2004), however, I found more B cells within the meninges in p75NTR-/- mice (Fig. 3.4). However, B cells and myelin specific antibodies are not considered important effectors in MOG35-55 induced EAE in C57 BL/6 mice. As such, we hypothesized that the B cells were attracted by the overall more severe inflammation in p75NTR-/- mice. Nevertheless, B cells could play important roles in antigen presentation and activation of antigen specific T cells (Hjelmström et al., 1998 a; Hjelmström et al., 1998 b; Oliver et al., 2003).

The question arises here, whether the increased disease severity and axonal loss in p75NTR-/- mice, while similar numbers of macrophages/microglia and T cells within the lesions, is due to a more aggressive immune response or due to increased axonal vulnerability in p75NTR-/- mice.

4.1.2 Similar cellular composition of infiltrates and only minor differences in the quality of inflammation in p75NTR-/- and wt mice

Flow cytometric analysis of inflammatory cells extracted from inflamed spinal cords of actively immunized mice revealed similar numbers of cytotoxic T cells (CD8+), T helper cells (CD4+), γδ T cells (γδ TCR+), NK cells (NK1.1+ DX5+), macrophages (CD11b+ CD45hi), microglia (CD11b+ CD45lo), and granulocytes (CD11b+ MHC class IIhi) at the peak of disease (score 3) (Fig. 3.8, Fig. 3.9 A, B, C) in p75NTR-/- and wt mice. This is different to what has been reported by Küst et al. (2006) who found decreased numbers of macrophages/microglia and neutrophils in lesions of p75NTR-/- mice, which stands in contrast to the increased disease course. Instead, Küst et al. showed increased T cell numbers in p75NTR-/- lesions, which we
could not confirm in our experiments. Quite similar to our approach, Küst et al. had compared score matched animals of p75NTR -/- and wt mice at the peak of disease.

With regard to the quality of inflammation, I found similar expression levels of MHC class II by microglia and macrophages in p75NTR -/- and wt mice (Fig. 3.9 D, E). Also, no significant differences in the mRNA amount of the cytokines/chemokines IL-1, IL-6, iNOS, CCL2, CCL5, and CXCL10 were measured (Fig. 3.11).

NGF was upregulated during EAE with highest amounts observed at the peak of disease (Fig. 3.12). This implies important regulatory functions of neurotrophins during EAE. However, I found no regulation at the level of the p75NTR -/- . At all time points the mRNA levels of NGF were lower in p75NTR -/- mice, which could indicate a lack of positive feedback loops. Also, in p75NTR -/- mice, NGF was not as strongly regulated during EAE progression as seen in wt mice, similarly pointing towards positive feedback loops in NGF - p75NTR interaction.

At the peak of disease no significant differences with regard to quantity and quality of inflammation within the spinal cord were found on the histological, cellular, and molecular levels (except for the number of meningeal B cells, as discussed above).

### 4.2 The role of p75NTR in the naïve immune system and during peripheral immune priming after active immunization with MOG35-55

p75NTR -/- mice suffered from a more severe disease after active immunization with MOG35-55 right from the start (Fig. 3.1). This clinical difference was not observed, when EAE was induced by adoptive transfer, completely circumventing peripheral activation and proliferation of antigen specific T cells (Fig. 3.46, chapter 4.3). This strongly suggests that p75NTR plays a role in the regulation of the immune response in peripheral lymph nodes after active immunization.

Naïve and immunized lymph nodes from p75NTR -/- and wt mice were examined on the cellular, protein, and mRNA levels before disease onset.
4.2.1 Similar cellular composition and activation states of naïve and MOG-35-55 immunized lymph nodes in p75NTR -/- and wt mice

Frequencies of cytotoxic T cells (CD8+), T helper cells (CD4+), γδ T cells (γδ TCR+), NK T cells (NK.1.1+), NK cells (DX5+), regulatory T cells (FoxP3+), and B cells (CD19+) within naïve and MOG35-55 immunized lymph nodes showed no significant differences between p75NTR -/- and wt mice.

Furthermore, no differences in the expression levels of adhesion molecules for trafficking into secondary lymphoid tissues (CD62L+) and target tissues (CD11a+) (Fig. 3.19), as well as early (CD69+) (Fig. 3.20) and late (CD29+, CD44+) (Fig. 3.22, Fig. 3.19) activation markers were found by flow cytometric analysis. Cell numbers of macrophages (CD45+ CD11b+) and their expression rates of CD80 and MHC class II were also similar (Fig. 3.27, Fig. 3.28). CD80 is a mandatory costimulatory molecule of the immunological synapse during antigen specific activation of T cells.

Also, no difference in the expression of cyto- and chemokines (IL-1, IL-6, CCL2, CCL5, CXCL10) and iNOS was found in p75NTR -/- and wt animals neither in naïve lymph nodes nor after immunization with MOG35-55 on the mRNA level (Fig. 3.11). Furthermore, no differences in the production of cytokines/chemokines were observed on the protein level in MOG35-55 immunized lymph nodes of p75NTR -/- and wt mice (Fig. 3.29).

In summary, no significant differences with regard to immune cell populations and activations states thereof were found in naïve and immunized lymph nodes of p75NTR -/- and wt mice that could explain the clinical difference between the strains in the acute stage of actively induced MOG35-55 EAE.

4.2.2 No differences in the numbers of αβ and γδ T111 and T1117 cells with and without PMA stimulation in immunized lymph nodes

At day 3 and 7 after active immunization with MOG35-55 the cell numbers of T111 (IFNγ+) and T1117 (IL-17+) cells were determined for αβ (CD4+) and γδ T cells (γδ TCR+), showing no significant differences between p75NTR -/- and wt animals (Fig. 3.23-Fig. 3.26).
Also, stimulation of these cells with PMA \textit{ex vivo}, revealed similar frequencies of IFN\(\gamma\) and IL-17 producing \(\alpha\beta\) and \(\gamma\delta\) T cells.

\subsection*{4.2.3 No role for p75\textsuperscript{NTR} in antigen-specific T cell proliferation}

MOG\(_{35-55}\) specific T cells did not exhibit altered proliferation rates when lacking a functional p75\textsuperscript{NTR} (Fig. 3.30 A). \textit{Vice versa}, p75\textsuperscript{NTR} deficient APCs stimulated wt MOG\(_{35-55}\) specific T cells as efficiently as wt APCs, leading to comparable T cell proliferation rates (Fig. 3.30 B). Thus, neither p75\textsuperscript{NTR} deficiency on T cells nor on APCs does have an effect on antigen specific T cell proliferation.

Furthermore, p75\textsuperscript{NTR} expression by T\(_{H1}\) cells was never detected \textit{in vitro} by TaqMan qRT-PCR analysis (Tab. 3.1.).

\subsection*{4.2.4 No role for p75\textsuperscript{NTR} in the production of proinflammatory mediators by bone marrow derived macrophages}

NO (Fig. 3.33), ROS (Fig. 3.34), and cytokine/chemokine (Fig. 3.35) production after various pre-treatments and stimuli \textit{in vitro} did not differ between p75\textsuperscript{NTR} \(-/-\) and wt bone marrow derived macrophages. Thus, with regard to mediators of axonal damage like NO and ROS, a role for p75\textsuperscript{NTR} deficiency on macrophages, leading to a more aggressive inflammation, could be ruled out.

Furthermore, FACS sorted macrophages (CD11b\(^+\) CD11c\(^+\)) from lymph nodes after immunization (dpi 3, 6, 14) showed no p75\textsuperscript{NTR} expression \textit{in vivo} measured by TaqMan qRT-PCR (Tab. 3.2).
4.2.5 **Constitutive p75\textsuperscript{NTR} expression by B cells in lymph nodes in vivo**

Of all FACS sorted immune cell populations from naïve and immunized lymph nodes at different time points, only B cells showed relevant expression levels of p75\textsuperscript{NTR} in vivo by TaqMan qRT-PCR (Tab. 3.2). Effector T helper cells (CD4\textsuperscript{+}), cytotoxic T cells (CD8\textsuperscript{+}), γδ T cells (γδ TCR\textsuperscript{+}), NK T cells (DX5\textsuperscript{+}, TCR\textsuperscript{+}), NK cells (NK1.1\textsuperscript{+}, DX5\textsuperscript{+}), DCs (CD11c\textsuperscript{+} CD11b\textsuperscript{−}), and macrophages (CD11b\textsuperscript{+}, CD11c\textsuperscript{−}) did not express p75\textsuperscript{NTR}.

4.3 **Similar inflammation but more axonal damage in EAE induced by adoptive transfer**

By using the adoptive transfer technique, the peripheral activation of the immune system can be circumvented. After i.v. injection, the encephalitogenic MOG\textsubscript{35-55} specific T\textsubscript{H}1 cell clone enters the CNS and causes CNS specific inflammation after a delay of around 6-7 days (Flügel et al., 2001 b). In contrast to EAE induction by active immunization, we could thereby exclude effects of p75\textsuperscript{NTR} deficiency on the so called “priming phase”, the activation and proliferation of autoimmune T cells in secondary lymphoid organs.

Consequently, in early disease, the disease courses were very similar in both mouse strains after adoptive transfer (Fig. 3.46), whereas active immunization of p75\textsuperscript{NTR} \textsuperscript{−/−} mice led to increased disease scores right from the beginning (Fig. 3.1). This finding suggests an important role of p75\textsuperscript{NTR} during the priming phase. Quantitative histological analysis, showing comparable numbers of lesions and infiltrating immune cells (T cells, macrophages, B cells) (Fig. 3.47), was in line with the similar disease course in the acute disease stage. Also, no differences in the quality of inflammation were found on the histological (numbers of S100A9\textsuperscript{+} early activated macrophages and iNOS expressing immune cells) (Fig. 3.48) and mRNA levels (cytokines, chemokines, inflammation related molecules) (Fig. 3.50).

Thus, despite the fact that we did not find any significant difference with regard to inflammation, p75\textsuperscript{NTR} \textsuperscript{−/−} mice suffered from significantly more acute axonal damage and axonal loss (Fig. 3.49). We interpreted these results as a hint that p75\textsuperscript{NTR} might play neuro- and/or axono-protective roles in the inflamed CNS.
This hypothesis is supported by the more severe disease course of p75\textsuperscript{NTR} --/- mice in the chronic disease stage after adoptive transfer (Fig. 3.51). Again, the quantity of inflammation and demyelination was similar (p75\textsuperscript{NTR} --/- spinal cords showed even less T cells) (Fig. 3.52, Fig. 3.53), but the degrees of axonal damage and loss were significantly increased in p75\textsuperscript{NTR} --/- mice (Fig. 3.55). Similar to the results obtained by active EAE induction, the levels of oligodendroglial damage were similar and therefore not contributing to the more severe disease course (Fig. 3.53).

Taken together, the similarities in the disease course as well as in the quantity and quality of inflammation in the early disease stage after adoptive transfer of encephalitogenic T cells contrast with increased axonal damage and loss in p75\textsuperscript{NTR} --/- mice, strongly suggesting a neuroprotective role for p75\textsuperscript{NTR} in CNS inflammation.

### 4.3.1 Constitutive expression of p75\textsuperscript{NTR} by astrocytes, but not by microglia

Since our \textit{in vivo} EAE results hinted at a neuroprotective role of p75\textsuperscript{NTR} within the CNS, p75\textsuperscript{NTR} expression by different CNS cell types was assessed \textit{in vitro}. Astrocytes were found to express p75\textsuperscript{NTR} constitutively (Tab. 3.4), whereas microglia did not Tab. 3.3). These findings are in line with previous publications (Hanbury \textit{et al.}, 2002; Oderfeld-Nowak \textit{et al.}, 2003). Therefore, astrocytes are candidates as mediators of the observed neuroprotection via p75\textsuperscript{NTR} after EAE induction. We can however rule out, that p75\textsuperscript{NTR} deficiency on astrocytes has effects on their ROS and cytokine/chemokine production \textit{in vitro} (Fig. 3.56, Fig. 3.57). Also, CCL5 secretion by astrocytes revealed no modulation by NGF. Therefore, according to the results obtained so far, the inflammatory response of reactive astrocytes is not altered by p75\textsuperscript{NTR}.

It is conceivable that astrocytes play alternative roles via p75\textsuperscript{NTR}, e.g. in the protection from excitotoxicity or in the integrity of the blood brain barrier (BBB). Astrocytic foot processes are important functional components of the BBB. Copray \textit{et al.} (2004) suggested a role of p75\textsuperscript{NTR} on the BBB integrity. However, they mainly considered an effect of p75\textsuperscript{NTR} on endothelial cells.

Future experiments will include an assessment of primary cell cultures of p75\textsuperscript{NTR} --/- and wt neurons with regard to their susceptibility to inflammatory mediators. A broad range of p75\textsuperscript{NTR} effects has been observed so far in neuronal cultures. For instance, neuroprotection
4 Discussion

4.4 Active EAE in bone marrow chimeras

by p75\textsuperscript{NTR} has been shown with regard to extracellular β-amyloid cytotoxicity (Zhang et al., 2003) and glutamate excitotoxicity (Kume et al., 2000; Culmsee et al., 2002).

Furthermore, inflammatory cells (T cells, B cells and monocytes) secrete high amounts of neurotrophins upon antigen specific stimulation (Stadelmann et al., 2002; Kerschensteiner et al., 1999; Kerschensteiner et al., 2003; Moalem et al., 2000), which is likely to play a role in CNS inflammation.

4.4 Dissecting the roles of p75\textsuperscript{NTR} in the immune system and the CNS: Active immunization of bone marrow chimeras

To further separate the effects of p75\textsuperscript{NTR} deficiency within the immune system and the CNS, a bone marrow chimera system was used. In these chimeras only cells of the immune system or the CNS, respectively, had a functioning p75\textsuperscript{NTR}.

We show on the molecular and histological levels that the irradiation procedure did not lead to immune cell infiltration of the CNS (Fig. 3.36 Fig. 3.37). Furthermore, cytokine/chemokine levels were not significantly increased except for CXCL10. This is in accordance with previous reports (Mildner et al., 2007). More importantly, no differences in the effect of CNS irradiation between p75\textsuperscript{NTR} \(-/-\) and wt mice were found.

4.4.1 Increased axonal damage in chimeras deficient for p75\textsuperscript{NTR} within the CNS

wt \(\rightarrow\) wt chimeras had a significantly milder disease course than p75\textsuperscript{NTR} \(-/-\) \(\rightarrow\) wt and p75\textsuperscript{NTR} \(-/-\) \(\rightarrow\) p75\textsuperscript{NTR} \(-/-\) animals. The latter tended to suffer from the most severe disease course. However, the disease courses of p75\textsuperscript{NTR} \(-/-\) \(\rightarrow\) wt and wt \(\rightarrow\) p75\textsuperscript{NTR} \(-/-\) chimeras lay right between the control groups at an early time point of disease (dpi 20) (Fig. 3.41 A). The cumulative score and inflammatory index were highest in p75\textsuperscript{NTR} \(-/-\) \(\rightarrow\) p75\textsuperscript{NTR} \(-/-\) and lowest in wt \(\rightarrow\) wt. p75\textsuperscript{NTR} \(-/-\) \(\rightarrow\) wt and wt \(\rightarrow\) p75\textsuperscript{NTR} \(-/-\) chimeras lay in between. Only 50% of the
wt → wt and 80% of wt → p75NTR -/- mice showed clinical signs, whereas all p75NTR -/- → wt and p75NTR -/- → p75NTR -/- chimeras developed EAE. Thus, a significantly lower disease incidence was found in animals with wt as compared to p75NTR-deficient immune systems. The more severe disease course early on of p75NTR -/- → wt compared to wt → wt and the lower incidence of wt → p75NTR -/- and wt → wt chimeras can be viewed as an indicator for a role of p75NTR during peripheral immune priming. As mentioned before, a down-modulatory role of regulatory B cells on T cells via p75NTR is a promising hypothesis, since B cells constitutively express high levels of p75NTR.

Of note however, animals deficient for p75NTR within the CNS (p75NTR -/- → p75NTR -/- and wt → p75NTR -/-) suffered from significantly more early axonal damage than CNS wt mice (p75NTR -/- → wt and wt → wt), independent of the level of inflammation (Fig. 3.45). At this early disease stage the acute axonal damage measured by APP⁺ spheroids does not correlate well with the disability score, since conduction block mediated by inflammatory cytokines is a more important contributor to the neurological symptoms (Bitsch et al., 2000; Rieckmann, 2005; Siffrin et al., 2010). However, the increased levels of acute axonal damage early on likely result in increased axonal loss in the chronic disease stage, in line with the increased disability observed in chronic actively induced and adoptive transfer EAE (Fig. 3.51, Fig. 3.55).

These findings strongly suggest a neuro- and/or axo-protective role of p75NTR within the CNS. This is also supported by the comparable disease severity in the acute disease stage and increased severity in chronic disease after EAE induction by adoptive transfer in p75NTR -/- mice compared to wt mice (Fig. 3.51, chapter 4.3). In these experiments, the mounting of a peripheral immune response was completely circumvented and inflammatory CNS infiltration was the same in wt and p75NTR -/- mice. As such, this model allows for the direct assessment of the role of p75NTR -/- in the CNS.

However, since p75NTR -/- → wt chimeras showed significantly increased scores compared to wt → wt mice, effects of p75NTR deficiency within the immune system are also indicated. Furthermore, the increased disease severity in p75NTR -/- mice at disease onset after active immunization (Fig. 3.1, chapter 4.1.1), but not after adoptive transfer, corroborates these findings.

Taken together, my data from active immunization experiments in bone marrow chimeras strongly hint at roles for p75NTR in both, the CNS (neuroprotective) and the immune system (anti-inflammatory / down-modulatory).
4.5 Is p75\textsuperscript{NTR} relevant to the human inflammatory demyelinating disease, MS?

The neurotrophin receptor p75\textsuperscript{NTR} attracted attention in the field of MS research, when increased levels of NGF were found in the cerebrospinal fluid of MS patients (Laudiero et al., 1992). Also, re-expression of p75\textsuperscript{NTR} by many different cell types in MS lesions was shown. Valdo et al. (2002) described p75\textsuperscript{NTR} expression by perivascular cells, reactive astrocytes, microglia / macrophages, and a subset of not otherwise specified inflammatory cells in chronic active lesions of MS patients. p75\textsuperscript{NTR} immunoreactivity was never co-localized with apoptotic cells. Thus, regulatory roles of neurotrophins and their receptors on supportive glia, but also on infiltrating immune cells during MS are suggested.

Furthermore, it has been shown that neurons of the CNS re-express p75\textsuperscript{NTR} under many pathological conditions (e.g. stroke, axonal transection, and also MS) (Ebadi et al., 1997; Dechant & Barde, 2002).

Single nucleotide polymorphisms (SNPs) have been described before to modulate the outcome of and susceptibility to MS. Mirowska-Guzel et al. (2008) found two SNPs in the BDNF gene that increase the risk of MS. For one of the described SNPs, carriers developed first neurological signs of the disease earlier than controls. This could emerge from a modified binding affinity or signalling of BDNF via p75\textsuperscript{NTR} leading to reduced pro-survival signals to neurons. In the light of my results, showing a more severe disease course and increased axonal loss in the chronic disease stage in p75\textsuperscript{NTR} \textsuperscript{-/-} mice after adoptive transfer of MOG\textsubscript{35-55} specific T cells, the p75\textsuperscript{NTR} deficiency could impede the pro-survival signal by BDNF.

Furthermore, a SNP in Exon 3 of the NGF gene of male patients with relapsing-remitting MS was found, resulting in a single amino acid substitution in the NGF protein (Akkad et al., 2008). Exon 3 encodes exclusively the precursor form of NGF, proNGF. ProNGF is only bound by p75\textsuperscript{NTR}, leading to cell death (Rabizadeh & Bredesen, 2003). This mutation could alter the proNGF-p75\textsuperscript{NTR}-interaction (e.g., the affinity) or the subsequent signalling. In view of the increased disease severity in p75\textsuperscript{NTR} \textsuperscript{-/-} mice after active immunization with MOG\textsubscript{35-55} shown in this study, p75\textsuperscript{NTR} mediated signalling by proNGF could differ during the peripheral immune response. This in turn could result in a more severe inflammation.

Only little is known about the function of p75\textsuperscript{NTR} on neurons, glia cells, and peripheral immune cells during CNS inflammation. Detailed elucidation of expression patterns, inflammatory response modulations, and the capacity to induce apoptosis or survival signals,
respectively, by \( p75^{\text{NTR}} \) could result in promising, novel treatments of MS. My study shows that B cells are potential targets for promising approaches. I found a high constitutive expression of \( p75^{\text{NTR}} \) in B cells that is downregulated during peripheral disease generation. Therefore, an immunomodulatory role of \( p75^{\text{NTR}} \) in B cells is indicated that could possibly be modulated by artificial activation.

This study shows also a beneficial role of \( p75^{\text{NTR}} \) within the CNS. Neuroprotective properties of \( p75^{\text{NTR}} \) in neurons have been shown before (Hamanoue et al., 1999; Gentry et al., 2000; Rabizadeh & Bredesen, 2003). Therefore, \( p75^{\text{NTR}} \) specific agonists could possibly prolong axonal survival and/or limit axonal loss, resulting in a milder disease course in the chronic disease stage.

Another interesting approach is the contribution of astrocytes via \( p75^{\text{NTR}} \) to the disease. I detected constitutively high expression levels of \( p75^{\text{NTR}} \) in astrocytes. Although, I can rule out effects of \( p75^{\text{NTR}} \) deficiency on astrocytic TNF\( \alpha \), IL-6, IL-10, CCL5, and ROS release \textit{in vitro}, it is conceivable that \( p75^{\text{NTR}} \) modulates the capacity of antigen presentation or the production of other cytokines/chemokines.

### 4.6 Conclusions

Taken together, we can rule out a major role of many different immune cell populations and inflammatory mediators, which do not contribute to the generation of a more aggressive inflammatory phenotype resulting in the more severe disease of \( p75^{\text{NTR}} \)-/- mice after active immunization with MOG\textsubscript{35-55}. This includes T helper cells, \( \gamma\delta \) T cells, cytotoxic T cells, \( T_{\text{reg}} \), NK T cells, NK cells, B cells, granulocytes, macrophages, and microglia in spinal cords and lymph nodes in the naïve state as well as during immune priming and disease. Also, \( p75^{\text{NTR}} \) does not play a role in antigen specific T cell proliferation, maturation, and activation, CD80 expression by macrophages, and MHC class II expression by macrophages and microglia. In line, significant differences for the key proinflammatory cytokines TNF\( \alpha \), IFN\( \gamma \), IL-1, and IL-6 and the chemokines CCL2, CCL5, and CXCL10 are also ruled out, as well as substantial differences in NO and ROS production. Furthermore, under the conditions examined, \( p75^{\text{NTR}} \) expression can be ruled out for T helper cells, \( \gamma\delta \) T cells, cytotoxic T cells, NK T cells, NK cells, DCs, macrophages, and microglia. This has been shown on histological, cellular, and molecular levels \textit{in vivo}, \textit{ex vivo}, and \textit{in vitro}. 

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To this point we have not found convincing evidence for substantial differences between p75\textsuperscript{NTR} \textasciitilde/- and wt mice in the naïve state or after immunization with regard to the quality and quantity of inflammation.

However, I show constitutive expression of p75\textsuperscript{NTR} on B cells, which is in accordance with Torcia et al., (1996). Also, p75\textsuperscript{NTR} expression was downregulated in B cells after active immunization with MOG\textsubscript{35-55}. It is conceivable that p75\textsuperscript{NTR} deficiency in B cells leads to altered activation, antigen presentation, or expression of surface molecules. This, in turn, could affect the quality of activation of other cell subsets, like T cells or macrophages. Therefore, p75\textsuperscript{NTR} deficiency could indirectly affect non-p75\textsuperscript{NTR} expressing cell lineages. Additional experiments should elucidate the role of B cells during the generation of the peripheral immune response in p75\textsuperscript{NTR} deficient mice (chapter 4.7).

While p75\textsuperscript{NTR} \textasciitilde/- animals suffered from a more severe disease course from the beginning on after active immunization with MOG\textsubscript{35-55}, the disease courses of p75\textsuperscript{NTR} \textasciitilde/- and wt mice were similar during disease onset after adoptive transfer of a MOG\textsubscript{35-55} specific T cell clone. This indicates a down-modulatory role of p75\textsuperscript{NTR} during peripheral priming.

Despite the similar disease onset, however, p75\textsuperscript{NTR} \textasciitilde/- mice subjected to adoptive transfer showed increased axonal damage and loss compared to wt. In addition, the similar disease course at onset but increased severity in the chronic disease stage in p75\textsuperscript{NTR} \textasciitilde/- mice after adoptive transfer of a MOG\textsubscript{35-55} specific T cell clone indicates a role for p75\textsuperscript{NTR} within the CNS. An influence of p75\textsuperscript{NTR} deficiency on the quantity and quality of inflammation can be ruled out for the acute and chronic disease stage after adoptive transfer EAE. While microglia did not express p75\textsuperscript{NTR} in our study, astrocytes did. However, p75\textsuperscript{NTR} deficiency had no effect on cytokine, chemokine, and ROS production by astrocytes \textit{in vitro}.

The more severe disease courses of p75\textsuperscript{NTR} \textasciitilde/- \textarrow{} wt as well as wt \textarrow{} p75\textsuperscript{NTR} \textasciitilde/- bone marrow chimeras compared to wt \textarrow{} wt hint at a role of p75\textsuperscript{NTR} in both compartments, the immune and central nervous system. Also, increased axonal damage in p75\textsuperscript{NTR} \textasciitilde/- \textarrow{} p75\textsuperscript{NTR} \textasciitilde/- and wt \textarrow{} p75\textsuperscript{NTR} \textasciitilde/- mice independent of the level of inflammation indicates an axonoprotective role for p75\textsuperscript{NTR} in the inflamed CNS.

In summary, the present study shows a role for p75\textsuperscript{NTR} in the immune system during the generation of the peripheral immune response, as well as in the CNS during EAE disease. Our data confine the range of possible functions of p75\textsuperscript{NTR} within the immune system and the CNS during EAE and emphasize the role of p75\textsuperscript{NTR} in B cells, astrocytes, and neurons.
### 4.7 Outlook

With regard to the role of p75\(^{NTR}\) in the immune system, our data point to a regulatory role of B cells via p75\(^{NTR}\). In a recent publication, Matsushita et al. (2010) describe the role of a relatively newly discovered B cell subset (B10 cells). This CD1d\(^{hi}\) CD5\(^{+}\) CD19\(^{hi}\) population produces anti-inflammatory IL-10 and exhibits regulatory roles during EAE initiation (Matsushita et al., 2008; Yanaba et al., 2008; Yanaba et al., 2009; Haas et al., 2010; Watanabe et al., 2010). Matsushita et al. (2010) also found that B10 cells do not directly influence T cell proliferation, which could be in accordance with our data showing similar T cell proliferation of p75\(^{NTR}\) \(-/-\) and wt lymph node cells. However, they describe that B10 cells affect IFN\(\gamma\) and TNF\(\alpha\) production by CD4\(^{+}\) T cells. Further experiments could include *in vitro* analysis of primary B cell cultures, e.g. achieved by FACS or MACS sorting of lymph nodes or spleens. Possible differences in the subpopulation of regulatory B10 cells could be examined. Effects of p75\(^{NTR}\) by B cells could be elucidated *in vivo* by inducing EAE in B cell depleted p75\(^{NTR}\) \(-/-\) and wt mice. This could be done by using a blocking CD19 or CD20 antibody or by reconstituting recombination activating gene (RAG) deficient mice with T cells only but not B cells. Furthermore, the use of B cell specific p75\(^{NTR}\) \(-/-\) will be helpful to assess the importance of p75\(^{NTR}\) as a modulator of B cell immune function.

A constitutive expression of p75\(^{NTR}\) on astrocytes, but no differences in TNF\(\alpha\), IL-6, IL-10, CCL5, and ROS production were found between p75\(^{NTR}\) \(-/-\) and wt astrocytes after stimulation *in vitro*. Thus, it would be interesting to assess whether p75\(^{NTR}\) deficiency contributes to an altered secretion pattern of other cytokines/chemokines or to a difference in the ability of astrocytes to present antigens. Effects on the BBB integrity are also imaginable.

Another critical experiment includes neuronal cell cultures to directly elucidate the role of p75\(^{NTR}\) on neurons. Readouts with regard to apoptosis, excitotoxicity, axonal growth and dendritic branching as well as electrophysiological measurements would be useful, preferably after stimulation with inflammatory mediators or even immune cells.

Furthermore, experiments with hippocampal slice cultures from p75\(^{NTR}\) \(-/-\) and wt animals would be promising. The addition of cytokines/chemokines or p75\(^{NTR}\) \(-/-\) and wt immune cells would let us examine immune system-CNS-interactions directly in a controlled system. E.g., it would be very interesting to assess differences in axonal damage or loss in p75\(^{NTR}\) \(-/-\) and wt hippocampal slices after antigen-specific T cell mediated inflammatory responses. This can be done by the addition to the culture medium of a MOG\(\text{35-55}\) specific T cell clone or immunized lymph node cells from p75\(^{NTR}\) \(-/-\) and wt mice. It is also
imaginable to sort out cell populations of interest of immunized p75NTR -/- and wt lymph nodes by using the MACS system and add them to the slice culture. These experiments would help to dissect the roles of p75NTR ex vivo and in vitro.

To further dissect the role of p75NTR not only by adoptive transfer experiments and bone marrow chimeras, transgenic conditional knock-out approaches would be highly valuable, e.g. by inducing EAE in astrocyte, B cell or neuron-specific p75NTR knock-out animals in vivo.
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**A Appendix**

**A.1 Common procedures**

**Cell culture incubations**

If not otherwise specified, all cells and cultures were long-term incubated at 37°C with 5% CO₂ in a cell culture incubator.

**Cell washing**

Filled up the tube with specified medium or buffer, centrifugation at 290g, 4°C, 6 min, discarded the supernatant, and resuspension of the pellet in an appropriate amount of specified medium or buffer.

**Determination of cell counts**

Stained a cell suspension sample with 10% trypan blue in PBS (1:10) to reveal dead cells. Counted in a Neubauer counting chamber and extrapolated to total cell counts (cells/ml).

**A.2 Reagents: Solutions, buffers, emulsions**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amounts</th>
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<tbody>
<tr>
<td>astrocyte medium</td>
<td></td>
</tr>
<tr>
<td>BME medium</td>
<td>2 drops</td>
</tr>
<tr>
<td>100units/ml penicillin/streptomycin</td>
<td>100ml</td>
</tr>
<tr>
<td>10% NHS</td>
<td>2 drops</td>
</tr>
<tr>
<td>2mM L-glutamine</td>
<td>100ml</td>
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<tr>
<td>Bielschowsky developer solution</td>
<td>65% nitric acid</td>
</tr>
<tr>
<td>20ml 37% formalin</td>
<td>citric acid buffer</td>
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<tr>
<td>0.5g citric acid</td>
<td>citric acid monohydrate</td>
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<tr>
<td>ad. 1000ml H₂O</td>
<td>pH 6</td>
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</table>
DAB solution
25mg DAB
20µl 30% H₂O₂
ad. 50ml PBS

HCl alcohol
175ml isopropanol
2.5ml 20% hydrochloric acid
75ml H₂O

ELISA wash buffer
0.05% Tween 20
ad. 1000ml H₂O
pH 7.4

LFB solution
1g LFB
1000ml 95% EtOH
5ml acetic acid

eosin solution
2g Eosin-G CertiStain
198ml 70% isopropanol
filtered
a little glacial acetic acid before use

macrophage medium
DMEM high glucose (4.5g/l), + L-glutamine, + sodium pyruvate
10% FCS
5% NHS
100units/ml penicillin/streptomycin
50µM β-mercaptoethanol

FACS buffer
1% FCS
ad. 1000ml PBS

microglia medium
DMEM high glucose (4.5g/l), + L-glutamine, + sodium pyruvate
10% FCS
100units/ml penicillin/streptomycin

FACS medium
2% FCS
500ml DMEM (high glucose)

MOG emulsion (per animal)
200µg MOG₃₅₋₅₅
100µl PBS
100µl CFA
5mg/ml killed mycobacteria tuberculosis
100µl incomplete Freund’s adjuvant
emulsified by sonication

Griess reagent A
0.1% naphtyl ethylene
in H₂O

Griess reagent B
1% sulphanilamide
6% H₃PO₄ (85%)
in H₂O

H₂O₂ ad. 50ml PBS

FACS medium
DMEM high glucose (4.5g/l), + L-glutamine, + sodium pyruvate
10% FCS
100units/ml penicillin/streptomycin

Griess reagent A
0.1% naphtyl ethylene
in H₂O

Griess reagent B
1% sulphanilamide
6% H₃PO₄ (85%)
in H₂O

H₂O₂ ad. 50ml PBS

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Griess reagent B
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H₂O₂ ad. 50ml PBS

FACS medium
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100units/ml penicillin/streptomycin

Griess reagent A
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in H₂O

Griess reagent B
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in H₂O

H₂O₂ ad. 50ml PBS

FACS medium
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10% FCS
100units/ml penicillin/streptomycin

Griess reagent A
0.1% naphtyl ethylene
in H₂O

Griess reagent B
1% sulphanilamide
6% H₃PO₄ (85%)
in H₂O

H₂O₂ ad. 50ml PBS

FACS medium
DMEM high glucose (4.5g/l), + L-glutamine, + sodium pyruvate
10% FCS
100units/ml penicillin/streptomycin
Appendix A.2 Reagents

**PBS**
9.55g PBS
ad. 1000ml H₂O

**PFA**
40g PFA
ad. 1000ml PBS
heating up to 60°C while constantly stirring to solve the PFA
some drops of 1M NaOH until the PFA is solved
pH 7.6

**T cell clone medium**
RPMI-1640 medium
40% FCS HyClone
20% dimethyl sulfoxide (DMSO)

**T cell clone freezing medium**
RPMI-1640 medium
40% FCS HyClone
20% dimethyl sulfoxide (DMSO)

**Tail lysis buffer**
100mM Tris
5mM EDTA
200mM NaCl
0.2% SDS
pH 8.5

**TBE**
10.8g Tris
5.5g boric acid
4mL 0.5M EDTA

**TBS**
9g NaCl
50ml 1M Tris/HCl

A.3 Chemicals / reagents manufacturers

- **³H-thymidine**  Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- **acetic acid**  Merck KgaA, Darmstadt, Germany
- **agarose**  StarPure, StarLab GmbH, Ahrensberg, Germany
- **ammonium chloride**  Pharm Lyse, 10x conc., BD Biosciences, Heidelberg, Germany
- **ammonium hydroxide**  Merck KgaA, Darmstadt, Germany
β-mercaptoethanol  
Sigma-Aldrich Chemie GmbH, Steinheim, Germany

BME  
Gibco, Invitrogen, Darmstadt, Germany

boric acid  
Roth, Karlsruhe, Germany

CD3/CD28  
Dynabeads, Dynal Biotech, Invitrogen, Darmstadt, Germany

citric acid  
Merck KgaA, Darmstadt, Germany

clodronate  
Calbiochem, Merck KgaA, Darmstadt, Germany

cloral hydrate  
Merck KgaA, Darmstadt, Germany

CM-H$_2$DCFDA  
Invitrogen, Darmstadt, Germany

copper sulphate  
Merck KgaA, Darmstadt, Germany

DAB  
Sigma-Aldrich Chemie GmbH, Steinheim, Germany

DePeX  
VWR international, Darmstadt, Germany

diethyl ether  
Sigma-Aldrich Chemie GmbH, Steinheim, Germany

DMEM  
High Glucose (4.5g/l), w/ L-Glutamine, sodium pyruvate, PAA, Cölbe, Germany

DMSO  
Sigma-Aldrich Chemie GmbH, Steinheim, Germany

DNA ladder  
gene ruler, Fermentas, St. Leon-Rot, Germany

DNase  
DNase I, Roche, Mannheim, Germany

dNTPs  
Fermentas, St. Leon-Rot, Germany

EDTA  
Roth, Karlsruhe, Germany

ELISA kit  
Quantikine Colorimetric Sandwich ELISA, R&D Systems, Minneapolis, MN, USA

Eosin-G CertiStain  
Merck KgaA, Darmstadt, Germany

ethidium bromide  
Sigma-Aldrich Chemie GmbH, Steinheim, Germany

EtOH absolute  
Merck KgaA, Darmstadt, Germany

FACS antibodies  
BD Biosciences, Heidelberg, Germany

Fc block  
BD Biosciences, Heidelberg, Germany

FCS (IHC)  
Biochrom AG, Berlin, Germany

FCS (T cell clone)  
HyClone, Thermo Fisher scientific GmbH, Schwerte, Germany

Fibrinogen  
R&D Systems, Minneapolis, MN, USA

formalin  
Merck KgaA, Darmstadt, Germany

Giemsa  
Merck KgaA, Darmstadt, Germany

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<td>H37 RA, Difco Laboratories, Augsburg, Germany</td>
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<td>NHS</td>
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<td>nitric acid</td>
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<td>nitroblue tetrazolium</td>
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<td>non-essential amino acids</td>
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<td>penicillin/streptomycin</td>
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<td>Qiagen DNAse</td>
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<td>Qiagen RNAlater</td>
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<td>qRT-PCR master mix</td>
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<td>w/o L-glutamine, PAA, Cölbe, Germany</td>
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<td>trypan blue (0.4%)</td>
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<td>trypsin / EDTA</td>
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<td>Zymosan</td>
<td>Sigma-Aldrich Chemie GmbH, Steinheim, Germany</td>
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### A.4 Equipment / instrument manufacturers

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-well plates (cell culture)</td>
<td>Greiner Bio-One GmbH, Frickenhausen, Germany</td>
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<td>96-well plates (qRT-PCR)</td>
<td>Applied Biosystems, Carlsbad, CA, USA</td>
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<tr>
<td>cell harvester</td>
<td>MicroBeta FilterMate-96 Harvester, Perkin Elmer, Rodgau, Germany</td>
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<td>cell irradiator</td>
<td>B+R Industrie-Elektronik GmbH, Bad Homburg, Germany</td>
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<td>Cell Quest Pro 5.2</td>
<td>BD Biosciences, Heidelberg, Germany</td>
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<tr>
<td>cell strainer (70 + 40 nm)</td>
<td>BD Biosciences, Heidelberg, Germany</td>
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<tr>
<td>cell^F program</td>
<td>Olympus Europa GmbH, Hamburg, Germany</td>
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<tr>
<td>centrifuge (cell culture)</td>
<td>5810 R, Eppendorf AG, Hamburg, Germany</td>
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<td>cytopsin</td>
<td>Shandon Cytospin 4, Thermo Fisher scientific GmbH, Schwerte, Germany</td>
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<td>ELISA reader</td>
<td>Model 680, Biorad, Munich, Germany</td>
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<td>FACS instrument</td>
<td>FACSCalibur, BD Bioscience, Heidelberg, Germany</td>
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<tr>
<td>FACS tubes</td>
<td>BD Bioscience, Heidelberg, Germany</td>
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<tr>
<td>fluorescence reader</td>
<td>Safire, Tecan, Mainz, Germany</td>
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<td>gel rack &amp; power supply</td>
<td>SubCell GT &amp; PowerPack 300, Biorad, Munich, Germany</td>
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<td>glass slides</td>
<td>Menzel, Braunschweig, Germany</td>
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<tr>
<td>incubator</td>
<td>Cellstar, Nunc GmbH, Wiesbaden, Germany</td>
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<td>linear accelerator</td>
<td>Clinac 3, 600 C – 446, Varian Medical Systems, Palo Alto, CA, USA</td>
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<td>microtome</td>
<td>Leica, Wetzlar, Germany</td>
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<td>microwave</td>
<td>Bosch, Gerlingen-Schillerhöhe, Germany</td>
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<td>NanoDrop</td>
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<td>Neubauer counting chamber</td>
<td>Brand GmbH &amp; Co KG, Wertheim, Germany</td>
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<td>PCR cycler</td>
<td>Thermocycler T3, Biometra, Göttingen, Germany</td>
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<td>Qiagen QiaShredder</td>
<td>Qiagen, Hilden, Germany</td>
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<tr>
<td>qRT-PCR cycler</td>
<td>StepOne Plus, Applied Biosystems, Carlsbad, CA, USA</td>
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<tr>
<td>scintillation counter</td>
<td>Wallac MicroBeta TriLux, Perkin Elmer, Rodgau, Germany</td>
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<td>sonicator</td>
<td>SonoPuls, Bandelin Electronics, Berlin, Germany</td>
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<td>Teflon coated pot</td>
<td>Roth, Karlsruhe, Germany</td>
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<td>tissue processor</td>
<td>TP 1020, Leica, Wetzlar, Germany</td>
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<tr>
<td>WinMDI 2.9</td>
<td>TSRI, La Jolla, CA, USA</td>
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</table>
Acknowledgements

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I am also very thankful to Dr. med. Stefan Nessler for great theoretical and practical support.

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Curriculum vitae

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Education and Qualifications

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Carl-von-Ossietzky University, Oldenburg, Germany
Studies in biology
Degree: Diploma, Grade: very good

1992 – 1999
Herbartgymnasium, Oldenburg, Germany
Abitur (A-levels)
Professional experience

05/2007 – present
Institute of Neuropathology, University Medical Center Göttingen, Germany
Ph.D. thesis: “The Role of the p75 Neurotrophin Receptor in Experimental Inflammation of the Central Nervous System”
Supervisor: Prof. Dr. med. Christine Stadelmann-Nessler

Carl-von-Ossietzky University, Oldenburg, Germany
Diploma thesis: “Morphological Characterization of the Retina in Connexin45 knock-out/Connexin 36 knock-in Mice”
Supervisor: Prof. Dr. Ulrike Janssen-Bienhold

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Carl-von-Ossietzky University, Oldenburg, Germany
student assistant in the laboratory of Neuroanatomy

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Institute for the Biosynthesis of Neural Structures, University Medical Center, Hamburg, Germany
Research internship

Publications

2010
“Wallerian degeneration: a major component of early axonal pathology in multiple sclerosis”
Brain Pathol 20(5):976-85
* equal contribution
Talks

2010


“The role of p75 neurotrophin receptor in axonal damage during CNS inflammation”

Ph.D. program CMPB, 2nd Ph.D. students seminar, Göttingen, Germany

2009


“The role of p75-neurotrophin-receptor in axonal damage during CNS inflammation”

NeuroWind, Motzen, Germany

2009


“The role of the p75 low affinity neurotrophin receptor in axonal damage during CNS inflammation”

German Society of Neuropathology and Neuroanatomy, Düsseldorf, Germany

Poster presentations

2010


“The role of the p75\textsuperscript{NTR} in axonal damage during CNS inflammation”

NeuroWoche, Mannheim, Germany

2010


“The role of the p75\textsuperscript{NTR} in axonal damage during CNS inflammation“

Inflammation in Brain Disease (NeurInfNet), Odense, Denmark
2009  
“**The role of the p75NTR in axonal damage during CNS inflammation**“
Ph.D. program GGNB Science Day, Göttingen, Germany

2009  
“**p75NTR-mediated axon protection in inflammatory CNS disease**”
European Committee for Treatment and Research In Multiple Sclerosis (ECTRIMS), Düsseldorf, Germany

2009  
**Dallenga T***, Escher A***, Nessler S, Boretius S, Brück W, Lühder F, Stadelmann C
“**MS-like cerebral inflammatory pathology in mice: A new experimental model for MS research**”
Meeting of the German Neuroscience Society, Göttingen, Germany
* equal contribution

2008  
**Tobias Dallenga**, Wolfgang Jäger, Stefan Nessler, Christine Stadelmann
“**The role of p75 neurotrophin receptor in axonal damage during CNS inflammation**“
Ph.D. program GGNB opening, Göttingen, Germany