Oligodendrocyte Cell Death Induced By Disialoganglioside GD3

Dissertation

Zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften des Fachbereichs für Biologie der Universität Konstanz

vorgelegt von
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Tag der mündlichen Prüfung: 1.12.2000
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ACKNOWLEDGEMENTS

Thank you very much, to Prof. Pierluigi Nicotera, for having created outstanding working conditions in his laboratory and for believing in the success of difficult projects. His mode to propagate fascination for science was a big source of motivation.

I am grateful to Dr. Dr. Thomas Hartung for being second reviser of this thesis.

I want to thank Dr. Marcel Leist for supervision of my work, stimulating discussion and proofreading of this thesis.

I am grateful to the Graduiertenkolleg Biochemische Pharmakologie for financial support during the time of my thesis and exceptional possibilities to enlarge my scientific education.

Special thanks are dedicated to Dr. Florence Malisan and Dr. Roberto Testi for initiating the GD3 project in fruitful collaboration. Thanks to Dr. Florence Malisan for performing ganglioside extraction and TLC analysis, to Oliver Speer for help with the mitochondria experiments, to Dr. Thomas Mürdter for GC-MS measurements, and to Gregor Pinski for carrying out LPS determinations.

I am indebted to Dr. Julio Castro-Palomino and Prof. Richard R. Schmidt for providing synthetic GD3.

Ein dickes **DANKE!!!** an alle Kollegen im Labor, für harmonisches Zusammenarbeiten, jederzeit freundliche Unterstützung, wenn es um Hilfe mit neuen Methoden, das Teilen von Ressourcen und Suchen von Material ging und für die netten spontanen und geplanten Grillaktionen! Ein extra liebes DANKE für Elvira Gawlitta-Gorka, die mich mit unermüdlichem Einsatz in der Dressur von Oligodendrozyten unterstützt hat. Herzlichen Dank an Heike, das Organisationstalent in Sachen Laborordnung und Fetenplanung.

Mille grazie a tutti i colleghi, agli ospiti ed ai cari amici di origine italiana conosciuti in questo laboratorio. Grazie per tutte le risate e per aver portato un po’ dello spirito mediterraneo alla “seria” Germania. Ci dovremmo “mescolare” un po’ di più! Un grandissimo ringraziamento va a Florence Malisan per le stimolanti discussioni via mail sugli sviluppi del progetto sul GD3 e per essere diventata un’ amica. Merci!

Un grazie grande come il mare per il mio dolce ¾ che ha vissuto tutti gli alti ed i bassi della vita scientifica in “prima linea” insieme a me. Grazie per il sostegno e per guardare sempre “at the bright side of life“!

Das grösste DANKESCHÖN ist für meine Familie reserviert, die immer für mich da ist. Besonders meine Eltern haben mir den langen Weg von Studium und Promotion erst ermöglicht und mich in allen meinen Plänen unterstützt.

This work was carried out in the time between January 1997 and October 2000 at the Chair of Molecular Toxicology of Prof. Pierluigi Nicotera at the University of Konstanz, Germany.

Publications:

Simon, B., Malisan, F., Testi, R., Nicotera, P., and Leist M. (2000) Disialoganglioside GD3 induced caspase-independent oligodendrocyte apoptosis. *Manuscript in preparation*.

Castro-Palomino, J.C., Simon, B., Speer, O., Leist, M., and Schmidt, R.R. (2000) Synthesis of Ganglioside GD3 and Comparison to Bovine GD3 with Regard to Oligodendrocyte Apoptosis and Mitochondrial Damage. *Chem Eur J. In press.*

Contributions to other studies:

Leist, M., Single, B., Naumann, H., Fava, E., Simon, B., Kuhnle, S. and Nicotera, P. (1999) Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis into necrosis. *Exp. Cell Res.*, **249**, 396-403.

Leist, M., Single, B., Naumann, H., Fava, E., Simon, B., Kuhnle, S. and Nicotera, P. (1999) Nitric oxide inhibits execution of apoptosis at two distinct ATP- dependent steps upstream and downstream of mitochondrial cytochrome c release. *Biochem Biophys Res Commun*, **258**, 215-221.

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ABBREVIATIONS

$\Delta\Psi_m$	mitochondrial membrane potential
Act D	actinomycin D
AIF	apoptosis-inducing factor
anti-CD95	agonistic anti-CD95 antibody
aSMacidic	sphingomyelinase
BSA	bovine serum albumin
CHX	cycloheximide
CNPase	2',3' cyclic-nucleotide-phosphodiesterase
CsA	cyclosporine A
CSF	cerebrospinal fluid
Cyt *c*	cytochrome *c*
DD	death domain
DDR	death domain receptor (CD95, TNF-R etc.)
EAE	experimental allergic encephalitis
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EH-1	ethidium homodimer
Fas	CD95
FasL	CD95 ligand
FCCP	carbonyl cyanide 4-trifluoro-methoxyphenylhydrazone
FCS	fetal calf serum
GD3	disialoganglioside GD3
IL-6	Interleukin 6
IFNγ	Interferone γ
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
MPTP	mitochondria permeability transition pore
MS	multiple sclerosis
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide
nSM	neutral sphingomyelinase
PBS	phosphate-buffered saline
PCD	programmed cell death, apoptosis
PS	phosphatidylserine
TMRE	tetramethylrhodamine ethylester
TNFα	tumor necrosis factor α
zVAD-fmk	N-benzyloxycarbonyl-Val-Ala-aspartyl-fluoromethylketone

1. INTRODUCTION

1.1. The physiologic function of the oligodendrocyte

Oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) support fundamentally the function of neuronal cells. Solely axons enveloped with several sheaths of oligodendrocyte or Schwann cell plasma membrane are capable of transmitting electrical signals with high velocity and without loss of signal strength. In the PNS each Schwann cell envelopes only one neuron by building up one single segment of myelin, while in the CNS, every oligodendrocyte envelopes the axons of 20–30 neurons, spanning an area of 40-80 μm with its multiple myelin segments (Knapp, 1997; Trapp *et al.*, 1997).

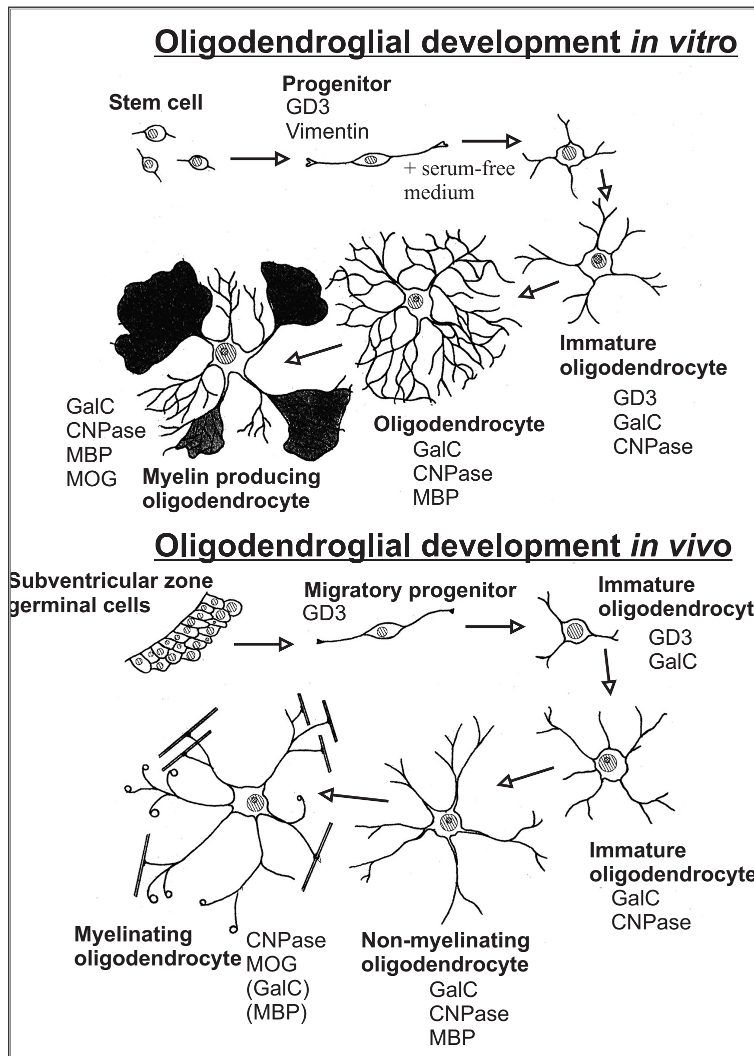

Figure 1: Maturation of oligodendrocytes. Adapted from (Hardy 1991).

Myelin is the main product of oligodendrocytes, composed of 70-75 % lipid and 25-30 % protein. It is composed of cholesterol, glycosphingolipids and complex phospholipids. The most abundant glycosphingolipid is galactosyl cerebroside, accounting for 32 % of all myelin glycolipids. Typical proteins embedded in the lipid bilayer are myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated protein (MAG), and myelin oligodendrocyte glycoprotein (MOG) (Cuzner and Norton, 1996; Ludwin, 1997).

Oligodendrocyte precursors evolve from the neuroepithel of the primitive neural tube as bipotential O-2A progenitors. After their migration to their decisive target zone they undergo final differentiation. The development and maturation from precursor cell to mature oligodendrocyte can be followed, as the differentiation states are characterised by the specific expression of typical glycolipids and myelin-proteins (Fig. 1). The bipolar O-2A cells do still have the potential to develop either into oligodendrocytes (O) or type-II-astrocytes (2A). *In vitro*, the developmental direction depends on the definition of the culture medium (Raff *et al.*, 1983). O2-A-progenitors are positive for several typical antigens, among them disialoganglioside GD3 and vimentin. Oligodendrocyte precursors dedicated to the oligodendrocyte lineage can be identified by positive staining for galactosylsulfatide, the antigen recognised by the O4-antibody (Sommer and Schachner, 1981). During maturation, oligodendrocyte morphology becomes more and more complex. The structure of the membrane processes changes from network-shape to the multi-layer composition present in myelin. Differentiating oligodendrocytes lose GD3 from their cell membranes and begin to express the cytoplasmic enzyme 2’3’-cyclic-nucleotide-phosphodiesterase (CNPase), followed by the appearance of galactosyl cerebroside (O1-antigen) on the outside of the cell membrane (Amur-Umarjee *et al.*, 1990). The ultimate state of maturation is defined by the expression of the myelin proteins MBP, PLP, MAG and finally MOG (Cameron and Rakic, 1991; Coffey and McDermott, 1997; Hardy and Reynolds, 1991; Kirchhoff *et al.*, 1997).

1.2. Demyelination and oligodendrocyte damage

1.2.1. *Demyelinating diseases in humans*

Myelin producing cells have an essential function in insulating neuronal processes. Damage of the myelin envelope around the axon results in slow or impaired conduction of electrical nervous signals. As a consequence of this, typical symptoms of demyelination are: disturbances of the vision, if the optic nerve is affected; weakness of the limbs or paraesthesia due to involvement of the spinal cord; ataxic gait and impairment of concentration and memory, if the disease attacks cerebellum or cortex (Pender, 2000). Demyelinating diseases are mainly caused by brain inflammation, which can develop into an autoimmune reaction against myelin components (e.g. multiple sclerosis, postinfectious encephalomyelitis) (Griffin, 1990; Kepes,

1993; Lucchinetti *et al*., 1996). Also, inherited mutations in oligodendrocyte genes (e.g. Pelizaeus-Merzbacher-disease) (Gencic *et al*., 1989; Watanabe *et al*., 1969) or metabolic dysfunction (thyroid deficiency in development, adrenoleukodystrophy) (Rosman *et al*., 1972; Sargent *et al*., 1994; Taneda *et al*., 1983) are responsible for myelin damage. The best known and probably most abundant "white matter disease" (demyelinating disease) is multiple sclerosis. Onset of symptoms is observed in persons with an average age of 30 years, with higher prevalence in women. About 90 % of all patients enter into disease with a relapsing-remitting course where acute phases are followed by full or partial recovery. Most of the MS-victims develop secondary progressive MS, with advancing deterioration interrupted by occasional relapses, minor remissions and plateau phases. A primary progressive MS, manifesting often in aggravating spastic paresis, affects 10 % of all MS patients (Coyle, 1996; Pender, 2000).

The pathologic features of MS are dominated by the multifocal degeneration of myelin in the CNS, mostly beginning in vicinity of the ventricles (Baum *et al*., 1994). During acute phases of MS, immune cells overcome the disturbed blood brain barrier and enter the nervous tissue. They begin to release inflammatory mediators, attracting more immune cells, and activating brain resident cells like microglia and astrocytes (Merrill and Benveniste, 1996; Merrill and Murphy, 1997; Patterson, 1995; Persidsky, 1999; Sharief, 1998). Activated microglia cells contribute to the production of inflammatory mediators and to phagocytosis of myelin and dying cells, while activated astrocytes show extensive hypertrophy and formation of a scar-like tissue, the sclerotic plaque (Zielasek and Hartung, 1996). The latter remains as demyelinated zone, with remyelination occurring, if at all, at the lesion edge. Remyelination is transient in typical MS, and although axons are not primarily injured in demyelinating diseases, they do not persist forever in the plaques devoid of myelin but degenerate by losing axon continuity (Mancardi *et al*., 2000; Prineas *et al*., 1993; Sobel, 1995; Trapp *et al*., 1998).

1.2.2. *Experimental allergic encephalitis: model of demyelination in rodents*

The cause of inflammatory demyelination has been a matter of intensive studies. The interactions of immune system and CNS are complex, thus complicating or even impeding investigations in patients. For this reason, experimental models for demyelination and oligodendrocyte death have been developed *in vivo* and *in vitro*.

An *in vivo* model to study demyelinating diseases was discovered accidentally in the 1890s by Louis Pasteur (Pasteur, 1885). By injecting a rabbit brain-derived rabies vaccine into humans, treated persons eventually developed encephalomyelopathy. Injection of brain homogenates into animals clarified, that brain compounds and not the virus induced the disease. By combining the administration of brain components and Freund's adjuvant (emulsion of aqueous antigen in oil,

containing killed mycobacteria), induction protocols were developed that cause an experimental demyelinating disease with symptoms very similar to inflammatory demyelination in humans (experimental autoimmune encephalitis, EAE).

To induce an immune reaction against CNS-myelin, initially homogenates of brain or spinal cord were administered together with Freud's adjuvant (Brown *et al*., 1982). Analysis of the antigenic compounds of myelin revealed that the isolated myelin-proteins MBP, PLP and MOG as well as short peptide sequences of these proteins were able to trigger an autoimmune response to CNS-myelin if they were injected into animals (Kerlero de Rosbo *et al*., 1995; Zaller and Sloan, 1996). The role of T-lymphocytes in the initiation EAE was investigated in adoptive transfer-studies, where the disease was induced by injection of expanded T-cell clones or splenocytes activated by myelin components (Ben-Nun *et al*., 1981; Mokhtarian *et al*., 1984). A large variety of induction protocols in different mouse strains has been developed until now. Comparison of results is often complicated due to differences in genetic background of animals, composition of the antigenic component or schedules of induction (reviewed in (Gold *et al*., 2000; Goverman and Brabb, 1996; Jafarian-Tehrani and Sternberg, 1999; Swanborg, 1995)).

EAE is mainly induced in rodents, where mice, in comparison to rats, show symptoms resembling more closely the pathogenesis in humans. While in rats mononuclear inflammation and paralysis, but often no demyelination is observed, mice display all these symptoms and show relapsing courses, depending on induction protocol and mouse strain used (Raine *et al*., 1980).

EAE pathology shares many similarities with MS. The initial trigger of an autoimmune reaction to myelin might be a viral infection, as was speculated for MS, and as it is working in the Theiler's murine encephalomyelitis virus (TMEV) model of demyelination (Dal Canto *et al*., 1995; Noseworthy, 1999; von Herrath, 2000). The blood-brain-barrier (BBB) becomes leaky and the CNS-endothelium activated, facilitating the infiltration of inflammatory cells from the circulation. Mainly mononuclear cells, i.e. monocytes and lymphocytes, accumulate in the so called "perivascular cuffs". CD4 T-cells, followed by other T-cells, B-cells, and monocytes are the first leukocytes to enter the brain tissues. T-cells become activated by recognising their specific antigen, and begin to produce cytokines, like TNFα and IFNγ. In response to IFNγ, antigens are presented on microglia and occasionally on astrocytes after upregulation of MHC-II (Cash *et al*., 1993). The inflammatory environment leads to recruitment of further immune cells from the circulation and to activation of more microglia and astrocytes (Coyle, 1996; Owens and Sriram, 1995). Many researchers addressed also an important role to autoantibodies in MS and EAE, as strongly elevated levels of autoantibodies are present in CSF and in plasma of MS patients (Amor *et al*., 1997; Archelos and Hartung, 2000; Wekerle, 1999). Studies on the importance of CD95-CD95-Ligand (CD95L) interactions in EAE revealed attenuation in mice deficient in CD95 or CD95L (Malipiero *et al*., 1997; Sabelko *et al*., 1997; Waldner *et al*., 1997). A contradictory study found EAE induction independent from the CD95 system (Dittel *et al*., 1999). The trigger for oligodendrocyte degeneration and myelin destruction has not been defined

yet, but possible candidates include inflammatory mediators, like cytokines and nitric oxide (NO) (Giovannoni *et al*., 1998); autoantibodies in combination with complement factors (Genain *et al*., 1999; Litzenburger *et al*., 1998); or direct cytotoxicity mediated by T-cells (Freedman *et al*., 1991; Jewtoukoff and Bach, 1988; Kawai and Zweiman, 1988; Linington *et al*., 1993).

1.2.3. In vitro *models of oligodendrocyte degeneration*

MS and its experimental model EAE are known as inflammatory brain diseases. Many different inflammatory cytokines have been detected in serum, CSF and in CNS-tissue, e.g. TNFα and TNFβ (lymphotoxin), IFNγ, IL-6, IL-1, but also anti-inflammatory cytokines like IL-4 or IL-10 (Correale *et al*., 1995; Frei *et al*., 1991; Hofman *et al*., 1989; Renno *et al*., 1995; Selmaj *et al*., 1991a; Vartanian *et al*., 1995). Therefore, testing the effect of cytokines on isolated oligodendrocytes was an obvious experiment. Since human-derived oligodendrocytes are rare, primary rat, murine or bovine oligodendrocytes were used. Additionally, a variety of cell lines exist. They originate either from rat and mouse oligodendrocyte progenitors (CG4, O4CB-neu) (Jung *et al*., 1995; Louis *et al*., 1992), which in part can be differentiated from progenitors to mature oligodendrocytes, or from human oligodendroglioma (e.g. MO3.13 (McLaurin *et al*., 1995b)).

First studies confirmed an oligodendrocytotoxic effect of TNFα, TNFβ and IFNγ (Louis *et al*., 1993; Selmaj *et al*., 1991c; Selmaj and Raine, 1988; Vartanian *et al*., 1995). In addition, the potential of IFNγ to induce apoptosis in developing oligodendrocytes or necrosis in mature cells, was described (Baerwald and Popko, 1998). But with increasing number of different culture systems tested, results became confusing. Induction of apoptosis seemed to depend on the differentiation status of oligodendrocytes, as TNFα was found to be toxic for progenitor cells, but not for mature oligodendrocytes (Scurlock and Dawson, 1999) or to be toxic only in combination with IFNγ in immature cells (Andrews *et al*., 1998). In another report, the combination of TNFα and IFNγ did neither induce cell death in progenitors, nor in mature oligodendrocytes. The inhibition of proliferation and maturation of cells by TNFα and IFNγ rather implicated a role of these cytokines in suppressing remyelination (Agresti *et al*., 1996).

The diverging results might be explained by differences in culture systems, e.g. the composition of the culture medium, where the presence of growth factors probably protects from toxic effects of cytokines. Analysis of TNF-receptor (TNF-R) expression in rodent oligodendrocytes disclosed expression of TNF-R I and TNF-R II *in vitro*, but of TNF-R II only *in vivo* (Dopp *et al*., 1997; Tchelingerian *et al*., 1995). TNF-R I belongs to the family of death receptors and triggers apoptosis upon ligand binding and subsequent trimerisation. TNF-R II is also involved in proliferative signalling and induces apoptosis in certain conditions (presence of

RIP, a protein Ser/Thr kinase required for NF-kappaB activation by TNFR1) (Pimentel-Muinos and Seed, 1999). Whether TNF or other cytokines can really trigger cell death *in vivo* in human oligodendrocytes remains to be confirmed.

Because ceramide had been discovered to be involved in the intracellular signalling of death receptors like TNFα and CD95 (see 1.4.3), the effect of ceramide on oligodendrocytes was investigated by several research groups. By adding C2-ceramide, the cell-permeable analogue of physiologic ceramide, apoptosis was induced in oligodendrocytes (Brogi *et al.*, 1997; Craighead *et al.*, 2000; D'Souza *et al.*, 1996b; Larocca *et al.*, 1997). However, a relevance of ceramide as mediator in demyelination has not been described.

In contrast to the known direct actions, inflammatory cytokines might cause cell death of oligodendrocytes indirectly by inducing contemporarily the expression of CD95 on oligodendrocytes and the production of CD95-Ligand (CD95L) e.g. by microglia. Sensitivity of oligodendrocytes to death induced by CD95 stimulation was previously reported (D'Souza *et al.*, 1996b; Hisahara *et al.*, 2000) as well as the expression of CD95 on human oligodendrocytes in situ (Bonetti and Raine, 1997). Very recently, the IFNγ-induced upregulation of CD95 on human oligodendrocytes *in vitro* and the sensitivity to CD95-mediated apoptosis has been shown (Pouly *et al.*, 2000).

Cytokines have not been the only inflammatory mediators investigated for their potential to induce oligodendrocyte death. Nitric oxide (NO) produced by microglia was proposed as death-inducing factor causing lysis of oligodendrocytes cocultured with microglia (Merrill *et al.*, 1993). In a rat oligodendrocyte cell line, NO triggered apoptosis (Boullerne *et al.*, 1999). Sensitivity of oligodendrocytes for reactive oxygen species, that might also be produced during inflammation, was shown by different studies investigating H_2O_2-induced apoptosis (Bhat and Zhang, 1999; Laszkiewicz *et al.*, 1999; Uberti *et al.*, 1999).

1.2.4. *Microglia and inflammatory demyelination*

A large variety of inflammatory mediators was found in inflammatory demyelination. Cells with potential to produce inflammatory mediators are infiltrating immune cells, mainly lymphocytes and monocytes, but also activated brain resident astrocytes and especially microglia (Zielasek and Hartung, 1996). Microglia descend from bone marrow derived precursors of the monocytic lineage. Migration of the microglia precursors to the brain occurs mainly during early fetal development and postnatally. Quiescent microglia are characterised *in vivo* by their ramified morphology (highly branched). Upon activation, cells retract their processes, become more compact ("ameboid") and gain phagocytotic activity in conditions of infection, trauma or neurodegeneration (Benveniste, 1997).

The mediators produced and released by microglia (Fig. 2) cover inflammatory cytokines (TNFα, IL-1, IL-6), chemokines for cell recruitment (MIP-1α, MIP-1β, MCP1 etc.) (Zhang *et al.*, 2000), eicosanoids (lipid mediators of the immune system) (Giulian *et al.*, 1996; Minghetti and Levi, 1998), reactive oxygen species (NO, H_2O_2 and others) (Parkinson *et al.*, 1997; Zajicek *et al.*, 1992) and proteases (matrix-metalloproteinases, elastase) (Nakajima *et al.*, 1992). Activation by lymphocyte-derived IFNγ induces the expression of MHC II, required for the presentation of antigens to lymphocytes, and B7-1, a costimulatory receptor for antigen-presentation (Benveniste, 1997). Inflammatory mediators lead to activation of more microglia cells and contribute to oligodendrocyte damage and demyelination. Astrocytes proliferate in response to the inflammatory environment (gliosis, Fig. 2) (Lee *et al.*, 1995).

The elementary function of microglia and macrophages and their mediators for pathogenesis of MS and EAE is emphasised by several intervention studies. Depletion of macrophages led to delayed onset or complete prevention of EAE (Huitinga *et al.*, 1990). The role of TNFα was investigated first by administration of anti-TNF antibody to mice induced for EAE, resulting in complete protection (Selmaj *et al.*, 1991b). Experiments with animals knocked out for TNFα, both TNFα and LTα, both TNF-R I and TNF-R II or TNF-R II exhibited decreased susceptibility for MOG-induced EAE, revealing an essential role for TNF-R I for induction of demyelination (Eugster *et al.*, 1999). In contrast, not all studies support an essential role for TNFα and LTα in demyelination (Frei *et al.*, 1997; Suen *et al.*, 1997). The importance of IL-6, another macrophage- or microglia-derived inflammatory cytokine, for EAE pathogenesis was implicated by protection of IL-6 deficient mice from MOG-induced EAE (Eugster *et al.*, 1998).

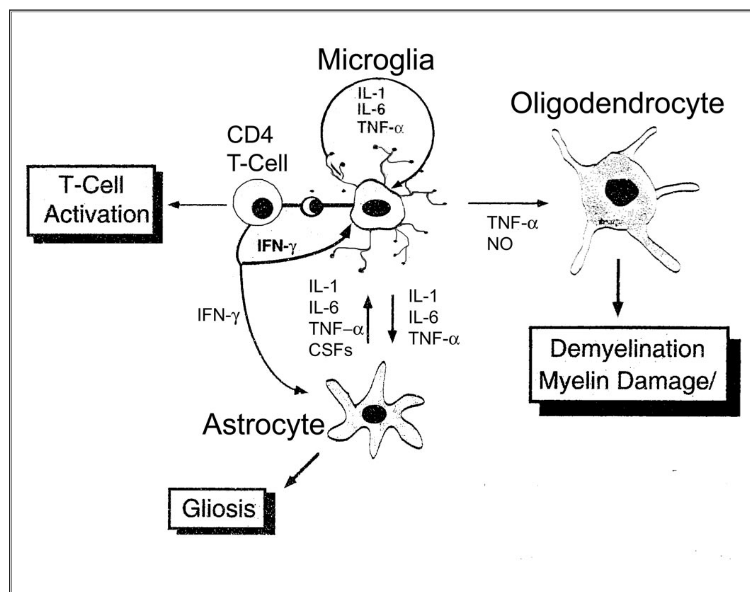

Figure 2: Microglia-derived inflammatory mediators.
IL interleukin, *CSF* colony stimulating factos, *TNF* tumor necrosis factor, *IFN* interfero (Benveniste, 1997).

A contribution of IL-6 to oligodendrocyte damage seems unlikely considering the studies reporting pro-proliferative effects of IL-6 on oligodendrocytes (Barres *et al.*, 1993; Kahn and De Vellis, 1994).

1.3. Apoptosis in pathology

Apoptosis, also called programmed cell death (PCD), is essential for ontogenetic tissue development (Meier *et al.*, 2000) and the homeostasis of cell populations like e.g. hematopoietic cells, that undergo quick changes in number depending on the physiologic demand . Aberrations in time or mode of apoptosis execution cause pathologies. Inhibition of apoptosis through mutations in death-determining genes is often a basis for neoplastic transformation. Resistance to stimuli inducing apoptosis in normal cells, like DNA-damage after irradiation, is characteristic for a variety of human cancers (Lu and Lane, 1993). Diminished apoptosis of both autoreactive T-cells during development, and activated T-cells after the completion of an immune response might be the cause for autoimmune diseases (Giordano *et al.*, 1995; Nakajima *et al.*, 1995; Watanabe-Fukunaga *et al.*, 1992). Numerous diseases are caused by excessive apoptotic death of discrete cell populations, e.g. CD4 T-cells during HIV-infection (Ameisen and Capron, 1991; Westendorp *et al.*, 1995). In many neurological diseases, specific subsets of neurons are lost. Neuronal apoptosis was demonstrated to occur in conditions of ischemia, Alzheimer's disease, Huntington's disease, Parkinson's disease, and also HIV-induced encephalopathy (Gelbard *et al.*, 1995; Gutekunst *et al.*, 2000; Lassmann *et al.*, 1995; Loo *et al.*, 1993; Mattson *et al.*, 1999; Portera-Cailliau *et al.*, 1995).

However, neurons are not the only cells affected by PCD in the CNS. Neuroinflammation (Lassmann, 1999), abnormal metabolism (Hughes *et al.*, 1999; Nagara *et al.*, 1986; Vanier, 1999), and secondary processes following ischemia (Pantoni and Garcia, 1997; Shibata *et al.*, 2000) lead to degeneration of oligodendrocytes, the myelinating cells in the CNS.

1.3.1. *Mechanisms of apoptosis*

The term "apoptosis" was defined by Kerr et al. (Kerr *et al.*, 1972), comparing the silent and selective death of single cells to leaves falling off from trees in autumn. Initial descriptions of morphologic changes observed in cells undergoing apoptosis were followed by a more and more detailed analysis of biochemical mechanisms involved.

Apoptosis can be triggered in cells by exogenous factors, like hormones, immune mediators and toxins, or by endogenous conditions, e.g. mitochondrial dysfunction, protein misfolding or imbalanced ion distribution. Despite the diversity in action of the mentioned stimuli, they share highly conserved intracellular pathways in death induction. Characteristic biochemical changes

in proteins, plasma membrane and chromatin cause typical morphological changes. Microscopically evident is the transformation of loosely packed and evenly distributed to condensed chromatin aggregated at the rim of the nucleus. Endonucleases are responsible for the cleavage of DNA first into large fragments of 50-300 kbp and finally into nucleosomal fragments of 180 bp (Oberhammer *et al*., 1993; Wyllie, 1980). Activation of proteases, in particular proteases of the caspase family, leads to cleavage of numerous structural and functional proteins (Cohen, 1997). Proteins are not only degraded, but some of them are typically redistributed during apoptosis. Mitochondrial proteins undergo the most evident changes. They have been shown to release a variety of proteins normally localised in the intermembrane space, like cytochrome *c* (cyt *c*) (Li *et al*., 1997; Zou *et al*., 1997) and apoptosis inducing factor (AIF) (Lorenzo *et al*., 1999; Susin *et al*., 1999b), into the cytoplasm. The cell membrane undergoes important changes, morphologically characterised by the extrusion of membrane vesicles („blebbing“). The loss of assymetric distribution of lipids in the bilayer leads to exposure of recognition structures. Within tissues, this might be the essential signal for phagocytes or neighbouring cells to engulf apoptotic cells. In this way the risk of releasing cytoplasm and subsequent inflammation within healthy tissue might be minimised (Fadok *et al*., 1992; Savill *et al*., 1993). In cell systems lacking phagocytes, apoptotic cells usually lyse instead of being taken up; a phenomenon called “secondary lysis”.

Although cell death can be initiated by defects in any organelle, mainly two executional apoptosis pathways have been proposed until now (Fig. 3; Hengartner, 2000):

Ligand binding to and subsequent trimerisation of a receptor of the death-receptor-class (characterised by their cytoplasmic so-called death domain (DD)) (Nagata, 1999) clusters together the cytoplasmic DDs and recruits the adaptor molecule FADD/MORT1, which also contains a DD. Binding of the cytosolic protein pro-caspase-8 to FADD completes formation of the death-inducing signalling complex (DISC) (Boldin *et al*., 1996; Kischkel *et al*., 1995). Autoactivation of caspase-8, classified as an “initiator caspase”, triggers the caspase-cascade with subsequent activation of further (“effector”) caspases and proetolytic degradation of intracellular substrates (reviewed in (Cohen, 1997; Nicholson, 1999)).

An alternative pathway is thought to start on the level of mitochondria and responds to a variety of endogenous and exogenous stimuli, e.g. DNA injury. Damaged by a primary insult alterating the mitochondria membrane, or through the action of proapoptotic members of the Bcl-2-family (e.g. Bid), proapoptotic proteins (cyt *c*, AIF) get released most likely via the opening of the mitochondria permeability transition pore (MPTP). Pro-caspase-9 is activated in the cytosol by recruitment to the apoptosome, a cytosolic aggregation of Apaf-1, cyt *c* and ATP (Hu *et al*., 1999; Zou *et al*., 1997). Subsequently, active caspase-9 cleaves a variety of cellular substrates, including other pro-caspases (“executioner caspases”, e.g. caspase-3), poly-ADP-ribose polymerase, nuclear lamins and many others.

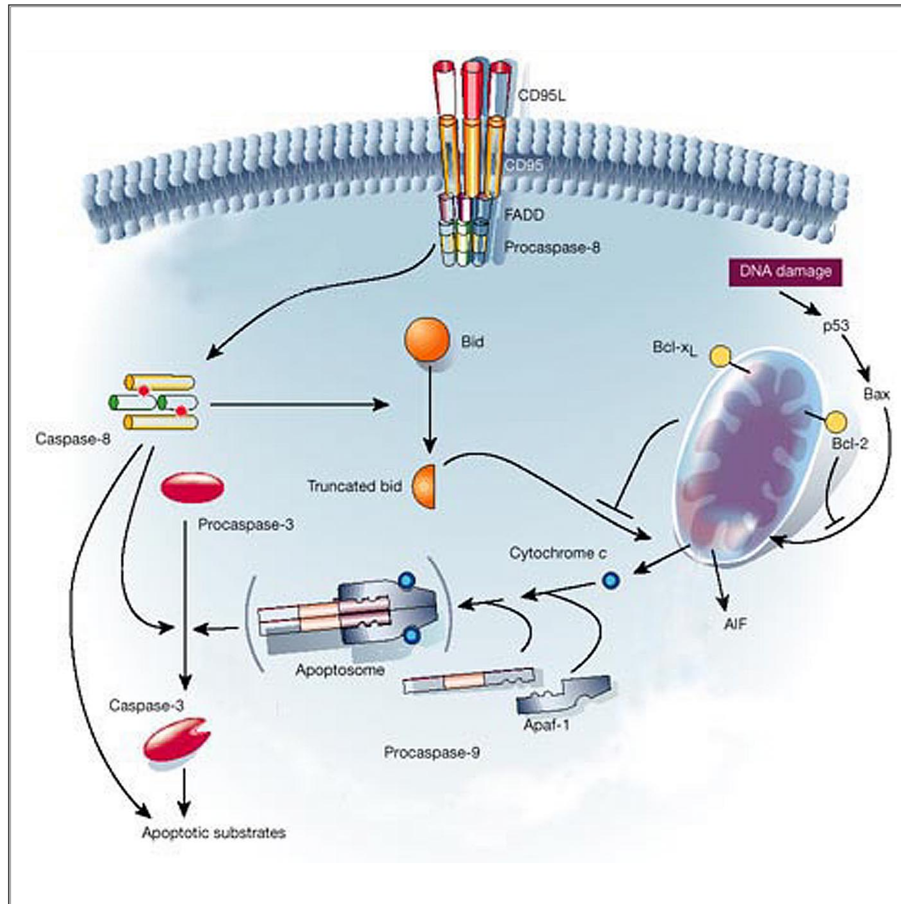

Figure 3: Pathways of apoptosis. Adapted from (Hengartner, 2000) .

Both pathways converge in activation of executioner caspases. Interactions between both pathways are possible e.g. by the action of Bid, a Bcl-2-family member inducing cyt c release from mitochondria upon caspase-8-mediated cleavage (Luo et al., 1998). Depending on cell type and apoptotic stimulus, either one of the two pathways dominates the apoptotic mechanism. Indicative for caspase-dominated cell death is the potential of caspase-inhibitors to block or delay cell death. In contrast, caspase inhibitors do not necessarily influence mitochondrial damage. An alternative caspase-independent mechanism can be triggered by AIF, inducing DNA-cleavage and apoptotic morphology also in absence of caspases (Susin et al., 1999b). Complex interactions of Bcl-2-like proteins and other molecules (e.g. cyclosporine A) with the MPTP can regulate the release of pro-apoptotic proteins and block or delay apoptosis (Halestrap, 1999; Shimizu et al., 1998; Susin et al., 1996; Yang et al., 1997).

1.3.2. *Apoptosis in oligodendrocytes*

Cell death can be induced both in physiologic and pathologic conditions in nearly all cell types, including oligodendrocytes. Up to 50 % of all initially generated oligodendrocytes are eliminated during development by apoptosis (Barres *et al*., 1992; Casaccia-Bonnefil, 2000; Ludwin, 1997). However, the occurrence of programmed cell death in oligodendrocytes in case of demyelinating disease, e.g. multiple sclerosis, is matter of controversial discussion. Several publications evidence apoptotic oligodendrocytes within lesion in MS and EAE, using the TUNEL staining as indicator for apoptosis (Dowling *et al*., 1997; Lucchinetti *et al*., 1999). Contrasting reports (summarised in (Raine, 1997)) favour cytolytic mechanisms to explain the prominent loss of mature oligodendrocytes from white matter lesions.

A clear answer to the question for the kind of cell death prevailing in oligodendrocytes cannot be given. Also *in vitro*, proof for apoptotic as well as for "lytic", probably necrosis-like, cell death can be quoted (D'Souza *et al*., 1996a; D'Souza *et al*., 1996b; McLaurin *et al*., 1995a; Selmaj *et al*., 1991c; Selmaj and Raine, 1988; Vartanian *et al*., 1995).

One possible explanation for these contradictory results comes from a recent publication, where mature oligodendrocytes are shown to be lost gradually from white matter lesions. Initial destruction of myelin, resembled by loss of oligodendrocyte membrane processes, left the perikarya of oligodendrocytes still intact. With progressing disease course, virtually all mature oligodendrocytes were lost from older lesions (Wolswijk, 2000). The difficulties of some researchers to find apoptotic oligodendrocytes might therefore be due to tissue damage proceeding over long time periods *in vivo.* This might decrease the probability to catch the cell exactly in the moment, when a cell death program of short duration is taking place.

1.4. Sphingolipids in demyelination and apoptosis

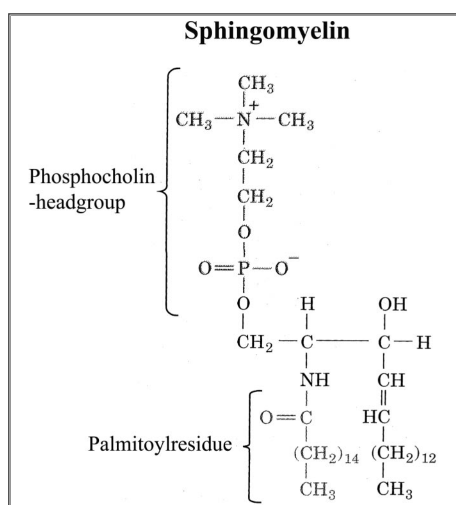

Figure 4: Chemical structure of sphingomyelin. (Voet, 1992).

The molecule class of sphingolipids contains complex lipid molecules like sphingomyelin, cerebrosides, gangliosides and sulfatides. Derived from N-acylsphingosine (ceramide) they occur in high concentrations especially in cell membranes in the nervous system. Sphingomyelin (Fig. 4) accounts for 4-12 % of all phospholipids in the brain, with higher concentrations in white matter, making up a major lipid component of myelin. The phosphodiesterbond of sphingomyelin is hydrolysed by the specific enzyme sphingomyelinase, yielding phosphocholine and ceramide (Sastry, 1985).

1.4.1. Sphingolipids in apoptosis

Sphingolipids have been known for long as important messenger molecules involved in proliferation, differentiation, and growth arrest (Spiegel *et al.*, 1998). A role of lipid molecules in apoptosis has evolved with studies investigating the intracellular signalling mechanisms that are activated during PCD (reviewed in (Malisan *et al.*, 1999)). Ceramide was the first sphingolipid molecule discovered to be important in apoptosis (Obeid *et al.*, 1993). An increased amount of it was found in cells after the application of a variety of stimuli, e.g. dexamethasone, ionising radiation or triggering of receptors of the "death-receptor-family" (DD-R), like TNFα-R, CD95 or NGF-R (nerve growth-factor receptor) (Edsall *et al.*, 1997; Kolesnick *et al.*, 1994).

Ceramide can be generated either by the cleavage of sphingomyelin, which is a component of the outer leaflet of the cell membrane, or by *de novo* synthesis (Perry, 2000). The liberation of ceramide from sphingomyelin is catalysed by sphingomyelin-specific type C phospholipases, the acidic or neutral sphingomyelinases (aSM, nSM). The signalling triggered by nSM-derived ceramide includes the activation of phospholipase A2 and the phosphorylation of Erk2 and is not

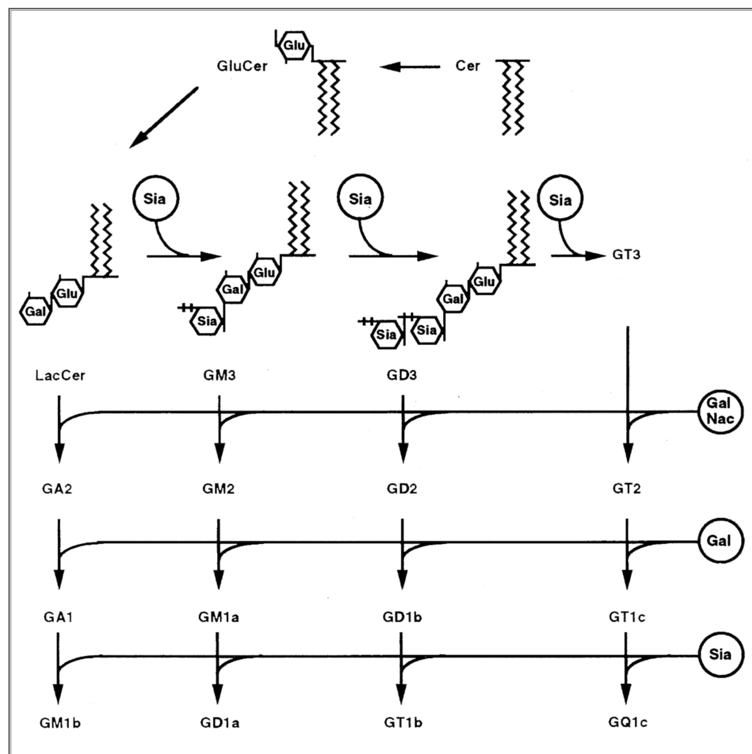

Fig. 5: Ganglioside biosynthesis.

Glu glucose; *GluCer* glucosylceramide; *Cer* ceramide; *Sia* sialic acid; *Gal* galactose; *LacCer* lactosylceramide; *GalNac* N-acetylgalactosamine; *GM* monosialogangliosides; *GD* disialogangliosides; *GT* trisialogangliosides. Adapted from (Malisan, 1999).

sufficient for induction of cell death. After the stimulation of DD-R, diacyl-glycerol produced by an upstream phosphatidylcholine-specific phospholipase C activates aSM and the ceramide liberated from sphingomyelin takes its part in the cell death cascade (Cifone *et al*., 1995).

Only recently it was discovered, that ceramide is not the ultimate lipid signal in the cell, but that it is transformed into a more complex metabolite. Released or newly synthesised ceramides are targeted to the Golgi apparatus, where the metabolism of sphingo- and glycolipids is regulated. It was shown, that after stimulation of the CD95 receptor, ceramide is liberated by aSM and metabolised via several intermediates to disialoganglioside GD3 (Fig. 5; De Maria *et al*., 1997; De Maria *et al*., 1998). In the last step of GD3-synthesis, α2,8-sialyltransferase (ST8) adds the second neuraminic acid to build up GD3. Inhibition of ST8 impaired the ceramide-induced cell death and desensitised hematopoietic cells to CD95 mediated apoptosis (De Maria *et al*., 1997; Rippo *et al*., 2000). The ability of cystein-protease-inhibitors to prevent the accumulation of GD3 and the protection from cell death in this way indicates that the two pathways activated by the stimulation of DD-R (caspase pathway and ceramide pathway) share a common upstream event.

After GD3 was mentioned the first time as apoptotic mediator, its involvement in cell death signalling was demonstrated in different cell types and *in vivo* (Farina *et al*., 2000; Stassi *et al*., 1999; Rippo, 2000). Consequently, the intracellular toxicity of GD3 was assigned to its effect on mitochondria. In isolated mitochondria and cells GD3 induces the opening of the permeability transition pore (MPTP); uncoupling of respiration; swelling of mitochondria; loss of $\Delta\Psi_m$; release of proapoptotic factors from the mitochondrial intermembrane space (Garcia-Ruiz *et al*., 2000; Kristal and Brown, 1999; Pastorino *et al*., 1999; Petronilli *et al*., 1999; Rippo *et al*., 2000; Scorrano *et al*., 1999b).

1.4.2. *GD3 in demyelination*

GD3 is a ganglioside highly expressed during development (see 1.1.) where it is found in oligodendrocyte progenitors, in microglia and reactive astrocytes (Cammer and Zhang, 1996; Ellison and de Vellis, 1995; Wolswijk, 1995). However, in adult rodents GD3 has been detected only in reactive astrocytes or neuronal subpopulations, e.g. Purkinje cells (Kawai *et al*., 1994; Molander *et al*., 2000). And although gangliosides are present in a relatively high amount throughout the nervous system, disialoganglioside GD3 makes up only 3 to 8 % of all gangliosides in cortex and white matter, respectively, of healthy adult humans (Svennerholm *et al*., 1994).

Particularly interesting is the finding that GD3 and other gangliosides accumulate in CSF in pathologic conditions, like white matter degeneration, HIV-caused neurodegeneration or medulloblastoma (Andersson *et al*., 1998; Ladisch *et al*., 1997; Miyatani *et al*., 1990; Tarvonen-Schröder *et al*., 1997). Significantly higher ganglioside concentrations have been found in lipid

extracts from EAE brain tissue (Zaprianova *et al*., 1998). Serious changes of ganglioside composition have been found in MS plaques. GD3 was heavily increased, while GM1, the major ganglioside in healthy brain, was reduced (Yu *et al*., 1974). GD3 is long known to be present in cancer cells, e.g. human malignant gliomas or soft tissue sarcomas (Chang *et al*., 1992; Wikstrand *et al*., 1992) and was found to be shed by medulloblastoma cells into the CSF (Ladisch *et al*., 1997). But the source of the elevated ganglioside levels in conditions of myelin and oligodendrocyte damage has not yet been identified. Several cell types are candidates for enhanced GD3 production in inflammatory demyelination.

Activated microglia cells produce elevated levels of GD3 as reaction to brain injury, as was reported *in vivo* before (Andersson *et al*., 1998; Reynolds and Wilkin, 1993). Lymphocytes infiltrating the CNS in EAE undergo pronounced apoptosis (Bauer *et al*., 1995; Bonetti *et al*., 1997; Schmied *et al*., 1993) and lymphoma derived cells have been shown on the other hand to accumulate GD3 upon stimulation of CD95 (De Maria *et al*., 1997). Probably also degenerating neurons might liberate parts of their membrane lipids during degeneration.

1.5. Aims of the study

Oligodendrocytes produce myelin and insulate neuronal axons in the central nervous system (CNS) to guarantee proper signal conduction. Myelin and oligodendrocytes are destroyed in demyelinating diseases, multiple sclerosis (MS) being one of the most abundant. The effector cell or mediator leading to oligodendrocyte damage has not been finally determined, although microglia cells and their products (inflammatory cytokines, nitric oxide, CD95-Ligand) might play an important role. The potential toxicity of single factors can be studied only *in vitro* due to the complexity of cellulare interactions *in vivo*.

The first aim of this study was to establish a murine *in vitro* culture of oligodendrocytes according to published protocols. Maturation of oligodendrocytes and purity of the culture had to be studied using common markers for cell types and oligodendrocyte differentiation.

Previous studies about oligodendrocyte death elicited by inflammatory mediators or stimulation of the CD95-receptor have been full of contradictory results. The sensitivity of murine oligodendrocytes both to inflammatory cytokines and CD95-induced cell death in the established culture system had to be tested.

Other mediators with potential to induce oligodendrocyte death had to be discussed. Attention was focussed on glycolipids, in particular on disialoganglioside GD3. GD3 was shown before to be elevated under conditions of demyelination and to induce apoptosis. GD3 was investigated with regard to cell type specific in comparison to other gangliosides. Different approaches had to be developed to assess specificity and purity of the GD3-effect.

The mode of oligodendrocyte degeneration *in vivo* has been described contradictory. In consequence to the findings about specific GD3 toxicity on oligodendrocytes, a closer characterisation of the cell death mechanism had to be carried out, paying attention to intracellular mechanisms, especially the involvement of caspases and mitochondria.

A new hypothesis about the involvement of GD3 in oligodendrocyte degeneration had to be worked out. To further emphasise the relevance of our working model, considering microglia as a key player, the potential of microglia to produce GD3 *in vitro* had to be investigated. An adequate protocol for the induction of GD3-production and detection of GD3 had to be set up.

2. MATERAL AND METHODS

2.1. Material

2.1.1. *Instruments and technical devices*

Centrifuges: Biofuge fresco and Megafuge 1.0 R (Heraeus Instruments, Hanau, Germany). **Confocal microscope system:** TCS 4D UV/VIS (Leica AG, Benzheim and Leica Lasertechnik, Heidelberg, Germany). **ELISA-Reader:** SLT Spektra (SLT Labinstruments, Crailsheim, Germany). **Imaging camera:** Dage-72 CCD camera (Dage-MTI, Michigan City, IN) and image analysis system MCID (Imaging Research Inc., St. Catherines, Ontario, Canada). **Incubator:** Model BB 6220 (Heraeus Instruments, Hanau, Germany). **Laminar Flow:** LaminAir® HB 2448 (Heraeus Instruments, Fellbach, Germany). **Microscopes:** Leitz DM IRB, Leitz DM IL (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). **Sonication bath:** Sonorex RK 102 H (Bender und Hobein, Ismaning, Germany)

2.1.2. *Chemicals and antibodies*

Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany: ECL Western blotting detection reagents. **Alexis, Läufelfingen, Switzerland:** Calpaininhibitor I and II. **Bachem Biochemica GmbH, Heidelberg, Germany:** z-VAD-fluoromethylketone (fmk), DEVD-CHO, YVAD-CHO, Calpaininhibitor III. **Bender MedSystems, Vienna, Austria:** recombinant murine TNFα and IFNγ. **BioClot, Aidenbach, Germany:** Lipopolysaccharide from *Salmonella abortus equi*. **BioRad Laboratories GmbH, München, Germany:** Pre-stained markers for SDS-PAGE. **Bio Whittaker, (Verviers, Belgium):** quantitative chromogenic *Limulus* assay QCL 1000. **ICN Jackson Immuno Research, West Grove, PA, USA:** goat anti mouse cy5 (λ_{ex} = 651 nm, λ_{em} = 674 nm). **Molecular Probes Europe BV, Leiden, Netherlands:** Alexa™ 488-conjugated anti-mouse and anti rabbit IgG-antibody (λ_{ex} = 488 nm, λ_{em} = 512 nm), Alexa™ 568-conjugated anti-mouse and anti-rabbit IgG-antibody (λ_{ex} = 577 nm, λ_{em} = 603 nm), ethidium homodimer (EH-1), Alexa™ 568 protein labelling kit, Hoechst 33342, Mitotracker Red, SYTOX, tetramethylrhodamine ethylester (TMRE), *Escherichia coli* (strain K12) inactivated. **Pharmingen, Hamburg, Germany:** monoclonal anti-cytochrome *c* antibody (clone 6H2.B4), rabbit antibody against active caspase-3 (clone C92-605), anti-murine-CD95 antibody (clone Jo2). **Polysciences, Warrington, PA, USA:** Aquapolymount. **Roche Biochemicals, Mannheim, Germany:** Annexin-V fluorescein-conjugated, DNAse I from bovine pancreas. **Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA:** polyclonal anti-Bcl-2 antibodies (N-19). **Sigma, Deisenhofen, Germany:** Monoclonal antibody against CNPase (clone 11-5B), monoclonal anti-Bcl-2 (clone Bcl-2-100), human apo-transferrine, disialogangliosides

GD3, GD1a, GD1b, monosialoganglioside GM3, trisialoganglioside GT1b, actinomycin D, bovine serum albumin (BSA), biotin, cycloheximide, digitonin, E 64 (Trans-Epoxysuccinyl-L-Leucylamido (4-guanidino) Butane), FCCP (Carbonyl cyanide 4-trifluoro-methoxyphenylhydrazone), goat anti-rabbit IgG-HRP, isolectin B4 FITC-labeled from *Bandeiraea simplicifolia*, leupeptin, progesterone, putrescine, monodansylcadaverine ((dimethylaminonaphthalene-sulfonyl)-1,5-pentanediamine), Na-selenite, L-thyroxin, tri-iodo-thyronin, Tris-Base, Triton X-100, Tween 20, trypanblue 0.4 %. **Vector Laboratories, Burlingame, CA, USA:** Vectastain Elite™ ABC kit**.**

All other reagents not further specified were from Riedel-de Haen (Seelze, Germany) or Roth GmbH & Co (Karlsruhe, Germany).

O4 and O1 antibody were a kind gift from Prof. C. Stürmer (Konstanz, Germany), anti-MOG-antibody (pure and biotinylated) was a kind gift from Dr. A. Iglesias (Max Planck Institute, München, Germany). Anti-GD3-antibody was produced by the R24 hybridoma cell line (ATCC no.HB-8445), which was a kind gift of Dr. R. Testi (University of Torvergata, Rome, Italy).

2.1.3.. *Cell culture material*

DMEM (Dulbecco's Minimal Essential Medium), trypsin, gentamycin and a mixture of penicillin and streptomycin were bought from Gibco BRL Life Technologies (Eggenstein, Germany). FCS was from Boehringer Mannheim (Mannheim, Germany). RPMI-1640 and horse serum were from Biochrom KG (Berlin, Germany).

Cell culture flasks and plates were obtained from Costar GmbH (Bodenheim, Germany) and Greiner GmbH (Frickenhausen, Germany).

2.1.4. *Buffers and media*

PBS: NaCl 137 mM, Na_2HPO_4 10 mM, KH_2PO_4, 3 mM, pH 7.4.

HBSS for Trypsin, DNAse: NaCl 137 mM, KCl, 5 mM, Na_2HPO_4 0.3 mM, KH_2PO_4 0.4 mM, $MgSO_4$ 0.8 mM, $CaCl_2$ 1 mM, Glucose 5.5 mM, $NaHCO_3$ 4.2 mM.

HIB buffer: NaCl 120 mM, KCl, 5 mM, HEPES 25 mM, Glucose 5.1 mM, pH 7.4.

Hibernation medium: KCl 30 mM, $MgCl_2$ 0.24 mM, NaH_2PO_4 11mM, Na_2HPO_4 5 mM, Glucose 5mM, pH 7.2, Sorbitol ad 295 mOsm.

DMEM for mixed brain culture: DMEM (high Glucose), 10 % FCS, 50 U/ml penicillin, 50 µg/ml streptomycin.

Sato Medium for primary cells (Bottenstein and Sato, 1979): DMEM (high Glucose), bovine Insulin 5 µg/ml, human apo-transferrin 100 µg/ml, progesterone 30 nM, Na-selenite 30 nM, putrescine 100 µM, 50 U/ml penicillin, 50 µg/ml streptomycin, BSA 100 µg/ml. Additional, 10 ng/ml biotin was added.

Sato medium for O4Cbneu cells: DMEM, bovine Insulin 10 µg/ml, humane apo-transferrine 10 µg/ml, progesterone 200 nM, Na-selenite 40 nM, putrescine 90 µM, tri-iodo-thyronine, 500 nM, L-thyroxine 450 nM, horse serum 1%, gentamycin.

2.1.5. *Cell lines*

HeLa-229 human cervix carcinoma cells were cultivated in DMEM with 5 % FCS, 50 U/ml penicillin *plus* 50 µg/ml streptomycin. WEHI-S (Jaattela *et al.*, 1992) cells were cultured in RPMI-1640 with 10 % FCS *plus* 50 U/ml penicillin and 50 µg/ml streptomycin. O4CB-neu cells were a kind gift of Dr. M. Jung. O4Cbneu (Jung *et al.*, 1995) cells were cultivated in a Sato medium with slightly different composition. N2A-FasL and N2A-Neo cells were a kind gift of Dr. M. Weller (Tübingen, Germany) (Rensing-Ehl *et al.*, 1995).

2.1.6. *Animals*

Primary brain cultures were prepared from BALB/c mice of either sex that were obtained from the internal animal breeding facility of the University of Konstanz. Mice were maintained under controlled specific pathogen free conditions (22°C, 55% humidity, 12 h day/night rhythm).

Bcl-2-transgenic mice overexpressing human *bcl-2* under the control of an ubiquitin promoter on background of C57BL/6 were a kind gift of Dr. J. Schenkel (Institute of Physiology and Pathophysiology, University of Heidelberg, Germany).

2.2. Methods

2.2.1. *Primary cell culture*

Mouse oligodendrocytes were isolated from a primary mixed brain cell culture (Trotter *et al.*, 1989). The primary mixed brain culture was prepared from BALB/c embryos at day 15 to 16 after gestation (E16). Cortex and cerebellum were excised from the skull and stored for the further preparation in cold HIB. After the removal of the meninges, brain tissue was incubated for 3 minutes in 1 % trypsin. Digestion was stopped by addition of 10 % fetal calf serum (FCS) and tissue was washed twice with HIB. Cells were dissociated by trituration in 0.05 % DNAse with three fire polished pasteur pipettes of decreasing diameters. The cell suspension was washed

with DMEM (10 % FCS). Cell culture flasks of 75 cm^2 surface were coated for 0.5 to 3 h with 100 µg/ml poly-L-lysin in sterile Millipore-H_2O. Flasks were washed once with PBS and twice with Millipore-H_2O. The cell suspension was plated on the coated culture flasks in DMEM with 10 % FCS in a density of 2 – 4 x 10^7 cells / flask. After seven days and every 3rd to 4th day from then, 50 % of the culture medium was exchanged to feed the cells. Fourteen to 20 days later in a first shaking step microglia cells were detached from the primary culture by mild shaking by hand. In a second step, by strong shaking of the culture flasks, the fraction of cells enriched in oligodendrocyte precursors was harvested. To further deplete this cell fraction of microglia, the suspension was incubated for 45 minutes in a 10 cm plastic petri-dish. Non-adherent cells were collected and plated as secondary culture on poly-L-lysin coated (100 µg/ml) coverslips (10 mm diameter). Cells were plated in 30 µl droplets on coverslips with 6 x 10^5 cells / ml. After 2 hours, plating medium was exchanged for DMEM containing a supplement according to Sato (see 2.1.4. Buffer and Media) (Bottenstein and Sato, 1979). Fifty percent of this medium had been conditioned by astrocytes for 24 h.

Microglia cells were obtained as 95 % pure culture from the first shaking step during the oligodendrocyte preparation. They were seeded on plastic culture dishes in DMEM with 10 % FCS and maintained in DMEM plus 1 % FCS, 50 U/ml penicillin and 50 µg/ml streptomycin.

Astrocytes were prepared by trypsinising (0.25 % trypsin) the feeder layer of the primary mixed cell culture and replating of the cells on PLL coated plastic dishes. Astrocytes were cultured in DMEM with 1 % FCS, 50 U/ml penicillin and 50 µg/ml streptomycin.

Murine cerebellar granule neurons (CGC) were cultivated as described before (Leist *et al.*, 1997a). Neurons were used 7 days after plating. To prevent excitotoxic mechanisms, cells were incubated with 1 µM MK-801, a non-competitive inhibitor of the NMDA-receptor.

2.2.2. *Culture of* bcl-2*-overexpressing oligodendrocytes*

To prepare cultures from *bcl-2*-overexpressing animals, C57BL/6 females (wt) were crossed with heterozygous *bcl-2*-tg males. Embryos were prepared at day 15 or 16 after gestation. Cortex and cerebellum were cleaned from meninges and maintained for 24 h in hibernation-medium. A part of the remaining brain was digested with proteinase K to obtain DNA (Schierle *et al.*, 1999). The genotype of each embryo was determined by PCR amplification of a 460 bp fragment of the human *bcl-2*-gene using the primers (5' to 3') act tgt ggc tca gat agg cac cca g and (5' to 3') cga ctt cgc cga gat gtc cag cca g. Brains from embryos carrying the *bcl-2*-transgene or not were pooled in two groups and processed like usual. To control *bcl-2*-expression on protein level, the astrocyte layer was scraped from the flask with Ripa buffer after harvesting of oligodendrocytes and the lysate was analysed by western blot (Leist *et al.*, 1997c). Briefly, cells were harvested in lysis buffer with protease inhibitors. Equal amounts of protein/lane were loaded

for wt and tg samples on a 12 % polyacrylamide gel. After blotting on nitro-cellulose, the membrane was blocked and incubated with an anti-Bcl-2 antibody (Sigma, clone Bcl2-100). Specifically stained bands were detected by chemiluminescence.

2.2.3. *Induction protocol for cytokine- and CD95-effects in oligodendrocytes*

To test the effect of different cytokines and Fas-Ligand on oligodendrocytes, cells were cultured for 3-6 days in Sato medium before incubation for various time periods (8 - 96 h with cytokines, 12 – 24 h with ActD or CHX pretreatment *plus* cytokines or FasL) with different stimuli (murine TNFα 10 - 200 ng/ml, murine IFNγ 200 – 2000 U/ml). Pretreatments with ActD (2 µg/ml), CHX (1 - 25 µM) were carried out for 30 to 60 min before addition of stimuli. To trigger Fas, two different approaches were used. Cells were stimulated either with an agonistic anti-CD95-antibody (200 ng/ml), or 10 to 50 Vol % of a cell culture (N2A cell line (Rensing-Ehl *et al*., 1995)) supernatant (SN), containing FasL, or the adequate control SN were added to the cells. Viability of cells was assessed by 3 different methods: incubation of cells for 60 min with MTT (3-(4,5-Dimethyldiazol-2-yl)-3,5-diphenyltetrazolium bromid, 1 mg/ml), subsequent lysis in isopropanol/formic acid (95:5) and measurement of absorption at 560 nm (Mosmann, 1983); staining of living cells by Sytox and H-33342 as described in 2.2.8.; determination of nuclear changes by immunostaining as described in 2.2.9. and scoring of damaged cells as described in 2.2.11.

2.2.4. *Induction protocol for ganglioside effects in oligodendrocytes*

To incubate cells with gangliosides, cells were incubated in small amounts of culture medium, by placing coverslips of 10 mm diameter on plastic petri-dishes and covering them with 75 µl of medium. Gangliosides were dissolved in 100 % MeOH for long term storage. To solve them in aqueous medium, the appropriate amount of solution was placed in an eppendorf cup and MeOH was either evaporated under an N_2-flow or under vacuum in a centrifuge. The gangliosides were then resuspended in culture medium in which oligodendrocytes were incubated to obtain the appropriate concentration. After vortexing and sonication (35 kHz) for 2 min in a sonication bath, medium containing gangliosides was placed on the cells.

2.2.5. *Pretreatment protocol for protease- and other inhibitors*

Treatments that should mediate protection from GD3 induced cell death were carried out 30 to 60 min before addition of GD3 to the cells and substances remained in the medium during the incubation. Inhibitors tested for their protective potency were caspase inhibitors (zVAD-fmk 100 µM, DEVD-CHO 100 µM, YVAD-cmk 100 µM), calpaininhibitors (calpaininhibitor I 25 µM,

calpaininhibitor II 30 µM, calpaininhibitor III 20 µM), cathepsin B inhibitor CH-074-Me (2 µM), general proteaseinhibitors (Leupeptin, 2 µM, TLCK 25 µM, E64 50 µM) and an inhibitor of receptor internalisation, monodansylcadaverine (25 µM) (Schutze *et al*., 1999).

2.2.6. *GD3 induction in microglia by inflammatory stimuli*

Microglia cells were plated in a density of 150 000 cells / well of a 12-well plate in DMEM with 10 % FCS. After 1-12 h medium was exchanged for DMEM with 1 % FCS and cells were stimulated with LPS, IFNγ, and TNFα, or inactivated *E. coli* for 24, 48, or 72 h. After this time, supernatants (SN) were collected from 2 wells that were incubated with the same stimulus. SN was centrifuged for 5 min at 13 000 rpm at 4°C and frozen at –20°C until TLC analysis.

2.2.7. *Ganglioside extraction and TLC analysis*

Gangliosides were extracted as previously described with minor modifications (De Maria *et al*., 1997). Briefly, supernatants (1ml) were treated for 30 min with trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml). They were then sonicated for 30 seconds at 10 Watt, and debris was removed by centrifugation for 10 min at 15000 g. Gangliosides were extracted from supernatants with chloroform/methanol (1:2) to obtain a ratio of 1:0.8:1.6 (supernatant/chloroform/methanol). After vortexing and 10 min centrifugation at 15000 g, the upper phase was recovered. Two volumes of methanol were added and evaporated to dryness under nitrogen gas. Gangliosides were then resuspended in chloroform/methanol (2:1) and loaded on a silica gel HPTLC plate (Merck, Darmstadt, Germany) and chromatographed in chloroform/methanol/$CaCl_2$ 0.2 % (2:1:0.2). Plates were treated with 0.5 % polyisobutylmetacrylate in hexane and dried. To carry out immunostaining for GD3, the plate was incubated for 1 hour with R24 monoclonal anti-GD3-antibody, followed by HRP-conjugated anti-IgG-secondary antibody. Specifically stained bands were detected by chemiluminescence.

2.2.8. *Viability assays*

To analyse living cells for apoptosis and necrosis, cultures were stained with a combination of the fluorescent chromatin dyes Hoechst-33342 (500 ng/ml; membrane permeant, stains all nuclei) and SYTOX (500 µM, membrane impermeant, stains nuclei of lysed cells) by adding the dye solution (100 x in PBS) 10 min prior to investigations. Using a Leica DM-IRB fluorescence microscope and lenses providing 400 x final magnification, cells with condensed or fragmented nuclei were scored as apoptotic. Lysed cells with non-condensed nuclei were scored as necrotic. Lysed and apoptotic cells together were counted as “dead cells” in some experiments.

Viability was also measured by determining the reduction of MTT by mitochondria. Cells were incubated with 1mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide) for 46-60 min. Then, supernatant was removed and cells were lysed for at least 10 min by a mixture of i-propanol : formic acid (95:5). Absorption was measured at 560/690 nm in an ELISA-reader.

2.2.9. *Immunostaining*

Immunostaining was performed on living cells to label galactosyl sulfatide (O4-antibody), galactosyl cerebroside (O1-antibody) or the glycoprotein that is recognised by the isolectin B4 from *Bandeiraea simplicifolia* (FITC-labeled) to stain microglia cells (Streit, 1990). Coverslips with living cells were briefly washed in prewarmed PBS, incubated with primary antibody (pure supernatant containing O4- or O1-antibody or IB4 1: 250 in PBS) for 15 min at 37°C, washed twice in PBS, 0.1 % BSA and incubated with secondary antibody in PBS, 0.1 % BSA for 15 min, 37°C. After two washing steps, cells were fixed in 4 % paraformaldehyde (PFA) in PBS for 10 min at RT and mounted in Aquapolymount.

To stain CNPase, cyt *c*, β3-tubulin, GFAP, active caspase-3 and GD3 cells were fixed in 4 % PFA in PBS for 10 min at RT, permeabilized with 0.1 % Triton X-100 (for GD3 with 20 µM digitonin) in PBS for 10 min at RT, blocked with 0.1 % BSA in PBS for 15 min at RT (with 5 % BSA for 30 min for GD3 staining) and incubated with the primary antibodies diluted in 0.1 % BSA in PBS (CNPase 1: 150, cyt *c* 1:400, β3-tubulin 1:1000, GFAP 1:250, active caspase-3 1:200, GD3 pure supernatant). Primary antibodies were detected with a goat-anti-mouse or goat-anti-rabbit antibody coupled to Alexa-488 or Alexa-568 (1:400 each). DNA was stained with H-33342 (50 µg/ml) and coverslips were mounted in Aquapolymount.

Staining for MOG was carried out after fixation of cells with 80 % MeOH at – 20°C for 5 min. After washing, cells were incubated with anti-MOG or anti-MOG-biotin for 60 min. Uncoupled MOG-antibody was detected with goat anti mouse Alexa-488, nuclei stained with Hoechst 33342. Biotin-coupled MOG was detected using the Vectastain Elite™ ABC kit together with a peroxidase substrate that results in a brown precipitation at the place of antibody binding. Procedures were carried out according to the instructions of the provider. Nuclei of POD-stained samples were coloured by methylgreen. Coverslips were incubated for 3 min at 60° C in a 1 % methylgreen solution in PBS, colour solution was washed away by quick washing in PBS and samples were mounted in mowiol.

2.2.10. *Multiple stainings*

To allow for double staining with two primary antibodies of murine origin, the CNPase antibody was coupled directly to Alexa™ 568 with a commercially available labelling kit. The reaction was carried out following the instructions of the provider.

For a simultaneous four colour staining for cyt *c*, CNPase, active caspase-3 and chromatin, reagents were applied in the following sequence: anti-active caspase-3 overnight, 4°C, anti-cyt *c* for 30 min RT, a mixture of goat-anti-rabbit Alexa-488 and goat-anti-mouse cy5 for 30 min RT. After washing, anti-CNPase coupled directly to Alexa-568 and H-33342 were added and samples were mounted in Aquapolymount.

For double staining for GD3 and CNPase, reagents were applied in the following sequence: fixation with PFA for 10 min, permeabilization with digitonin (20 µM) for 15 min, blocking with 5 % BSA in PBS, pure R24 supernatant (containing anti-GD3) for 45 min RT, goat-anti-mouse Alexa-488 for 30 min RT, anti-CNPase coupled directly to Alexa-568 and H-33342.

2.2.11. *Scoring of oligodendrocyte damage*

To identify oligodendrocytes in the enriched oligodendrocyte culture, cells were stained for CNPase. To assess the detachment of oligodendrocytes from the coverslip, the total number of oligodendrocytes was counted in 10 randomly chosen fields of 310 x 310 µm and normalised to the oligodendrocyte number on untreated coverslips.

For quantification of nuclear damage, CNPase positive cells with a nuclear structure different from the control cells were scored as damaged. For each condition, 9 fields of 310 x 310 µm were counted.

2.2.12. *Microscopy*

Immunostainings were analysed with a Leica DM-IRB fluorescence microscope equipped with appropriate filter sets or a confocal laser scanning microscope (TCS-4D UV/VIS) equipped with an UV-laser and an Ar-Kr-laser with a maximum of four different channels. Images of the inverse microscope were acquired by using a Dage-72 CCD camera and image analysis system MCID.

2.2.13. *Measurement of mitochondrial respiration*

Mitochondria were isolated from 3 months old rats (Costantini *et al.*, 1995). Livers were homogenised in ice cold isolation buffer A (250 mM sucrose, 10 mM Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4, 1 mM glutathione (GSH), 1 mM EGTA, 1 % BSA). The homogenate was centrifuged for 10 min at 700 x g and the supernatant was recentrifuged for 10 min at 1000 x g. After resuspending the pellets in isolation buffer B (125 mM KCl, 10 mM Hepes, pH 7.4, 1 mM GSH, 0.1 mM EGTA), they were centrifuged for 10 min at 700 x g. The supernatant was recentrifuged for 10 min at 1000 x g and the resultant pellet was used as mitochondrial fraction. Isolated rat liver mitochondria (protein concentration 0.4 mg/ml)

were incubated in medium containing 125 mM KCl, 10 mM Hepes, 1 mM GSH, 2 µM rotenone, 5 mM Mg^{2+}-phosphate, 5 mM succinate, pH 7.2. Oxygen consumption was measured at 25° C in an oxygraph (E.Geiger and A.Para, Innsbruck, Austria) equipped with thermostatic control and magnetic stirring. GD3 from bovine brain or synthetic GD3 was added to mitochondria after intense sonication in buffer. The increase of respiration due to uncoupling effects of GD3 was measured as described (Garcia-Ruiz *et al*., 2000; Scorrano *et al*., 1999a). Mitochondrial function was controlled at the end of every experiment by adding 100 nM FCCP (Carbonyl-cyanide-p(trifluoromethoxy)phenyl hydrazone) to stimulate maximal respiration rate. Initial oxygen consumption of mitochondria energised with succinate (31 $\pm$ 55.6 nmol O_2/min x mg protein) was used as 100 % reference value.

2.2.14. *Detection of phosphatidylserine translocation and mitochondrial membrane potential*

Surface phosphatidylserine-expression was analysed by annexin-V-staining and confocal microscopy. Ten min before annexin-V staining cells were incubated in the original culture medium with EH-1 (0.5 µg/ml), H-33342 (1 µg/ml) and the fluorescent indicator TMRE (5 nM, λex = 568 nm, λem = 590 nm) for 10 min to visualise plasma membrane integrity, chromatin structure and mitochondrial membrane potential ($\Delta\Psi_m$). Cells were then shortly washed in annexin-binding-buffer (140 mM NaCl, 10 mM Hepes/NaOH, pH 7.4, 2.5 mM $CaCl_2$, filtered through a 0.2 µm pore filter) and were stained with annexin-V (FITC-conjugated) 1:50 diluted in binding-buffer for 3 min at room temperature in the dark. Then, cells were washed once with binding buffer to remove excess annexin-V. After washing, cells were observed with a fluorescent microscope (Leica, DM-IRB) or by confocal microscopy (Leica, TCS) and three channel confocal microscopy (blue, chromatin structure; red, membrane integrity and $\Delta\Psi_m$; green, annexin-V binding) using a 63 x /NA 1.32 UV-corrected lens. To score annexin-V-positive cells or cells that lost $\Delta\Psi_m$, cells with oligodendrocyte-like morphology in phase contrast were counted in 6 fields (300 x 300 µm) for each condition. To test the specificity of the TMRE-staining, cells were incubated with FCCP, an uncoupler of the mitochondrial membrane potential, and staining disappeared within few seconds.

2.3. Statistics

All experiments were repeated in at least three cell preparations. Statistical significance was calculated on the original data sets using the Student's t-test, or two-way ANOVA and subsequent Tukey's multiple comparison test. When variances within the compared groups were not homogeneous, the Welch test was applied.

3. RESULTS

3.1. Separation of different glia cell types

An enriched culture of murine oligodendrocytes was established according to a published protocol (Trotter *et al.*, 1989). Changes were introduced in using embryos at day 15 and 16 after gestation in order to increase the yield of cells. The cultivation of the primary culture was prolonged from 14 to 16-20 days before preparation of the secondary enriched oligodendrocyte culture, since in this way more oligodendrocytes survived the isolation procedure.

Fourteen to 20 days after preparation of the primary mixed brain culture, a confluent feeder layer of astrocytes had formed (Fig. 6A). Bipolar oligodendrocyte progenitors, microglia and some neurons were sitting on top of the astrocyte layer (Fig. 6A). Microglia cultures (95 % pure) were obtained by moderate shaking of the mixed primary cultures and harvesting of the easily detaching microglia cells. In pure culture, microglia displayed an ameboid phenotype (Fig. 6B). Oligodendrocytes were detached from the astrocyte layer by more intense shaking. Astrocytes were obtained by trypsinisation of the feeder layer and replating (Fig. 6C). After

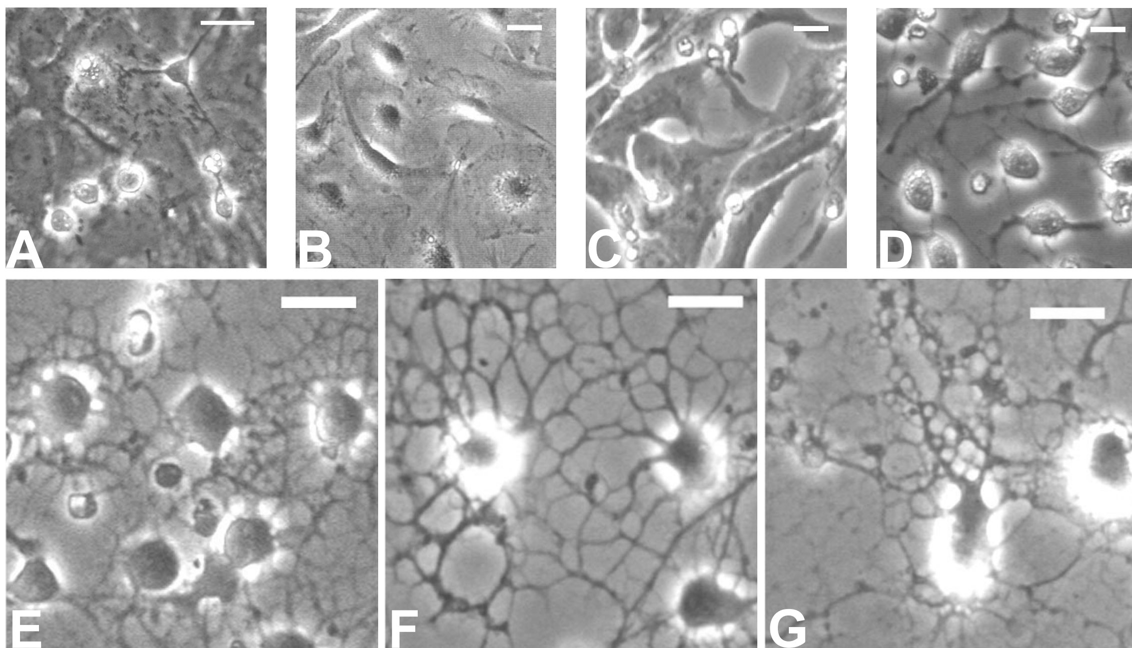

Figure 6: Isolation of oligodendrocytes, microglia and astrocytes from a primary mixed brain culture.

(**A**) Primary mixed brain culture after 14 DIV. Feeder layer of astrocytes and microglia and oligodendrocyte precursors on top. (**B**) Secondary culture of pure microglia. (**C**) Secondary culture of pure astrocytes. (**D-G**) Secondary culture of enriched oligodendrocytes. (**D**) Few hours after plating, (**E**) 2 DIV, (**F**) 4 DIV, (**G**) 6 DIV. Scale bar (**A**) 50 µm, (**B-G**) 20 µm.

isolation from the primary mixed brain culture, oligodendrocytes underwent maturation, showing typical morphologic changes. Few hours after plating, oligodendrocyte progenitor cells became bipolar (Fig. 6D). The developing network of membrane processes became visible 2 days after plating (days *in vitro*, DIV) (Fig. 6E). After 4 DIV, oligodendrocytes had enlarged their network of membrane processes (Fig. 6F) and had obtained an even more complex structure of membrane extensions at 6 DIV (Fig. 6G).

3.2. Characterisation of the enriched culture of murine oligodendrocytes

3.2.1. *Maturation of oligodendrocytes*

To control maturation of oligodendrocyte precursors to differentiated oligodendrocytes, cells were stained with antibodies against marker molecules that characterise differentiation states of oligodendrocytes (see 1.1). O4-antigen contains a characteristic sulfatide residue, recognised by the O4-antibody and appears in progenitor cells (O-2A cells) that can give rise to either oligodendrocytes or type II astrocytes (Raff *et al*., 1983). Galactosyl cerebroside is stained by the O1 antibody and appears in the cell membrane of early oligodendrocytes that have already begun the differentiation to oligodendrocytes. The oligodendrocyte specific cytoplasmic enzyme 2’,3’cyclic-nucleotide-phosphodiesterase (CNPase) is found in early as well as in mature oligodendrocytes, while myelin oligodendrocyte glycoprotein (MOG) is one of the latest antigens expressed in mature oligodendrocytes *in vitro* and on the outer layers of myelin *in vivo* (Coffey and McDermott, 1997). The expression of the above-mentioned antigens was investigated in the established enriched oligodendrocyte culture. In Fig. 7 typical staining patterns for the O1-antigen (Fig. 7A), CNPase (Fig. 7B) and MOG (Fig. 7C) are displayed. As the staining patterns of the O4 and O1-antibodies are similar, only the O1-staining is shown.

Oligodendrocyte precursors, which were initially positively labelled for the O4-antigen but negatively for O1-antigen or MOG, underwent differentiation to mature oligodendrocytes (Fig. 7D). While the amount of O4-positive cells remained relatively constant around 20 to 30 %, the percentage of cells that expressed O1-antigen, CNPase or MOG increased continuously over the investigated period. MOG as the latest marker of mature oligodendrocytes was present in 30 % of all cells after 5 to 8 days. This indicates that oligodendrocytes, that had already been positive for CNPase, were fully differentiated after this time.

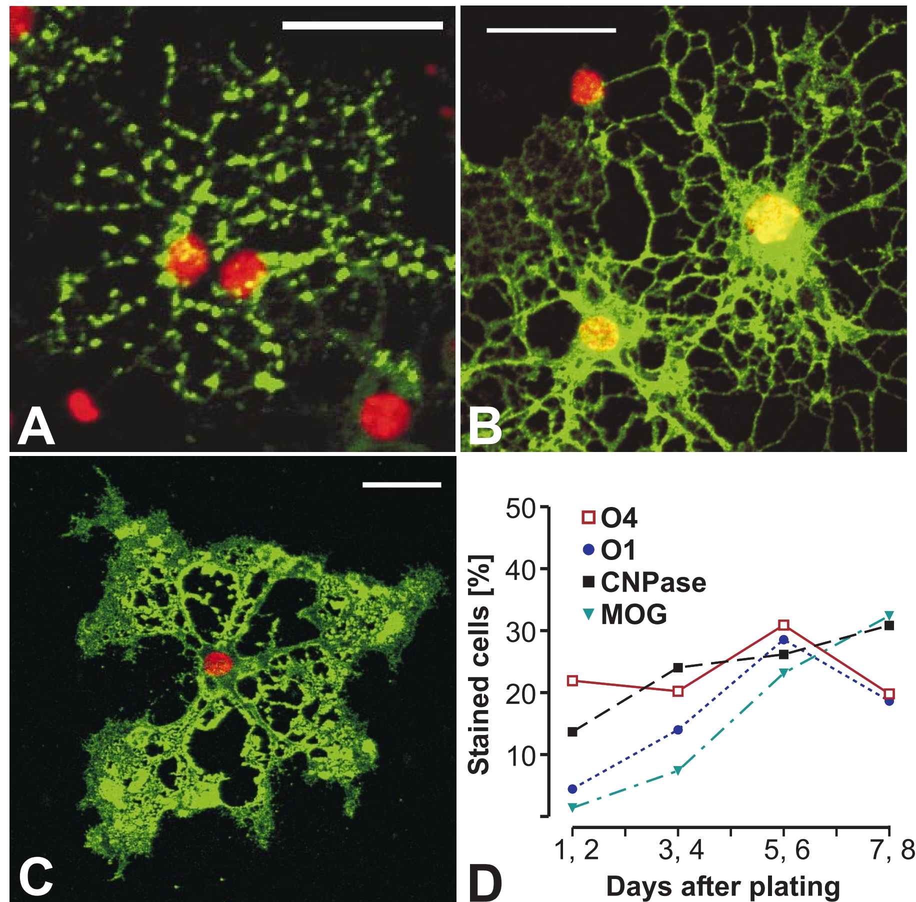

Figure 7: Maturation of oligodendrocytes in enriched secondary culture of oligodendrocytes.
Cells were stained by immunocytochemistry with antibodies against O4-antigen, O1-antigen, CNPase and MOG (green). Nuclei were counterstained with H-33342 (red). (**A**) Oligodendrocyte, 5 DIV, O1-antigen. (**B**) CNPase positive oligodendrocyte, 4 DIV. (**C**) MOG staining on a mature oligodendrocyte, 8 DIV. All scale bars 20 µm. (**D**) Quantified expression of maturation markers, average data from 2-7 experiments.

3.2.2. *Non-oligodendrocyte cells in the enriched oligodendrocyte culture*

To characterise non-oligodendroglial cells in the oligodendrocyte-enriched secondary culture, cells were stained with an antibody against glial fibrillary acidic protein (GFAP) to identify astrocytes (Jacque, 1991). Neuronal cells were identified by positive β3-tubulin staining (Fournier and McKerracher, 1997; Lee *et al.*, 1990). Microglia cells were stained specifically with a FITC-coupled lectin (IB4) (Streit, 1990). In Fig. 8 morphological characteristics of non-oligodendroglial cells are demonstrated: astrocytes, exhibiting either a compact shape with large nuclei (Fig. 8A) or a star-like form (“astro”) with smaller nuclei (Fig. 8B); neurons characterised by a compact cell body and extended processes (Fig. 8C); microglia, with a round and relatively regular shape (Fig. 8D).

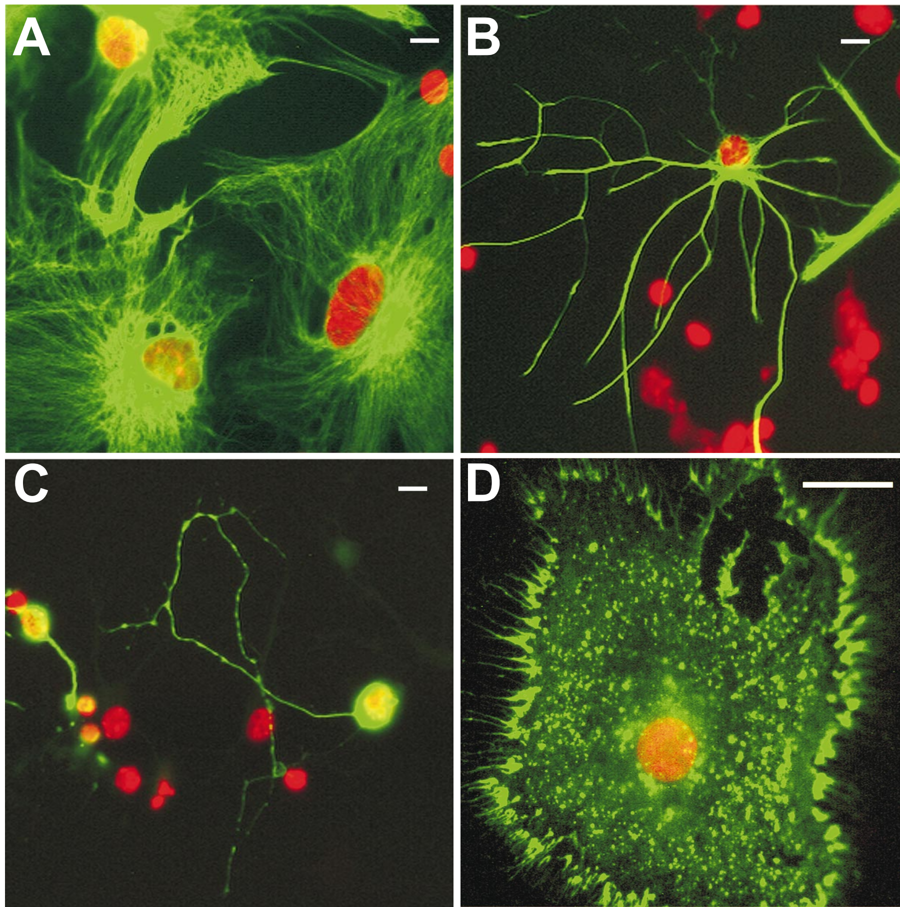

Figure 8: Non-oligodendroglial cells in the enriched oligodendrocyte culture.
Cells were stained by immunocytochemistry for different antigens (green) and analysed by confocal microscopy. Nuclei were counterstained with H-33342 (red). (**A, B**) GFAP staining in astrocytes. (**C**) β3- tubulin in neuronal cells. (**D**) IB4 on a microglia cell. All scale bars 20 µm.

DIV	GFAP	IB4	3-tubulin
	Av [% ± SEM (n)]		
1,2	**5 ±** 2 (3)	**10 ±** 3 (6)	**26 ±** 4 (6)
3,4	**6 ±** 1 (5)	**13 ±** 3 (6)	**22 ±** 4 (7)
5,6	**8 ±** 3 (3)	**21** (1)	**n.d.**
7,8	**13 ±** 1 (3)	**30 ±** 8 (3)	**19 ±** 5 (4)

Table 1: Non-oligodendroglial cells in the enriched oligodendrocyte culture.
Cultures were stained separately for GFAP (astrocytes), β3-tubulin (neuronal cells), and IB4 (microglia). Total cell number and number of stained cells were scored by counting cells in 5 randomly chosen fields (310 x 310 µm) of a coverslip. Values are given as % of total.

Cells positive for GFAP, IB4 and anti-β3-tubulin were scored at different days after plating (Tab. 1). The number of astrocytes and microglia cells, that are still able to proliferate in culture, increased with duration of the culture. The amount of neuronal cells, identified by positive anti-β3-tubulin staining slightly decreased over cultivation time, indicating that neuronal cells probably died due to suboptimal culture conditions. After 7 to 8 days, more than 40 % of all cells were microglia cells or astrocytes. To avoid excessive presence of other glial cells in the oligodendrocyte culture, most experiments were carried out at 3 to 6 DIV, when the major part of oligodendrocytes were differentiated, but the amount of astrocytes and microglia was only between 20 and 30 %.

3.3. Effect of inflammatory cytokines on murine oligodendrocytes

Contradictory results exist about sensitivitiy of oligodendrocytes to inflammatory cytokines *in vitro* (see 1.3.2.) (Andrews *et al*., 1998; Baerwald and Popko, 1998; Louis *et al*., 1993; Vartanian *et al*., 1995). To test, whether cytokines would induce cell damage in the enriched culture of oligodendrocytes, cells were exposed to murine TNFα and murine IFNγ (TNFα 10 - 200 ng/ml, IFNγ 200 – 2000 U/ml) for 8-96 h. The effect of the cytokine-treatment was monitored by measuring MTT reduction and/or condensation of nuclei. Nuclear changes were investigated either by staining with Sytox and H-33342 or by immunostaining for CNPase and chromatin staining by H-33342. No cytotoxic effect of these cytokines alone or in combination was found.

ActD and CHX were reported to sensitise cells for cytokines by impairing mRNA and protein synthesis, respectively (Leist *et al*., 1997b; Scanlon *et al*., 1989; Wallach *et al*., 1988). Experiments were repeated with additional preincubation of the cells for 30-60 min with actinomycin D (ActD, 2 μg/ml) or cycloheximide (CHX, 1 - 25 μM). None of the experimental conditions tested resulted in cell death induction in oligodendrocytes.

TNFα and IFNγ were reported before to stimulate production of NO in macrophages and microglia (Corradin *et al*., 1993; Nacy *et al*., 1991). Functionality of TNFα and IFNγ was controlled by incubation of microglia cells with TNFα and IFNγ and measuring nitrite formation in the SN in a Griess test (Misko *et al*., 1993). Incubation of microglia with the two cytokines (concentrations like mentioned above) induced NO-synthase activity and nitrite formation.

3.4. Effect of CD95-ligand on murine oligodendrocytes

The sensitivity of oligodendrocytes to CD95-stimulation induced cell death was tested using two different ligands for CD95. Cells were either stimulated with agonistic anti-Fas-antibody (200 ng/ml) or by using the supernatant (SN, 10 to 50 vol %) of a transfected cell line secreting CD95L into the culture medium (Rensing-Ehl *et al.*, 1995). Control SN was obtained from cells transfected with the plasmid coding for the neomycin-resistence. Cell death induction was assessed as described for cytokine experiments. Neither the agonistic anti-CD95-antibody nor CD95L containing SN were effective in inducing cell damage. Pretreatment with ActD or CHX (concentrations as in cytokine experiments) did not sensitise for the toxic effect of the anti-CD95-antibody or CD95L.

Functionality of FasL was controlled by scoring apoptosis in Jurkat cells after stimulation with FasL SN (Zipp *et al.*, 1997). FasL SN (10 vol %) induced nuclear condensation in 70 % of the Jurkat cells within 2 h while the control SN did not. After 6 h, MTT reduction of Jurkat cells treated with 10 vol % of FasL SN was decreased to 40 % of control, while the MTT reaction of cells treated with the control SN was even elevated. The potential of anti-CD95-antibody to induce cell death was tested on mouse hepatocytes (Leist *et al.*, 1996). Anti-CD95-antibody (200 ng/ml) induced LDH-release in 70 % of the hepatocytes within 8 h (control cells 36 % LDH release).

3.5. Ganglioside effects in oligodendrocytes and other cells

Cytokines and NO are mediators released by microglia. They have been considered in many studies to be relevant in inflammatory demyelination and oligodendrocyte death. The molecule class of gangliosides has rarely been looked at with respect to involvement in pathologic mechanisms of demyelinating diseases. However, descriptive studies have been published that report unusually high levels of gangliosides in cerebrospinal fluid of multiple sclerosis patients and in brain tissue of mice induced for EAE (Miyatani *et al.*, 1990; Zaprianova *et al.*, 1998). Therefore, we were interested to investigate potential toxic effects of gangliosides in our oligodendrocyte culture.

3.5.1. *GD3 toxicity in oligodendrocytes*

To test the effect of a ganglioside that already has been reported to have the potency to induce cell death, we used disialoganglioside GD3 (De Maria *et al.*, 1997). Oligodendrocytes (4 DIV) were incubated for 24 h with 100-300 µM GD3. Concentrations higher than 100 µM induced cell degeneration within 24 h (Fig. 9). Cell damage manifested in retraction of cellular processes and breakdown of the membrane network. In the late phase of degeneration cells

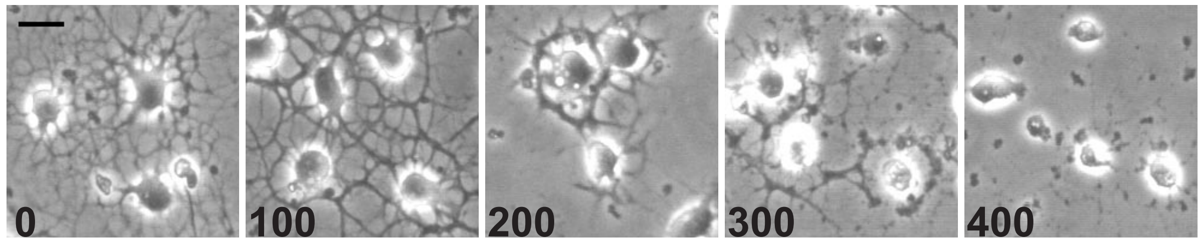

Figure 9: GD3 induced degeneration of processes and cell detachment.
Oligodendrocytes (4DIV) were incubated for 24 h with GD3 at the given concentrations [µM]. Concentrations > 100 µM induced retraction of membrane processes, fragmentation of the membrane network and rounding up of the cells. At high GD3 concentrations, cells were prone to detach. Scale bar 20 µm.

easily detached from the substrate. This was quantified by counting the oligodendrocytes remaining on the coverslip after immunostaining for CNPase (Fig. 10). About 40 to 50 % of all oligodendrocytes detached from their substrate within 24 h at the two highest concentrations of GD3. Within 48 h up to 80 % of the cells detached. After long term exposure, a concentration as low as 50 µM of GD3 caused cell loss. After 72 h of incubation with 50 µM GD3, over 60 % of all cells were lost from the substrate.

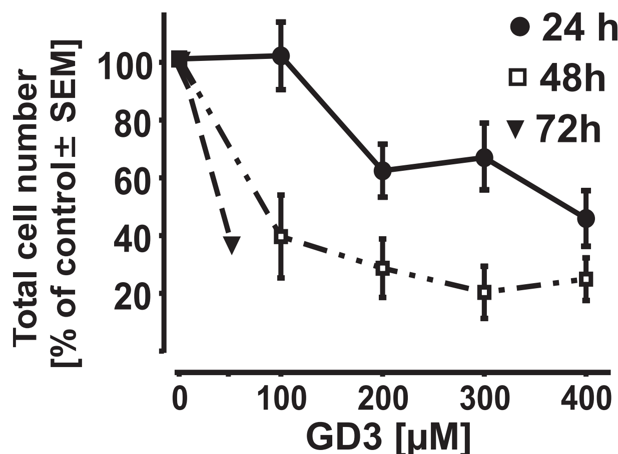

Figure 10: GD3 triggered oligodendrocyte loss.
Cells were incubated with the given concentrations of GD3 for the indicated times, then fixed and stained for CNPase. Total oligodendrocyte number in 10 randomly chosen fields was determined. To normalise for differences in plating density, values for the number of oligodendrocytes remaining attached to the culture dish are given as % of control. When oligodendrocytes were exposed to GD3 for > 24 hours a toxic effect was already observed at 50 µM GD3 . Data are from 3-6 cell preparations.

3.5.2. *Effect of GD3 on brain cells*

GD3 has a strong toxic effect in oligodendrocytes. To evaluate the cell type specificity of the GD3 effect, the main cell types residing in the brain were compared for their sensitivity to GD3. Murine astrocytes, microglia cells, cerebellar granule neurons (CGC) as representatives of neuronal cells, and oligodendrocytes were exposed to GD3. Cell death was quantified in microglia cells, astrocytes and CGC by scoring condensed or Sytox-positive nuclei (all damaged cells). Oligodendrocytes were stained for CNPase and DNA after GD3-exposure. Nuclei showing abnormal shapes in comparison to control cells (chromatin condensation, clumping like described in detail below, see 3.7.3.) were counted. Neither microglia cells nor

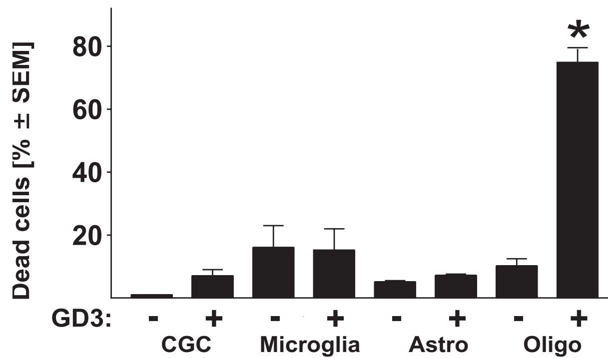

Figure 11: Selectivity of GD3-mediated oligodendrocytotoxicity.
Cultures of cerebellar granule neurons (CGC), microglia, astrocytes or oligodendrocytes were incubated for 24 h with 400 µM GD3. Cell death in CGC, microglia and astrocytes was evaluated after staining for 10 min with the two chromatin dyes Sytox (1 µM) and H-33342 (1 µg/ml). Cells with permeabilised plasma membranes and cells that had condensed nuclei were scored as dead. Oligodendrocyte death was evaluated from cultures co-stained with H-33342 and anti-CNPase. CNPase positive cells with condensed chromatin were scored as dead oligodendrocytes. Data are from 2-9 experiments with evaluation of at least 6 scoring fields per experiment. Oligodendrocytes: Student's t-test of control *vs* GD3: *= $p < 0.01$.

astrocytes showed sensitivity for GD3 at a high concentration (400 µM) that induced 80 % cell death in oligodendrocytes within 24 h (Fig. 11). In contrast, GD3 triggered cell death in a small population of CGC. However, even incubation with concentrations up to 600 µM of GD3 for 24 h induced only a maximum of 22 % cell death in CGC (Tab. 2). Other gangliosides that are structurally similar to GD3 or that could be precursors or metabolites of GD3 did not induce cell death in CGC.

Dead cells [%[	Av [% ± SEM (n)]
Control	**3 ±** 1 (4)
GD3 400 µM	**6 ±** 3 (3)
GD3 500 µM	**12 ±** 7 (2)
GD3 600 µM	**22 ±** 5
GD1a 600 µM	**9** (1)
GD1b 600 µM	**5 ±** 2
GM3 600 µM	**12** (1)
GT1b 600 µM	**15** (1)

Table 2: Cerebellar granule neurons are insensitive to gangliosides.
Cultures of CGC (7DIV) were incubated for 24 h with gangliosides. Cells were preincubated with 1 µM of the non-competitive NMDA-receptor-inhibitor MK-801 for 1h to avoid excitotoxic conditions. Cell death was evaluated after staining for 10 min with the two chromatin dyes Sytox (1 µM) and H-33342 (1 µg/ml). Cells with permeabilised plasma membranes and cells that had condensed nuclei were scored as dead.

3.5.3. *Effect of disialoganglioside GD3 on cell lines*

The effect of GD3 was also tested on several cell lines. Neither HeLa-229 cells, nor WEHI-S cells were sensitive to GD3 (data not shown). Also O4CB-neu cells, that descend from O4-positive oligodendrocyte progenitors (Jung *et al.*, 1995), were not damaged by GD3 (control = 7 % dead cells, 400 µM GD3 = 7 % dead cells after 24 h; quantified by staining with Sytox and H-33342; data from 2 experiments).

3.6. Specificity of GD3 induced toxicity

3.6.1. *Differential toxicity of structurally similar gangliosides on oligodendrocytes*

To exclude that oligodendrocytes are in general more sensitive to exogenous gangliosides than other brain cells, oligodendrocytes were incubated with GD3-precursors or GD3-metabolites. Neither gangliosides with a structural similarity to GD3, like GD1a or GD1b, induced the pronounced toxicity that was observed with GD3, nor was the precursor of GD3, GM3 toxic (Fig. 12). The possibility that GD3 might exert a non-specific toxic effect on oligodendrocytes acting like a detergent was excluded by this comparison of GD3 to structurally similar gangliosides.

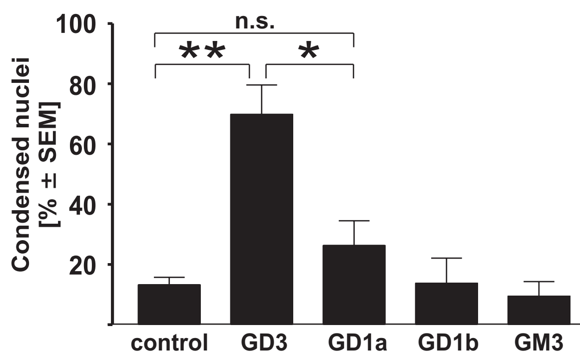

Figure 12: Specificity of GD3 induced oligodendrocyte toxicity
Oligodendrocytes were incubated for 24 h with 400 µM of GD3, GD1a, GD1b or GM3. Then, cells were stained for CNPase and condensed nuclei in CNPase-positive cells were scored as described above. Data from 5 experiments were subjected to one-way ANOVA followed by Tukey's multiple comparison test. Control *vs*. GD3: $p < 0.001$, control *vs*. GD1a: $p > 0.05$ (n.s.), GD3 *vs*. GD1a: $p < 0.01$.

3.6.2. *Investigation of ceramide contamination*

To determine the possible presence of ceramides in the GD3 extracted from bovine brain, we measured acetyl-, hexanoyl-, palmitoyl-, oleyl-, stearyl-, behenoyl-, lignoceroyl-, and nervonyl-sphingosine by combined gas chromatography and subsequent mass spectrometry. No traces of the mentioned ceramides were detected (Data not shown).

3.6.3. *Investigation of LPS contamination*

Lipopolysaccharide is a component of the bacterial cell wall and is known to be a strong activator of cells of the macrophage and monocyte lineage (Kielian and Blecha, 1995; Ulevitch *et al*., 1990). An eventual LPS contamination in the commercial GD3 preparation may therefore induce an inflammatory reaction in microglia cells that are present in the oligodendrocyte culture (see 3.1.2.). Inflammatory mediators like cytokines or NO could be produced by microglia and in this way cause the death of oligodendrocytes after incubation with GD3. The amount of LPS was determined by a *Limulus* assay, that specifically recognises LPS. LPS was

detected in none of the tested GD3 preparations (two different lots of bovine brain-derived GD3, Sigma). When also GD1a and GD1b stocks were tested, 10 pg / ml of LPS were found in the GD1b preparation.

3.6.4. *Comparison of biologic preparations and synthetic GD3 on oligodendrocytes*

As bovine brain derived GD3 (bb GD3) might contain many other remnants apart from ceramides, comparative experiments with chemically synthesised GD3 were carried out. Synthetic and bb GD3 in concentrations between 100-800 µM and 100-400 µM, respectively were tested in the same experiment on oligodendrocytes. Both preparations of GD3 proved to induce the same cellular changes like process degeneration, nuclear changes and cell detachment. After 24 h of incubation, the plasma membrane was still intact in oligodendrocytes stimulated with either bb or synthetic GD3. Maintenance of cell integrity was assessed by staining with calcein, as demonstrated in Fig.13A. Cells still showed calcein staining 24 h after addition of GD3, indicating that unspecific lysis was not induced by either GD3 preparation.

To obtain an equivalent effect of synthetic and bb GD3, slightly higher concentrations of synthetic GD3 had to be used (Fig.13B). This might be due to the different composition of fatty acid residues in bb GD3 and synthetic GD3. In bb GD3 a mixture of fatty acid residues forms the lipophilic moiety, while synthetic GD3 contains uniform fatty acid residues (C18). The different length of fatty acid residues might influence the formation of micelles.

In order to find out, if only bb GD3 or also other biologic GD3 preparations could induce cell death in oligodendrocytes, GD3 isolated from bovine milk (m GD3) was tested. M GD3 induced nuclear condensation in the same way as bb GD3 (cells with nuclear changes in %: control = 0, m GD3 200 µM = 35, m GD3 300 µM = 63, m GD3400 µM = 52, data from one experiment).

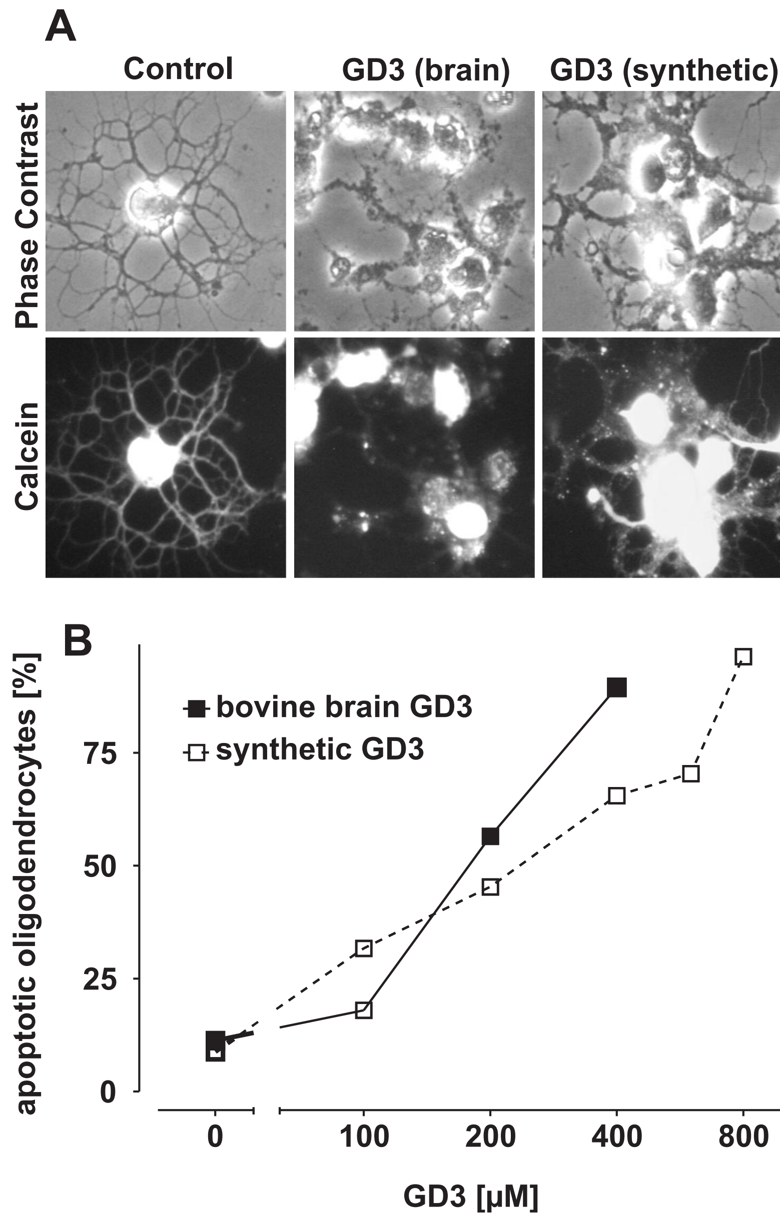

Figure 13: Brain derived and chemical synthesised GD3 induce oligodendrocyte degeneration.

Murine oligodendrocytes were incubated with GD3 extracted from bovine brain or synthetic GD3 to compare the effects. (A) 24 h after addtion of 400 µM GD3, cells were incubated for 10 min with 1 µM calcein-AM, a fluorescent probe that is only retained in cells with functional esterases and intact cell membrane. (B) Cells were incubated for 24 h with different concentrations of either synthetic GD3 or bb GD3 . Cells were then fixed and stained for CNPase and condensed nuclei in CNPase-positive cells were scored as described above. One representative experiment of 5 is shown.

3.6.5. *Comparison of bovine brain-derived and synthetic GD3 on isolated liver mitochondria*

As a second test system for the comparison of bb and synthetic GD3 isolated liver mitochondria were used. GD3 already has been shown to induce an uncoupling effect in isolated mitochondria which increased mitochondrial oxygen consumption (Kristal and Brown, 1999; Scorrano *et al*., 1999b). Liver mitochondria stimulated with either bb GD3 or synthetic GD3 showed a concentration dependent increase in respiration (Fig.14). Two representative experiments are shown. Comparable to the published experiments, the effect of bb GD3 (Fig.14A) as well as the effect of synthetic GD3 (Fig.14B) was blocked by the preincubation of mitochondria with cyclosporin A (CsA), which is thought to interfere with the opening of the mitochondria permeability transition pore.

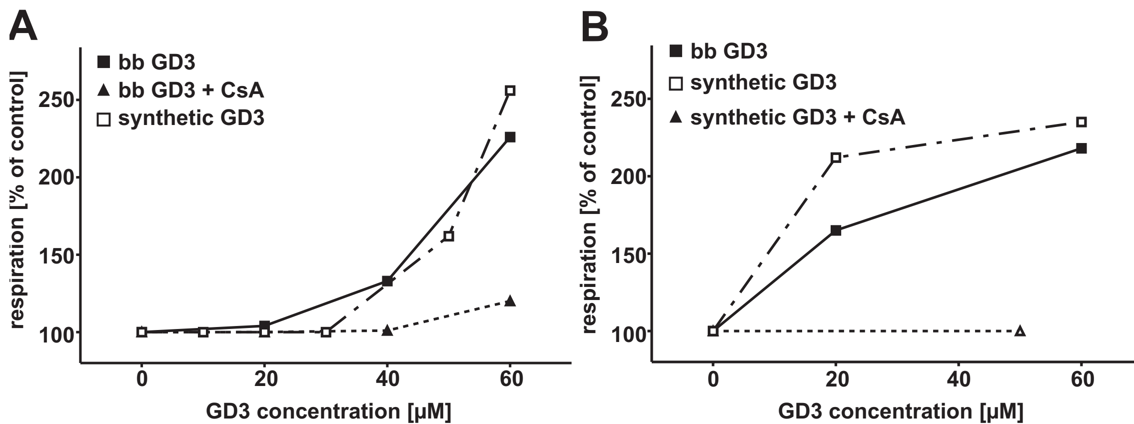

Figure 14: Synthetic and bb GD3 induce uncoupling of mitochondrial respiration.
Isolated rat liver mitochondria were incubated in an oxygraph chamber. Succinate stimulated respiration was set to 100 %. Mitochondria were incubated with increasing concentrations of GD3. Accelerated respiration due to the uncoupling effect of GD3 was measured. Uncoupling was inhibited by preincubation with cyclosporine A (CsA, 2 µM, 2 min preincubation). Mitochondrial function was controlled at the end of every experiment by adding 100 nM FCCP, which induced full uncoupling in intact mitochondria. Two representative experiments are shown.

3.7. Characterisation of GD3 induced oligodendrocyte degeneration

3.7.1. *Time course of GD3-triggered oligodendrocyte degeneration*

After the investigations about the specificity of the GD3 effect on oligodendrocytes, a more detailed characterisation of the GD3 induced cell death was begun. As already mentioned above, GD3 induced oligodendrocyte degeneration at concentrations higher than 100 µM within 24 h. By following an experiment over 24 h, changes in the cell morphology became visible by phase contrast microscopy after 6 to 12 hours (Fig. 15). After 6 h, first changes in the membranes became visible and after 12 h the membrane network was heavily damaged. After 18 to 24 h, the contact between cellular processes and cell bodies was lost in most of the cells resulting in the detachment of cells from the substrate (see also 3.5.1.). As after 24 h of incubation already up to 50 % of the oligodendrocytes had detached, shorter incubation periods and a concentration of 300 µM GD3 were used for most of the subsequent experiments.

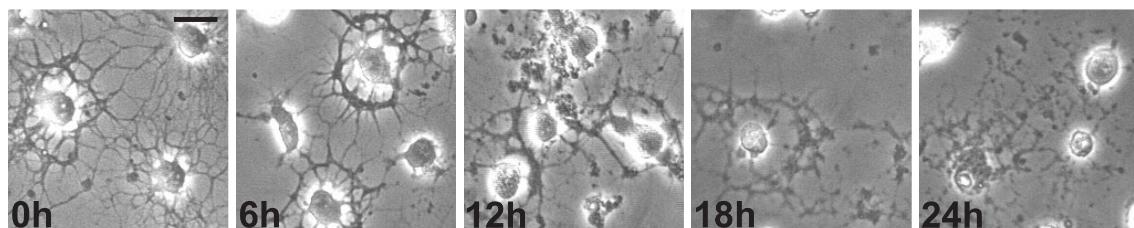

Figure 15: Time course of GD3 triggered oligodendrocyte degeneration.
Oligodendrocytes were incubated with 300 µM GD3 for the given times and cell degeneration was followed by phase contrast microscopy. First changes in the morphology of the network of membrane processes became visible after 6h, while obvious changes could be seen after 12 h. At 18 to 24 h, cells were easily detaching from the coverslip. Scale bar 20 µm.

3.7.2. *GD3 induced damage is independent from the differentiation state of the oligodendrocyte*

For the relevance of GD3 as an oligodendrocytotoxic factor it could be important to know, if GD3 triggers cell death in the same way in immature and mature oligodendrocytes. For this reason, oligodendrocytes at 2, 4, and 6 DIV were incubated with 100-400 µM GD3. No differences in the shape of the concentration response curves were observed (Fig. 16). In young oligodendrocytes (2 DIV), the curve was shifted to a lower amount of condensed nuclei at 200 and 300 µM. This difference was not observed at the highest concentration. Some experiments were also carried out with oligodendrocytes at 7 and 10 DIV, where GD3 was working with the same efficiency (7 DIV: cells with nuclear changes in %: control = 6, GD3 100 µM = 8, GD3 200 µM = 47, GD3 300 µM = 88, GD3 400 µM = 90; 10 DIV data not shown).

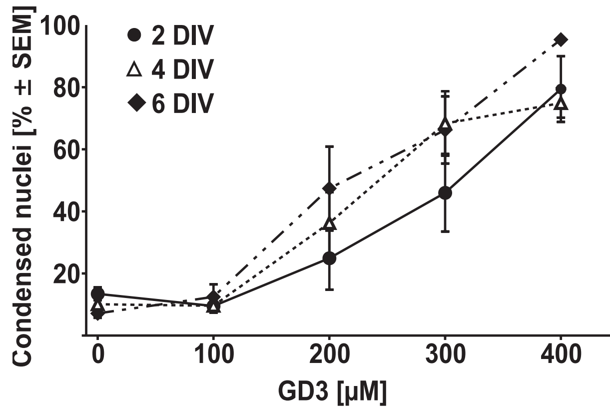

Figure 16: GD3 damages oligodendrocytes in different maturation states.
Oligodendrocytes were incubated with 100-400 µM GD3 for 24 h at 2, 4, and 6 DIV. Cells were stained for CNPase and chromatin as described above and apoptotic nuclei were scored. 2 DIV: n = 6, 4 DIV: n = 7, 6 DIV: n = 5.

3.7.3. *GD3 causes chromatin condensation*

Oligodendrocytes were incubated with 100 to 400 µM of GD3 for 24 h and stained for CNPase and chromatin. In oligodendrocytes, identified by their positive CNPase staining, GD3 induced nuclear hyperchromasy and chromatin condensation as well as DNA clumping in a concentration-dependent mode (Fig. 17A). These changes were counted as “condensed nuclei”. Examples of two nuclei with typical chromatin changes how they can be observed in early and late phases of cell death, are shown (Fig. 17B). The chromatin condensation in the late nucleus resembles a phenotype found in many cells undergoing apoptosis (Fig. 17B, late).

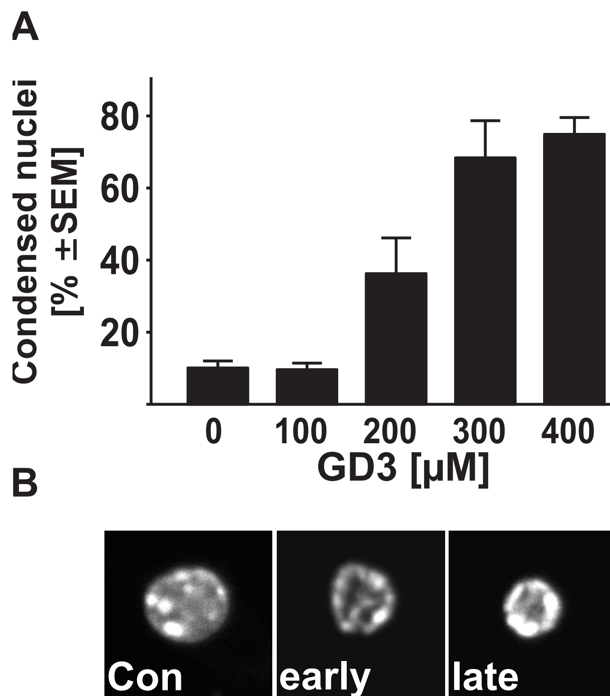

Figure 17: GD3 stimulated chromatin changes.
Oligodendrocytes were incubated with 100-400 µM GD3, fixed and stained for CNPase and chromatin. (**A**) Concentrations > 100 µM induce typical changes in chromatin structure. (**B**) Representative examples of nuclei with condensed chromatin are shown. Positive counts included both peripheral hyperchromasy (early) and serious condensation/ fragmentation of the chromatin (late). Image size: 20 µm x 20 µm.

3.7.4. *Exposure of phosphatidylserine and changes of mitochondria membrane potential after stimulation with GD3*

Phosphatidylserine (PS) exposure to the outer leaflet of the cell membrane as a typical feature of apoptosis (Fadok *et al*., 1992; Martin *et al*., 1995) was investigated next. The upper panels in Fig. 18A show examples for the typical staining of PS in oligodendrocytes. In many control cells a peripheral staining of membrane processes with annexin-V was observed (Fig. 18A, upper left). After induction of apoptosis by GD3, migration of the staining towards the cell body occurred (Fig. 18A, center up), until the whole cell was stained by annexin-V (Fig. 18A, upper right). Contemporaneously, nuclei began to change shape and finally condensed their chromatin (Fig. 8A, arrow). Mitochondria stained by TMRE, a dye sensitive for the mitochondria membrane potential ($\Delta\Psi_m$), changed morphology to a round shape (arrow) and finally lost membrane potential (Fig. 18A, lower panels).

A quantification of the changes visible in Fig. 18A is given in Fig. 18B. Over a period of 18 h, the exposure of phosphatidylserine on the surface of oligodendrocytes increased and was accompanied by changes in nuclear morphology. Secondary lysis took place after 12 to 18 hours when damaged cells began to lose membrane integrity. In consequence, nuclei were stained by impermeable chromatin dyes like ethidium homodimer (Fig. 18B).

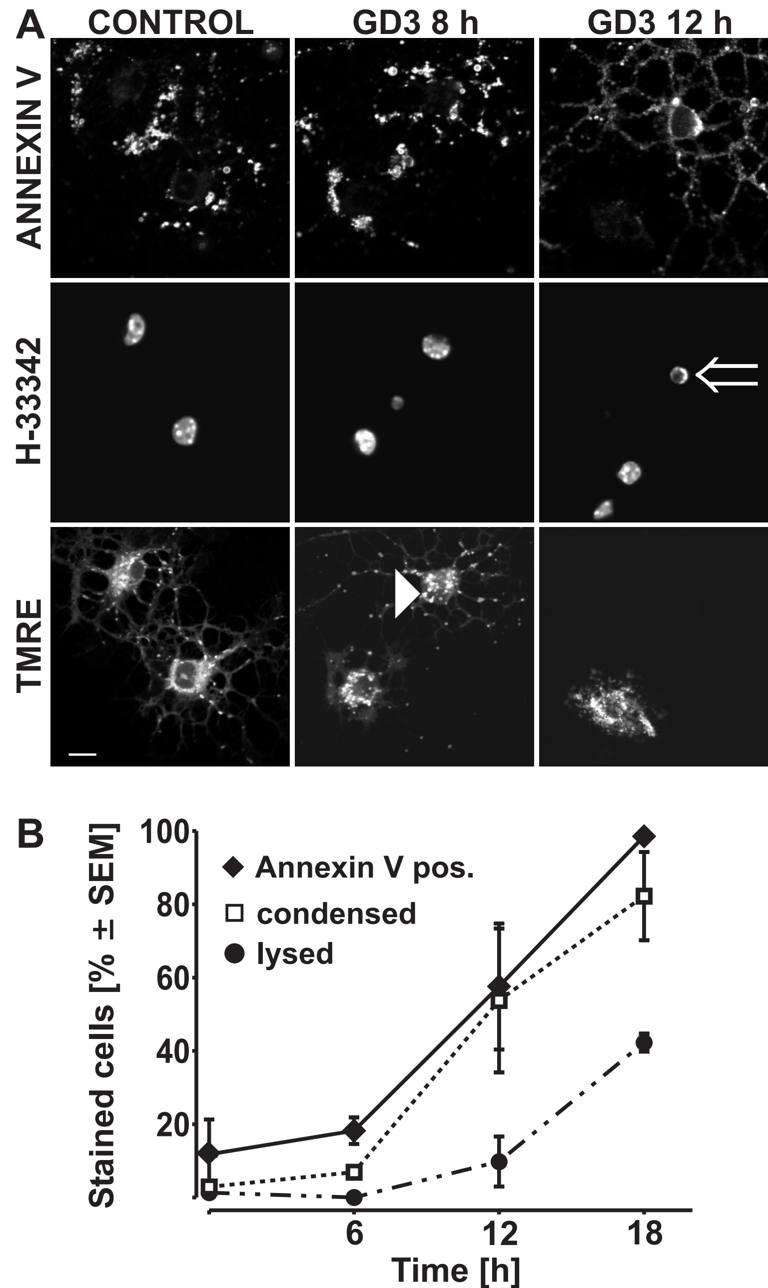

Figure 18: GD3 induced PS-exposure and loss of mitochondrial membrane potential.
Oligodendrocytes were exposed to 300 µM GD3 and analysed at different time points. Cells were stained for 15 min with a mixture of H-33342 (1 µg/ml), EH-1 (0.5 µM) and the $\Delta\Psi_m$ sensitive dye TMRE (10 nM). Then, cells were stained with fluorescent-labelled annexin-V and analysed in cold buffer. (A) By multi-channel confocal microscopy, the individual fluorescent channels were recorded and are shown separately for a representative group of cells. Note the annexin-V-staining at the tip of the myelin extension of control cells, the morphological change and the clustering of the initially longish mitochondria prior to the loss of $\Delta\Psi_m$ (▸), and the condensation of chromatin and loss of TMRE-staining in the annexin-V-positive cell (arrow). Scale bar: 10 µm. (B) To quantify the effect of GD3, oligodendrocytes were scored for positive annexin-V-staining on the cell body, for membrane permeability (lysed), and for condensed chromatin (condensed). Data are from 3 cell preparations.

3.8. Involvement of downstream caspases in GD3 toxicity

Since cell death induced by GD3 in oligodendrocytes showed features of apoptosis, such as chromatin condensation, phosphatidylserine exposure, and maintenance of the cell membrane integrity for a relatively long time after stimulation, we were asking for the involvement of caspases in the cell death mechanism.

3.8.1. *Caspase-3 is activated during GD3 induced apoptosis and is inhibited by zVAD-fmk*

To measure the activity of executioner caspases, a staining for active caspase-3 was established. The presence of active caspase-3 should indicate if activation of downstream caspases occurred after GD3 stimulation. By simultaneous staining, activated caspase-3, cyt *c*, and nuclear morphology were assessed in CNPase-positive cells (Fig. 19A). The lowest panels illustrate GD3-triggered activation of caspase-3. ZVAD completely abolished the occurrence of cells stained for active caspase-3. Notably, staining for active caspase-3 was usually present in cells with fully condensed chromatin, but rarely in cells that showed early forms of DNA condensation like hyperchromasy (not shown). This indicates that caspases are activated later in the GD3 triggered cell demise but most likely not in the initial phase of apoptosis.

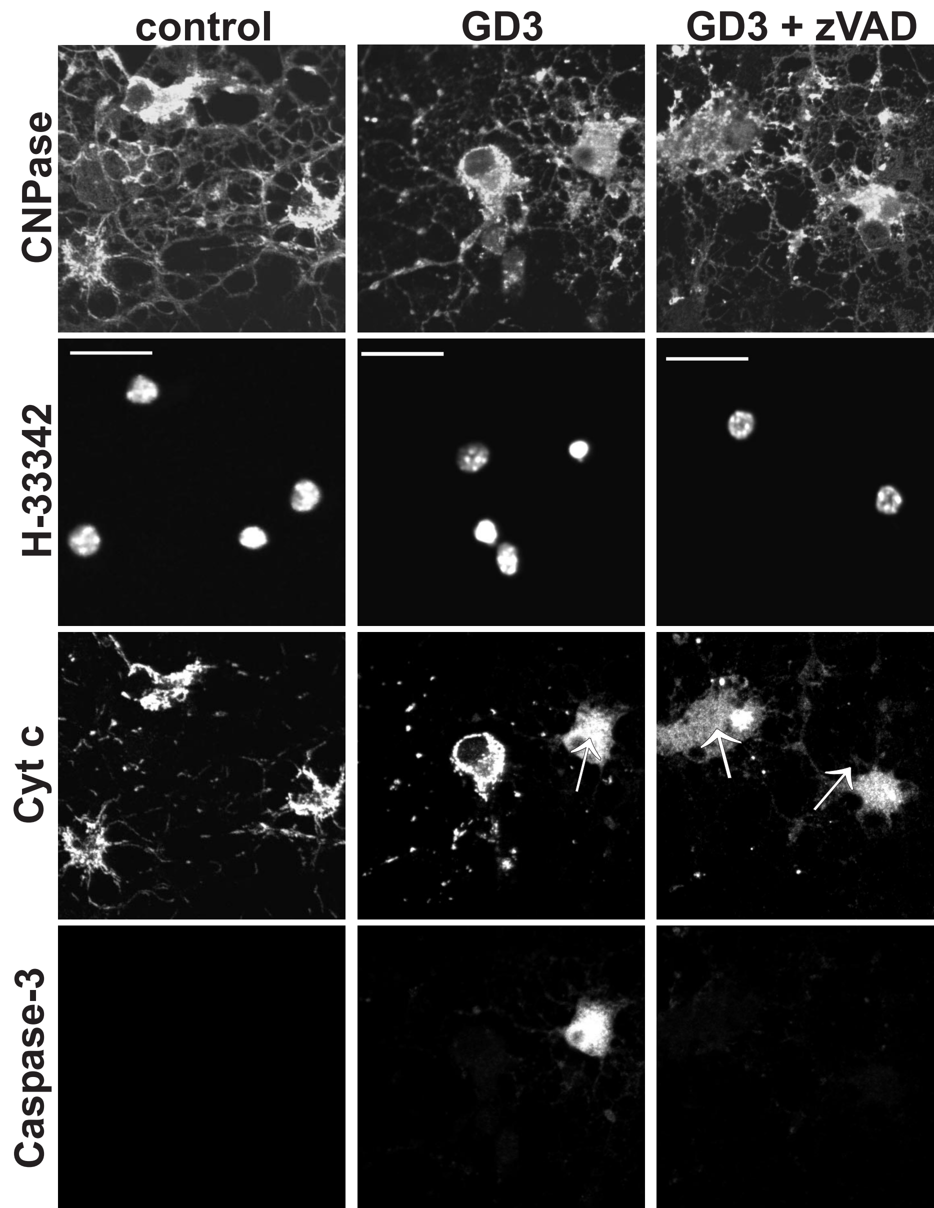

Figure 19: GD3-induced activation of caspase-3.

Oligodendrocytes were exposed to GD3 (300 µM) with and without zVAD-fmk (100 µM) for 12 h and then fixed. They were simultaneously stained for CNPase to identify oligodendrocytes, cytochrome *c*, active caspase-3 and DNA (H-33342). Fluorescence was recorded with a four channel confocal scanning microscope. Control cells show the typical distribution of cyt *c* in mitochondria, while GD3-exposed cells treated with or without zVAD show release of cyt *c* (arrows). Activation of caspase-3 is evident in the GD3 treated cells with condensed chromatin and released cyt *c*. Scale bar: 20 µm.

3.8.2. *ZVAD neither prevents GD3 induced oligodendrocyte death nor PS exposure nor release of cyt* c

Since caspase-3 was clearly activated by GD3, and zVAD effectively prevented this activation, we tested next, if zVAD might increase survival of GD3 challenged oligodendrocytes. Oligodendrocytes were incubated with 300 µM GD3 and the time courses of PS exposure and chromatin condensation were analysed. Neither the appearance of PS on the cell surface nor the nuclear condensation of chromatin were reduced or delayed by zVAD (Fig. 20).

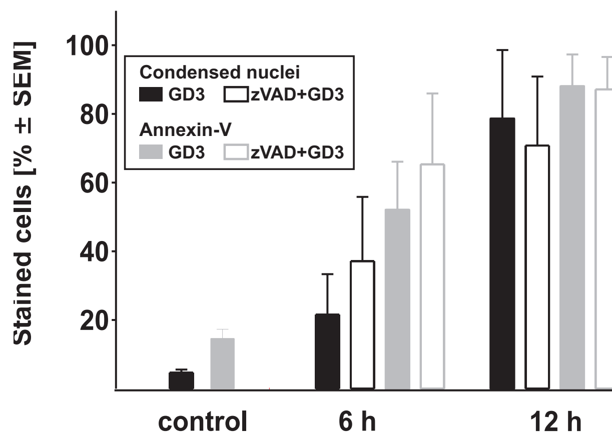

Figure 20: No inhibition of cell death and PS-exposure by zVAD.
Oligodendrocytes were treated with 300 µM GD3 or GD3 plus 100 µM zVAD-fmk for the indicated times. The number of nuclei with partially or fully condensed chromatin and cells with annexin-V staining were scored. Data are from 3 experiments.

The same held true for cyt *c* release from mitochondria. In Fig. 19A control oligodendrocytes are shown that exhibit the classical cyt *c* staining resembling the localisation of mitochondria, as stained e.g. by mitotracker (not shown). Cells stimulated with GD3 with or without zVAD-preincubation displayed a delocalised staining for cyt *c*. Cyt *c* was homogeneously distributed over cytosol and nucleus. A quantification of the number of oligodendrocytes with cyt *c* released into cytosol and nuclei confirmed this observation (Tab. 3): zVAD did neither delay nor inhibit release of cyt *c* induced by GD3.

Cells with cyt *c* released [%[	Time [h]	Av ± SEM (n)
Control		3 ± 1 (4)
GD3	6	6 ± 3 (3)
GD3+zVAD	6	12 ± 7 (2)
GD3	12	22 ± 5 (2)
GD3+zVAD	12	9 (1)

Table 3: zVAD does not prevent cyt *c* release.
Cells were pretreated or not with 100 µM zVAD for 30 min and stimulated with 300 µM GD3. After 6 and 12 h, cells were fixed and stained for CNPase and cyt *c*. CNPase positive cells with cyt *c* released into the cytosol or the nucleus were counted.

3.8.3. *ZVAD modulates the morphology of chromatin condensation and the loss of membrane integrity*

Although zVAD did not prevent PS exposure, cyt *c* release, and cell death, some differences in cells treated with zVAD and GD3 were found. The first observation was, that zVAD changed the GD3-triggered nuclear changes by preventing complete chromatin

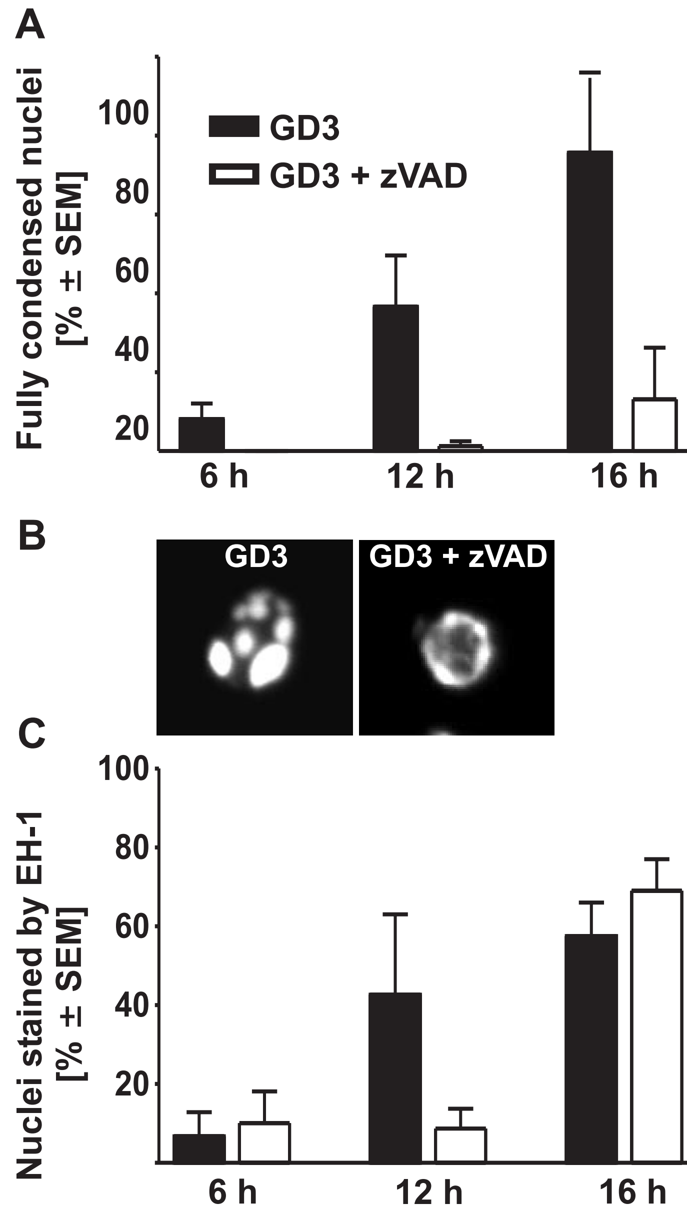

Figure 21: zVAD changes GD3 induced chromatin condensation and delays cell lysis.

Oligodendrocytes were treated with 300 µM GD3 or GD3 plus 100 µM zVAD-fmk for the indicated times. (**A**) The number of nuclei with fully condensed chromatin was scored. Data from 3 experiments. Two-way ANOVA yielded $p < 0.001$ for the effect of zVAD-fmk on chromatin condensation over time. (**B**) Nuclei of cells *plus*GD3 show full condensation; with zVAD and GD3 chromatin is hyperchromatic but not completely condensed. Image size 20 x 20 µm. (**C**) Non-fixed cells stained with EH-1. Cells with positive EH-1 staining (as parameter for membrane lysis) were scored. Data from 4 cell preparations.

fragmentation (Fig. 21A). Hyperchromasy and clumping of DNA occurred also in the presence of zVAD. Fig. 21B gives examples of typical oligodendrocyte nuclei after induction of cell death with GD3 in the presence or absence of zVAD pretreatment.

Additionally, zVAD influenced the permeability of the cell membrane after GD3 treatment. ZVAD-preincubation reduced the amount of secondary necrosis in oligodendrocytes observed after 12 h, while this effect was not present anymore at later time points (Fig. 21C).

3.9. Effect of other protease inhibitors on GD3 induced cell death

In order to exclude an important role of other proteases in GD3 induced oligodendrocytotoxicity, inhibitors of other proteases were tested for their potency to inhibit or reduce cell death. No effect was obtained by preincubation with DEVD-CHO, YVAD-cmk, calpaininhibitors I, II and III, the cathepsin B inhibitor CH-074-Me, TLCK and E64 (for concentrations see 2.2.5.).

Some protection from cell death was found with leupeptin (60 µM) preincubation before addition of GD3 (300 µM). While at earlier time points (12 h) GD3 induced nuclear changes were not inhibited (26 ± 42 % inhibition), after 18 h a protective effect became evident (47 ± 27 % inhibition, n=4).

3.10. Role of mitochondria in GD3 induced oligodendrocytotoxicity

Already several studies have given evidence for direct GD3 effects on isolated mitochondria (Garcia-Ruiz *et al*., 2000; Kristal and Brown, 1999; Petronilli *et al*., 1999). For this reason, attention was focussed on the fate of mitochondria in oligodendrocytes exposed to GD3.

3.10.1. *Loss of mitochondrial membrane potential after GD3 exposure*

As already described in chapter 3.7.4, GD3 impairs the function of mitochondria. Fig. 18A, (down), demonstrates examples for mitochondrial changes in living cells, stained by TMRE, a dye staining mitochondria with intact membrane potential. Mitochondria of control oligodendrocytes displayed a characteristic longish shape, with few mitochondrial staining in the membrane processes and an asymmetric accumulation around the nucleus. In the early phase of cell degeneration (Fig. 18A, lower center, 8 h), mitochondria changed their shape. They seemed to round up, while $\Delta\Psi_m$ was still present, according to the TMRE staining. Later, $\Delta\Psi_m$ was completely lost (Fig. 18A, lower right, 12 h), although the cells continued to maintain plasma membrane integrity (no EH-1 staining). Note that in Fig. 18A at 12 h, a cell

with oligodendrocyte morphology shows annexin-V-staining, nuclear condensation and loss of $\Delta\Psi_m$, while another cell nearby without oligodendrocyte morphology is annexin-V-negative, shows a normal nuclear morphology and normal TMRE staining.

A quantification of these observations was performed by counting oligodendrocytes that had lost TMRE staining, and at the same time, cells with nuclear changes. In parallel incubations, cells were fixed and stained for cyt *c* to determine the amount of oligodendrocytes that showed delocalised cyt *c* staining. Already after 6 h of GD3 exposure, some oligodendrocytes had lost $\Delta\Psi_m$. after 12 h, about 70 % of the cells were TMRE- negative (Fig. 22). It is important to note, that the release of cyt *c* and also the first evident changes in chromatin structure were detectable already before the clear loss of $\Delta\Psi_m$. Nevertheless, it must be considered that smaller disturbances of $\Delta\Psi_m$ could not be evaluated by the all-or-nothing-score used.

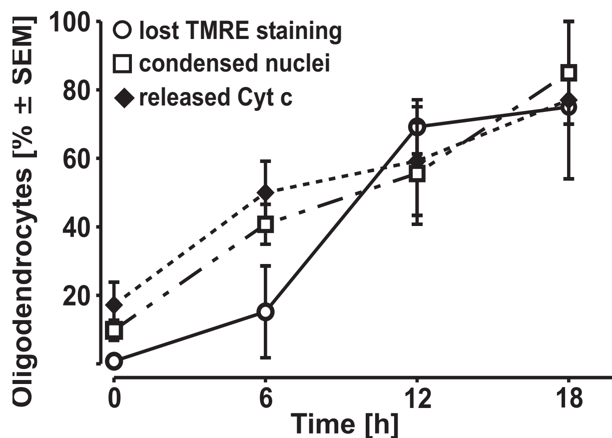

Figure 22: The role of mitochondria in GD3-induced oligodendrocyte apoptosis.
Cells were incubated with 300 µM GD3 for the indicated times and then fixed. CNPase positive cells with delocalised cyt *c* staining (as in Fig. 18 A) or with condensed chromatin were scored. Data are from five cell preparations. In parallel non-fixed cultures, the percentage of oligodendrocytes (oligodendrocyte-morphology) with lost TMRE staining was counted.

3.10.2. *Effect of* bcl-2*-overexpression on GD3 triggered oligodendrocyte degeneration*

If the major pathway of GD3 induced apoptosis involved mitochondria as a crucial signalling element, the anti-apoptotic protein Bcl-2 might influence the cellular reaction to GD3. To investigate this, primary oligodendrocytes were prepared from animals overexpressing a human *bcl-2*-transgene and cultures from non-transgenic littermates on background of C57/B6 as control. Cells were incubated with 100-300 µM GD3 for 18 h, and analysed after immunostaining for CNPase as usual. Fig. 23 shows the concentration response curves to GD3 of *bcl-2*-transgenic and non-transgenic cells. A first effect was observed in control cells, where the basal apoptosis rate was reduced about 50 % under *bcl-2*-overexpression. When cells were treated with > 200 µM GD3, protection by *bcl-2* was even

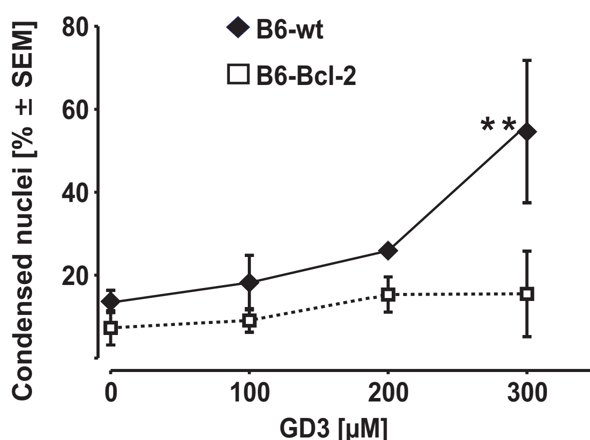

Figure 23: Bcl-2 protects from GD3 induced cell death.
Oligodendrocyte cultures were prepared from C57Bl/6 mice (B6-wt) or from mice expressing a human *bcl-2-* transgene (B6-Bcl2). Cells were stimulated with the indicated GD3 concentrations for 18 h, fixed and stained for CNPase and chromatin. Data are from 3 cell preparations. Condensed nuclei were counted and two-way ANOVA was performed with respect to genotype and GD3 concentration: $p < 0.001$ for the effect of the genotype(**).

more obvious. The amount of cells with nuclear changes was decreased from about 55 % in wt to 16 % in *bcl-2*-transgenic cells. By testing the curves in a two way ANOVA, the difference with respect to the genotype was considered highly significant ($p = 0.001$).

3.10.3. *Effects of cyclosporin A and bongkrekic acid on GD3 induced cell death and cytochrome* c *release*

It was mentioned before that GD3 is believed to stimulate opening of the permeability transition pore (MPTP) in mitochondria (see 3.6.5.). Since Bcl-2 was shown above to protect oligodendrocytes from GD3-induced death, the involvement of MPTP in the death mechanism is likely. Cyclosporin A (CsA) and bongkrekic acids are substances demonstrated to interact with the pore complex on the mitochondrial membrane and to prevent permeability transition (see also 3.6.5.) (Zamzami *et al*., 1996). For this reason we studied if CsA might interfere with GD3-toxicity.

Cells were preincubated with 2 µM CsA for 30 min, incubated with 300 µM GD3 for various times, fixed, immunostained, and scored for nuclear damage as usual. In contrast to the CsA-mediated protection of mitochondria from GD3-triggered uncoupling, only a minor protective effect was observed in oligodendrocytes. After 12 h, GD3-toxicity was inhibited by 13 ± 9 % and after 24 h by 33 ± 10 %.

3.11. Microglia and GD3

Microglia cells are among the key players in inflammatory demyelination. In these conditions, GD3 was found to be one of the gangliosides present in excess in CSF of patients suffering from white matter diseases. Microglia cells possess the potency to produce a broad palette of potential oligodendrocytotoxic mediators (see 1.2.4.). Furthermore, microglia cells were described to be positively stained for GD3 after brain injury (Reynolds and Wilkin, 1993). To find out if microglia cells are a potential source for GD3, the following experiments were carried out.

3.11.1. *GD3-positive cells in the enriched oligodendrocyte culture*

GD3 was not only reported to be increased in activated microglia, but it is known as a marker molecule for oligodendrocyte progenitors. In order to learn about the possible presence of GD3 in immature oligodendrocytes or other cells, costainings for CNPase and GD3 were carried out.

Fig. 24A displays two CNPase-positive oligodendrocytes, one of them costained for GD3 and probably not fully differentiated. Fig. 24B shows a CNPase-negative cell (according to its morphology a microglia cell) positively stained for GD3. For quantification of GD3-stained cells, cells positively labelled for GD3, for CNPase, and for both antigens, were counted (Tab.

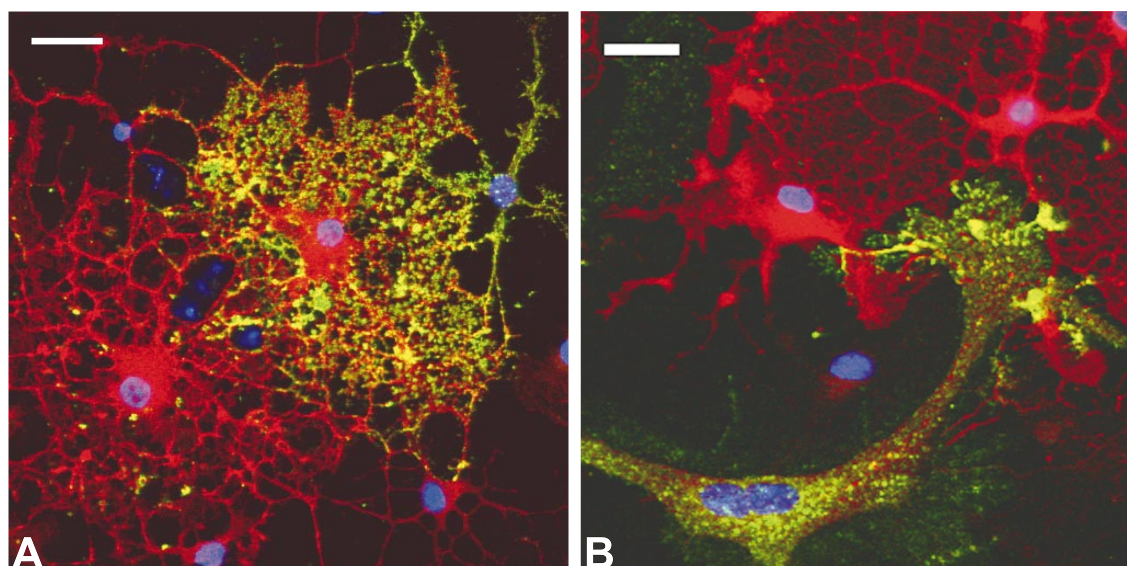

Figure 24: Oligodendrocytes and other cells are positive for GD3.
The oligodendrocyte-enriched secondary culture was stained simultaneously for GD3 (green) and CNPase (red). Nuclei counterstained by H-33342 (blue). (**A**) Two CNPase-positive oligodendrocytes, one also positive for GD3. (**B**) Two oligodendrocytes (upper half of the image) and one CNPase-negative, GD3-positive cell. According to its morphology most likely microglia. Scale bars 20 µm.

DIV	GD3	CNPase	GD3+CNPase
	Av [% ± SEM (n)]		
1,2	**15 ±** 10 (3)	**11 ±** 6 (3)	**2 ±** 2 (3)
3,4	**19 ±** 7 (4)	**19 ±** 5 (4)	**7 ±** 3 (4)
7,8	**9 ±** 3 (3)	**24 ±** 7 (3)	**3 ±** 2 (3)

Table 4: GD3 positive oligodendrocytes and other cells.
Cells were labeled by a double-immunostaining for GD3 and CNPase. Cell number was scored by counting cells positive for GD3 or CNPase or for both antigens simultaneously on 5 randomly chosen fields (310 x 310 µm) on each coverslip.

4). While the amount of CNPase-positive cells increased with longer cultivation, the percentage of GD3 positive cells decreased. The total number of oligodendrocytes stained positive for GD3 remained below 10 %. Since GD3 was described to be present mainly in oligodendrocyte precursors, and oligodendrocytes matured *in vitro* (see 3.2.1.), the latter finding is in line with data from the literature. The presence of GD3-positive cells with microglia-morphology gave a first indication that microglia cells have the potential to synthesise GD3 *in vitro*.

3.11.2. *GD3-production by murine microglia cells* in vitro

The investigations about GD3 induced oligodendrocytotoxicity initiated the search for potential sources of GD3 in case of demyelinating diseases. Levels of GD3 are elevated in acute phases of demyelinating diseases in CSF (Miyatani *et al*., 1990; Tarvonen-Schröder *et al*., 1997) and in sclerotic plaques of MS patients (Yu *et al*., 1974). Even though the source of gangliosides in liquor is not known, some evidence comes from the observation that ganglioside levels are increased in conditions of microglia activation (Andersson *et al*., 1998; Zaprianova *et al*., 1998).

Starting from these reports and from our own findings *in vitro*, we exposed isolated microglia to stimuli that are commonly used to trigger the inflammatory cytokine production in cells of the monocyte or macrophage lineage. Cells were stimulated with 1 or 10 µg/ml LPS, LPS and IFNγ (1 µg/ml / 200 U/ml) or with various concentrations of inactivated *E. coli* (0,8 or 4 µg/ml). LPS, LPS and IFNγ as well as inactivated bacteria triggered an inflammatory response in microglia characterised by TNFα-release and NO-production (data not shown). In a first attempt, we tried to immunostain stimulated microglia cells for GD3. But no increased amount of GD3-positive cells was observed after activation of microglia for 12, 24, 48 or 72 h of incubation.

As intracellular GD3-detection might be complicated due to release of GD3 by microglia we measured GD3 in the SN of stimulated microglia. Supernatants were analysed by TLC for their GD3-content. Within 24–72 h GD3 accumulated in the supernatant of cells stimulated with LPS (10 µg/ml) and *E. coli* (Fig. 25). No increased GD3 level in SN was detected in incubations with LPS and IFNγ (Data not shown). From calibration with an external standard it was calculated that GD3 in the supernatant reached concentrations of up to 250 nM after stimulation with LPS or inactivated *E. coli*. Any changes of viability of microglia were controlled at the end of the experiment within the same cultures used for GD3 production. Unimpaired capacity of the cells to reduce MTT showed that neither microglia cells stimulated with LPS or inactivated bacteria nor control cells had an impaired viability after an experimental period of 72 h.

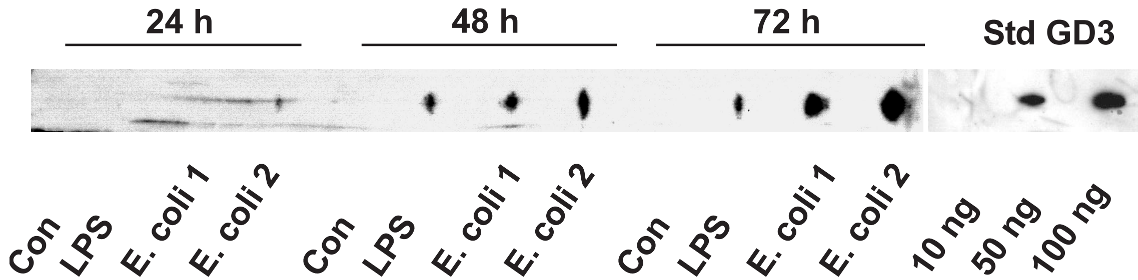

Figure 25: GD3 production by microglia
Murine microglia cells were stimulated or not (con) with 10 µg/ml LPS (LPS), 0.8 µg/ml of E. coli (1), or 4 µg/ml of E. coli (2) for the indicated times. Supernatants of cells were analysed for GD3 by chloroform / methanol extraction, subsequent TLC and immunostaining.

4. DISCUSSION

4.1. Oligodendrocyte demise in inflammatory demyelination

Although neurons are considered to be the basic units in the CNS, signaltransduction is impossible without glial cells. In particular oligodendrocytes, enveloping axons with multilamellar layers of myelin, essentially support the rapid conduction of electrical signals in axons of minimal radius. Therefore, degradation of myelin by inflammatory reactions results in severe impairment of neuronal function like in the case of multiple sclerosis and its experimental animal model EAE. Although myelin is obviously damaged under these conditions, and even lost from affected brain regions, the question on whether oligodendrocyte death has to occur has not been answered satisfyingly. Like for many other details concerning pathogenesis and propagation of inflammatory demyelination, results are inconsistent. A number of studies reported the presence of apoptotic oligodendrocytes in or at the border of MS lesions (Dowling *et al*., 1996; Lucchinetti *et al*., 1996; Vartanian *et al*., 1995), identifying up to 40 % of all apoptotic cells as oligodendrocytes (Dowling *et al*., 1997). Other researchers observed apoptosis exclusively in infiltrating lymphocytes (Bonetti *et al*., 1997) proposing a lytic mechanism for oligodendrocyte demise (Raine, 1997). Lately, the gradual loss of oligodendrocytes from MS-lesions was documented. While regions of acute or recent demyelination still contained mature oligodendrocytes devoid of membrane processes and with condensed nuclei (indicating an apoptotic morphology), presence of oligodendrocytes was restricted in older lesions to edges of the sclerotic plaque (Lucchinetti *et al*., 1999; Wolswijk, 2000). No matter how oligodendrocytes might degenerate, they are lost from MS plaques without doubts. The crucial mediator eliciting degeneration during brain inflammation is known just as little as the mode of oligodendrocyte death.

4.2. Cytokines and FasL as mediators of oligodendrocyte death

During inflammatory demyelination, different cytokines are produced in brain tissue by infiltrating blood-derived lymphocytes, activated microglia, and astrocytes (Renno *et al*., 1995; Selmaj *et al*., 1991a). TNFα, LTα, and IFNγ are probably the best-studied cytokines regarding the relevance for induction of EAE and oligodendrocyte death. The first approaches investigating TNFα in EAE used anti-TNFα-antibody or anti-LTα-antibody (Ruddle *et al*., 1990; Selmaj *et al*., 1991b) or the phosphodiesterase-inhibitor rolipram (Sommer *et al*., 1995) to block the action of TNFα, and observed attenuated or inhbited demyelination, respectively. Also the administration of soluble TNF-receptor prevented inflammation and demyelination (Korner *et al*., 1997). A study in LTα$^{-/-}$ mice proposed an important role for LTα, but no role

for TNFα in EAE (Suen *et al*., 1997). However, some demyelination was still observed in LTα$^{-/-}$ (-/- = knock out) mice (Suen *et al*., 1997) and another report found no relevance for TNFα and LTα in the development of EAE (Frei *et al*., 1997). Overexpression of TNFα in brain tissue under control of the MBP-promotor did not induce demyelination *per se*, but aggravated MBP-induced EAE (Taupin *et al*., 1997). Even an antiinflammatory property of TNF was stated as conclusion from EAE induction in TNFα$^{-/-}$ deficient mice. In this case, substitution of TNFα was decreasing severity of symptoms (Liu *et al*., 1998). Also for IFNγ, both a crucial role and in another case no importance for EAE-induction has been suggested (Ferber *et al*., 1996; Horwitz *et al*., 1997).

Similar confusion about cell death induction dominates the *in vitro* results (see 1.2.3.). Both, cell death induction, or not, of TNFα and IFNγ were observed, in part depending on the differentiation status of cultured oligodendrocytes. Our own investigations about the potential of IFNγ and TNFα to induce oligodendrocyte damage revealed insensitivity of murine oligodendrocytes to the two cytokines alone or in combination, even after long term incubation. For several *in vitro* systems it is known that cytokine toxicity manifests only in conditions of inhibited mRNA-transcription or protein-translation (Leist *et al*., 1997b; Scanlon *et al*., 1989; Wallach *et al*., 1988). In order to test, if oligodendrocytes might be sensitised by ActD (inhibitor of transcription) or CHX (inhibitor of translation), we pretreated cells with ActD and CHX before adding cytokines. Still, no cytotoxic effect of the cytokines was noticed. One possible reason for insensitivity of oligodendrocytes to TNFα and IFNγ might lie in the culture system itself, where 30 % of all cells are oligodendrocytes, surrounded by mainly microglia and astrocytes. Astrocytes are able to release growth factors, e.g. PDGF and TNFα was shown to stimulate PDGF-production (Silberstein *et al*., 1996). PDGF was described to protect oligodendrocytes from chemically induced cell death *in vitro,* and thus might probably prevent also cell death induction by TNFα and IFNγ (Fressinaud *et al*., 1996). Further, the relevance of *in vitro* data in this field has to be evaluated considering reports about differential expression of cytokine receptors *in vitro* and *in vivo* (see 1.2.3.). Although cytokine toxicity might be observed in some cases *in vitro*, it has still to be shown, whether cytokine-receptors are functionally expressed in oligodendrocytes under conditions of demyelination.

A similar result was obtained when oligodendrocytes were stimulated with agonistic anti-Fas-antibody or with soluble Fas-ligand. Neither treatment with anti-Fas-antibody or soluble Fas-ligand alone, nor combination with ActD or CHX induced cell death in oligodendrocytes. This findings correspond to a report, where rat oligodendrocytes were not sensitive for Fas-mediated cytotoxicity (Malipiero *et al*., 1997). Also if we do not know, if oligodendrocytes in our culture system really express CD95, it was shown before, that cells carrying the receptor are not necessarily sensitive to CD95-induced death (Becher *et al*., 1998). It is not clear yet, whether CD95 might really play a role for oligodendrocyte death during inflammatory demyelination (see also 1.2.2. and 1.2.3.). Several studies have demonstrated the mitigation of

EAE in mice defect in Fas- or FasL-expression (Dittel *et al.*, 1999; Sabelko *et al.*, 1997; Waldner *et al.*, 1997), but EAE-induction is still possible in these mice with disease scores reaching eventually high levels (Malipiero *et al.*, 1997). The Fas/FasL-system might have more importance for the initiation of the disease and down-regulation of the immune response than for demyelination and oligodendrocyte death (Sabelko-Downes *et al.*, 1999).

Despite the fact that no final inducer of oligodendrocyte death has been identified until now, one conclusion can be drawn from the results described above. Inflammatory cytokines and the Fas/FasL-system are not necessarily the key mediators of oligodendrocyte death. Similar to cytokines, NO has been discussed controversially and was several times presumed to be deleterious for oligodendrocytes, but seemed to have also protective effects (reviewed in (Parkinson *et al.*, 1997; Smith *et al.*, 1999)). Rather, these mediators represent constituents of a redundant system with many components, like cytokines, NO, autoantibodies, infiltrating lymphocytes, activated phagocytosing microglia, and macrophages. New components of this complex system contributing to demyelination are discovered continuously, like glutamate that was found to be toxic in oligodendrocytes. Lately it has been shown that blockage of glutamate receptors mediated protection from demyelination in EAE (Pitt *et al.*, 2000). It may be, that not single factors, but a combination of them is responsible for oligodendrocyte damage. But it has to be considered, that also other, not yet described mechanisms or mediators, can contribute to demyelination.

4.3. Ganglioside-induced oligodendrocytotoxicity *in vitro*

After investigating about cytokine-toxicity on oligodendrocytes, we focussed our attention to gangliosides and their effects on glial and neuronal cells. Changes in the ganglioside content of the CNS have been described for several types of neurodegeneration, e.g. Tay-Sachs and Sandhoff diseases, caused by deficiency of GM2-metabolising hexosaminidase and subsequent lysosomal accumulation of the ganglioside (Huang *et al.*, 1997). Demyelinating diseases with documented changes in ganglioside patterns are Niemann-Pick Disease, Guillan-Barrè syndrome, leukoaraiosis and multiple sclerosis (Hughes *et al.*, 1999; Miyatani *et al.*, 1990; Tarvonen-Schröder *et al.*, 1997; Vanier, 1999; Yu *et al.*, 1974). So far, elevated levels of gangliosides have been considered as diagnostic markers of disease course, but no studies about a potential toxicity of gangliosides on brain cells had been performed.

4.3.1. *GD3 induces oligodendrocyte death* in vitro *with high specificity*

Gangliosides were reported before to be increased in conditions of demyelination, and might therefore be interesting for pathogenesis. Disialoganglioside GD3 was the first ganglioside shown to be involved in intracellular cell death signalling (De Maria *et al.*, 1997). Apoptosis induction by GD3 was demonstrated on the lymphoma cell line Hut78, the U937 myeloid leukemia cell line, primary hepatocytes and the MH1C1 hepatoma cell line (De Maria *et al.*, 1997; Petronilli *et al.*, 1999; Scorrano *et al.*, 1999b).

Starting from these findings, we tested the effect of various gangliosides on brain-derived cells. Oligodendrocytes were heavily damaged by GD3, while GM3, GD1a and GD1b did not induce cell death in the same mode. This excludes damage of oligodendrocytes by GD3 via an unspecific detergent effect, as such would have been elicited in the same way by the structurally related gangliosides GM3, GD1a and GD1b (for structures see 1.4.1). High cell type specificity of GD3 on brain derived cells was unveiled by comparing the reaction to GD3 of astrocytes, microglia, cerebellar granule neurons, and oligodendrocytes. Oligodendrocytes were the only cell type being killed within a time frame in which GD3 did not induce any degeneration in microglia and astrocytes and only a small amount of cell death in CGC. The latter were investigated closer by incubation with higher concentrations of GD3. Nevertheless, GD3 did not induce cell death in CGC with an intensity comparable to the effect on oligodendrocytes, proposing relative insensitivity of CGC to GD3. Similar to the insensitivity of astro- and microglia, none of the cell lines tested (HeLa-229, Wehi-S, O4CB-neu) were killed by GD3. This result is interesting in particular for O4CB-neu, a cell line generated by immortalising oligodendrocyte progenitors. This could be a sign of differential GD3-sensitivity of immature compared to mature oligodendrocytes. To confirm this possibility, experiments with primary immature oligodendrocytes will have to be carried out in the future.

The observation of GD3-toxicity in oligodendrocytes rises the question, why cells that are full of GD3 during their development, become sensitive for the same substance after maturation. Yet, no definite answer has been found, but similar examples for differential effects of the same molecule have been reported. Ceramide liberated by the activity of neutral sphingomyelinase is not sufficient to trigger cell death in lymphoma-like cells. Activation of acidic sphingomyelinase is necessary to induce apoptosis (De Maria *et. al.*, 1998). It seems likely that not a certain substance itself, but its intracellular compartimentalisation decides, which reactions are induced in the cell. Analogies exist in proteins, that initiate fatal sequences of events after being delocalised from one compartment to the other (e.g. cyt *c*, from the mitochondrion to the cytosol, see 1.3.1.). Little is known about the targeting of lipid molecules to organelles and their transport between the compartments. Therefore, one interesting topic to investigate in the future is the intracellular distribution of GD3 and other lipid molecules.

After the first results about GD3-toxicity on oligodendrocytes, a closer examination of the GD3-effect was begun. Bovine brain-derived GD3 preparations were obtained from a commercial source without an exact analysis of contents provided by the supplier. To exclude artefacts due to eventual ceramide contaminants in the preparation, bb GD3 was subjected to GC-MS. No relevant amounts of different ceramide-subtypes were detected proving that oligodendrocytes incubated with GD3 were not killed by ceramide contamination. The presence of LPS in the commercially available GD3 was excluded next. LPS contamination of GD3 might have implicated the risk of microglia activation in the enriched oligodendrocyte culture and an indirect effect: activated microglia could have attacked oligodendrocytes directly or by release of cytokines or reagent oxygen species. However, no LPS was detected in the two GD3 lots tested. A small amount of LPS was found in the GD1b preparation, but did not result in particular toxicity of GD1b to oligodendrocytes.

Direct comparison of chemically synthesised – virtually pure – and bb GD3 finally excluded an artefact effect in two different experimental systems. First, synthetic and bb GD3 were tested on oligodendrocytes in the same incubation. Both GD3 preparations were equally potent in inducing degeneration of oligodendrocytes. The morphologic changes (process retraction, nuclear condensation) caused by both GD3 preparations did not differ. In order to compare the two preparations of GD3 in another experimental set up, isolated liver mitochondria were chosen. GD3 was discovered already before to induce uncoupling of mitochondria, probably by opening of the mitochondrial permeability transition pore (Garcia-Ruiz *et al.*, 2000; Kristal and Brown, 1999; Rippo *et al.*, 2000; Scorrano *et al.*, 1999b). Synthetic and bb GD3 behaved identically inducing enhanced mitochondrial respiration. The inhibition of the uncoupling effect elicited by synthetic or bb GD3 by CsA gave additional evidence for the specific action of GD3 on mitochondria. The only difference between synthetic and bb GD3 was found in the concentrations necessary to damage oligodendrocytes. To obtain an equivalent effect of synthetic and bb GD3 on oligodendrocytes, slightly higher concentrations of synthetic GD3 in comparison to bb GD3 had to be used. This difference might be caused by the different content of lipid residues in bb GD3 and synthetic GD3. In bb GD3 a mixture of fatty acid residues forms the lipophilic moiety, while synthetic GD3 contains uniform fatty acid residues (C18). The latter might form micelles more easily than a mixture of lipophilic residues with different length and saturation level of the carbon chain. After prolonged incubation of GD3 in an aqueous environment, uniform lipophilic GD3-molecules might form micelles, reducing in this way the effective free concentration of ganglioside. Fewer molecules of GD3 would reach the cells at a given concentration of synthetic GD3 compared to bb GD3. This effect was not observed in the experiments measuring mitochondrial respiration. Probably sonication of the ganglioside stock solution immediately before the experiment and the relative short duration (20 min) of the experiment compared to the longer incubation periods in cells did not permit the formation of micelles.

The last attempt to ensure specific action of GD3 on oligodendrocytes was made by comparing biologic GD3 from two different sources. GD3 derived from bovine milk was equally effective as bb GD3 in triggering oligodendrocyte death. This emphasised again that no other brain-derived substance, but GD3, was responsible for cytotoxicity.

It was shown by different approaches, that GD3 is able to kill oligodendrocytes *in vitro* with high specificity and that GD3 and not any other component of the biological preparation is responsible for this effect. Oligodendrocytes were the brain-derived cells with the highest susceptibility to GD3 induced degeneration. Until now, gangliosides, were known because of their anti-apoptotic effects (Koike *et al.*, 1993; Ryu *et al.*, 1999). Our results propose a new role for gangliosides, especially for GD3, in pathogenesis.

4.3.2. *Apoptotic features of GD3 induced oligodendrocyte death*

The shape of oligodendrocyte death has been described both as apoptotic or "lytic"/necrotic *in vivo* and *in vitro* (see 1.3.2.). GD3 induced oligodendrocyte death in a relatively slow mode, beginning with degeneration of membrane processes. Subsequently, connections between cell body and network were lost. Changes in the membrane composition became obvious by positive phosphatidylserine-staining on the outer leaflet of the plasma membrane. Interestingly, many control oligodendrocytes in different cell preparations showed some PS-staining in the periphery of their process network. Especially cells that had already built up a complex network structure (comparable to the cells at 6 DIV, Fig. 6G) exposed PS on the peripheral cell membrane in a ring-like-shape. As oligodendrocytes were described to synthesise myelin-like structures *in vitro* (D'Urso *et al.*, 1997; Solly *et al.*, 1996), PS-staining might occur in membrane-areas with high synthetic activity and turnover of membranes. PS-staining changed after stimulation of oligodendrocytes with GD3. PS-exposure increased on the cell membrane, until the whole network and cell body were stained. PS-positive cells kept membrane integrity over 12 to 18 h, evidenced by the late increase of cells stained by an impermeable DNA-dye. Maintained impermeability of cell, together with PS-exposure are arguments indicating an apoptotic cell death mechanism elicited by GD3 (Fadok *et al.*, 1992; Martin *et al.*, 1995).

Support for this hypothesis comes from the shape of the observed nuclear changes. GD3-challenged cells underwent typical changes in chromatin formation. In control cells, chromatin was homogeneous in the nucleus, with lucent appearance of nucleoli. In GD3 treated oligodendrocytes, chromatin was first clumping together, while the nuclear size was already slightly decreased. In late phases of apoptosis, DNA was hyperchromatic (highly fluorescent clumps at the rim of the nucleus) or condensed to one or few fragments, and the regular round shape of the nucleus was lost. This kind of nuclear transformation is a typical feature of apoptosis.

4.3.3. *Activation of caspase-3 during GD3-induced apoptosis*

GD3 was mainly investigated before in its role as intracellular mediator, but less as intercellular signalling molecule. In oligodendrocytes we were interested to investigate, which intracellular mechanism was induced by GD3. Therefore, the involvement of caspase-3, as representative downstream caspase, and of mitochondria was studied.

The role of caspases was examined by two different approaches. First, the influence of caspase-inhibitors on GD3-toxicity was looked at, using zVAD-fmk, DEVD-CHO and YVAD-cmk. ZVAD is used as general caspase-inhibitor, while DEVD-CHO interferes with the activity of caspases-3, 7, and 8. YVAD preferentially inhibits caspase-1 (Hisahara *et al.*, 1997). Cell death was neither prevented nor delayed by any of the mentioned inhibitors. In a second attempt, activation of caspase-3 was determined by immunostaining with an antibody selective for the proteolytically active processed caspase-3. Caspase-3 is known to become activated by caspase-9-catalysed cleavage. Staining for active caspase-3 was positive in those GD3-treated oligodendrocytes, exhibiting an advanced state of chromatin condensation. Staining was not observed in oligodendrocytes with early changes in chromatin. All oligodendrocytes pretreated with zVAD were negative for active caspase-3, evidencing penetration of zVAD into cells. It could be argued that low levels of caspase-3 activation might not be detected by the immunostaining. This possibility cannot be excluded totally. But in CGC treated with colchicine, zVAD abolished staining for active caspase-3 (E.Fava, personal communication), and at the same time prevented cell death, as apoptosis induced by colchicine is caspase dependent. This indicates that GD3-activated caspases might not be the only element in the cell death mechanism.

GD3 is known to induce liberation of cyt *c*, AIF and other proteins from mitochondria (Rippo *et al.*, 2000). Caspase-activation following GD3-treatment in cells might be due to cyt *c*-release from mitochondria and subsequent formation of the apoptosome (see 1.3.1.). Blocking of caspase-activity by zVAD or other inhibitors was reported before, not to prevent cell death, but to change features of cell death (Daugas *et al.*, 2000). Especially, condensation of chromatin was arrested in an early phase. This observation was confirmed in the enriched oligodendrocyte culture. Caspase-inhibitors did not influence commitment of oligodendrocytes to die, but changed the cell death pattern. Chromatin was not completely condensed to clumps, but remained in a state of hyperchromasy, concentrated at the borders of the nucleus. Probably, the inhibitors inactivate specific proteases responsible for the final break down of nuclear structure proteins and chromatin is impeded to be transformed into a compact mass. Additionally, caspase-inhibition caused a delay in secondary lysis of oligodendrocytes. This might be as well a consequence of caspase inhibition, but the effect was only observed at early and not at late time points.

ZVAD was entering GD3-treated cells but did not protect from toxicity. From this finding we concluded, that caspases are not the only relevant death pathway in GD3-triggered oligodendrocytotoxicity. Caspases had been considered for some time to be absolutely essential parts of the cell death machinery. This opinion has been overcome by various models of caspase-independent apoptosis (Borner and Monney, 1999; Dumont *et al*., 2000; Green and Kroemer, 1998).

4.3.4. *Role of mitochondria during GD3-induced apoptosis*

GD3 was described as final component of the ceramide signalling-pathway activated by CD95-stimulation (De Maria *et al*., 1997). Subsequent to this first report, the intracellular effects of GD3 were studied intensively. GD3 was demonstrated in several studies to directly influence mitochondria. GD3, but not structurally similar gangliosides, was proposed to induce the mitochondrial permeability transition (Garcia-Ruiz *et al*., 2000; Kristal and Brown, 1999; Pastorino *et al*., 1999; Petronilli *et al*., 1999; Rippo *et al*., 2000; Scorrano *et al*., 1999b). Permeability transition is a process observed during apoptosis, when the mitochondrial inner membrane, which is characterised by highly selective permeability to solutes, undergoes important changes causing characteristic features: swelling of mitochondria due to imbalanced ion distribution (Vander Heiden *et al*., 1997); dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) (Zamzami *et al*., 1996); induction of the release of proteins, that are localised in the matrix or intermembrane space of mitochondria ("proapoptotic" proteins, cytochrome *c*, apoptosis inducing factor (AIF) (Daugas *et al*., 2000; Lorenzo *et al*., 1999), adenylate kinase (Single *et al*., 1998), procaspases-2 and 9 (Susin *et al*., 1999a; Susin *et al*., 1996)), to the cytosol.

Several theories try to explain the phenomenon of increased mitochondrial permeability (reviewed in (Green and Kroemer, 1998; Kroemer and Reed, 2000)). One favours to explain mitochondrial changes by conformational alterations in the MPTP complex. MPTP is a protein complex localised in the mitochondrial contact sites. It consists mainly of VDAC (voltage dependent anion channel, porin, outer membrane), ANT (adenin nucleotide translocase, inner membrane), and associated proteins: mitochondrial creatine kinase, peripheral benzodiazepine receptor, hexokinase II, cyclophilin D. Conformational changes of this complex are believed to be induced by altered interactions between the complex components. Disturbance of the complex causes formation of pores, and subsequent loss of $\Delta\Psi_m$, release of proapoptotic proteins, and induction of apoptosis. According to one theory, one possibility is interaction between ANT and cyclophilin D (CyD) (Halestrap, 1999). ANT normally functions as specific antiporter transporting ATP in exchange with ADP. Binding of CyD converts ANT into an unspecific uniporter for molecules > 1500 Da, changing the permeability of the inner mitochondrial membrane and inducing probably the opening of the MPTP (Kroemer and Reed,

2000). Pore formation, release of proapoptotic factors and propagation of apoptotic mechanisms is prevented by binding of CsA to CyD, impeding contact between ANT and CyD or by bonkrekic acid (BA), that directly stabilises ANT in its closed conformation. Other regulators of MPTP are members of the Bcl-2-family of proteins (e.g. Bax, Bcl-2, Bcl-X_L) (Marzo *et al*., 1998a; Marzo *et al*., 1998b; Tsujimoto and Shimizu, 2000).

As discussed above, GD3 was studied intensively for its effect on mitochondria. The control of GD3-induced mitochondrial changes by Bcl-2 has been shown (Rippo *et al*., 2000). Before, it had been described, that Bcl-2 can inhibit the release of mitochondrial proteins, like cyt *c* and AIF (Susin *et al*., 1996; Yang *et al*., 1997). *Bcl-2*-overexpression in cells was preventing apoptosis, loss of $\Delta\Psi_m$, production of reactive oxygen species and activation of caspase-9 (Ghafourifar *et al*., 1999; Rippo *et al*., 2000). In our *in vitro* culture of oligodendrocytes, we observed GD3-induced mitochondrial changes similar to the ones described above. Mitochondria released cyt *c* to cytosol and nucleus and completely lost membrane potential. The protection of *bcl-2*-overexpressing oligodendrocytes from GD3-induced death observed in our system is perfectly in line with recent findings (Rippo *et al*., 2000). Therefore, in oligodendrocytes GD3 might be taken up into the cell without further metabolisation and might be targeted directly to mitochondria.

AIF was shown before to be one of the proapoptotic factors released from mitochondria. It was also demonstrated, that AIF induces DNA-condensation in isolated nuclei, causing incomplete fragmentation, but DNA-condensation at the nuclear periphery (Susin *et al*., 1999b). The general caspase-inhibitor zVAD was not inhibiting AIF-release from mitochondria in STS-triggered apoptosis, and chromatin condensation was arrested at an early stage (Daugas *et al*., 2000). Whether AIF is involved also in GD3-induced apoptosis in oligodendrocytes, will be matter of future studies.

Although CsA and BA are known to interfere with MPTP-opening, and to prevent apoptosis in many cases, no substantial protection from GD3 was observed in our system with CsA and no effect at all with BA. One possible reason might be the pronounced sensitivity of oligodendrocytes for both substances. The particular sensitivity of oligodendrocytes for CsA was reported before (McDonald *et al*., 1996). CsA and BA alone induced oligodendrocyte death within 24 h in concentrations higher than 2 µM and 50 µM, respectively. CsA is known to have additional effects other than binding to CyD, like interaction with intracellular transport, inducing intracellular calcium increase, and inhibition of ATP-dependent export carrier (Bohme *et al*., 1994; Gordjani *et al*., 2000; Kusunoki *et al*., 1998). Probably, such an effect might negatively affect oligodendrocytes and cause cell damage in concentrations that are necessary to block the opening of the permeability transition pore.

Caspases have been demonstrated to be involved in GD3-induced oligodendrocyte death, but seem to play a secondary role in cell death execution. We have also found a protective effect of the unspecific protease-inhibitor leupeptin, which might indicate the activation of other subclasses of proteases. In contrast, mitochondria are an important check point in GD3-toxicity and maybe the only control and intervention point. Future investigations will have to address the possibilities to intervene with GD3 toxicity, either by modulating the MPTP or by abrogating the activity of yet unknown proteases involved in GD3-mediated oligodendrocyte apoptosis.

4.4. Release of GD3 by microglia

Microglia cells are strongly involved in the pathogenesis of demyelination. Activated by cytokines like TNFα and IFNγ, they are able to release inflammatory cytokines, NO, and other reactive oxygen species (see 1.2.4.). Resident directly in the brain, microglia have important functions during development, when clearance of in excess created, apoptotically dying cells is necessary. In embryonic rats, ameboid – i.e. activated- microglia cells were found in regions of oligodendrogenesis, probably taking part in phagocytosis of myelin debris (Ellison and de Vellis, 1995; Milligan *et al*., 1991). In response to demyelination induced by ethidium-bromide-injection into the ventricle, microglia cells became activated (Reynolds and Wilkin, 1993). In both conditions of microglia activation, GD3 was detected in microglia (Ellison and de Vellis, 1995; Reynolds and Wilkin, 1993).

Also in the enriched cultures of oligodendrocytes, different cell types were positive for GD3. Not only some CNPase-positive cells were stained for GD3, but also cells with microglia-morphology, giving evidence for the potential of microglia cells *in vitro* to synthesise GD3.

In order to simulate inflammatory conditions in isolated cultures of microglia, cells were challenged with LPS with and without IFNγ, or with inactivated *E. coli*. Analysing the cells by anti-GD3 immunostaining did not reveal any increase of intracellular GD3 in microglia. Probably, microglia might not accumulate GD3 intracellulary upon activation *in vitro*, but release the ganglioside. Similar problems to stain GD3 in ameobid microglia in culture were previously reported (Reynolds and Wilkin, 1993). In contrast, GD3 was clearly augmented in the supernatant of cells incubated with high concentrations of LPS or bacteria. Stimulating cells with low concentrations LPS or LPS *plus* IFNγ alone did not increase GD3. The reason might lie in the lower LPS-concentration used in the costimulation with IFNγ.

Although LPS and bacteria normally are not present during inflammatory demyelination, our data emphasise the possibility not only of GD3-production by microglia, but of ganglioside-release to the extracellular space. The GD3-concentrations in the SN reached up to 250 nM. In CSF of MS-patients concentrations up to 600 nM GD3 have been measured (Miyatani *et al*.,

1990). Which concentrations of GD3 can be reached in the tissue, is not known, but in the intercellular space GD3 might be more concentrated. Using more sensitive techniques in the future to measure gangliosides in SN or CSF might help to detect the presence of lower GD3-concentrations and elucidate dynamics of GD3 secretion.

4.5. GD3 as mediator in demyelinating diseases

In this study, disialoganglioside GD3 was demonstrated to be toxic for oligodendrocytes, damaging this cell type with high selectivity in comparison to other neural cells. Further, microglia activated by inflammatory stimuli were shown to increase GD3-production and to release GD3. These results document for the first time toxicity of a ganglioside released by activated microglia on oligodendrocytes.

Oligodendrocytes are target of the inflammatory attack against myelin during MS and other diseases of the white matter. The changes in ganglioside composition of brain tissue during demyelination, and elevated CSF-levels of GD3 in multiple sclerosis and leukoaraiosis (selective incomplete white matter infarction) suggest a role of gangliosides, especially GD3, in this conditions (Miyatani *et al.*, 1990; Tarvonen-Schröder *et al.*, 1997; Yu *et al.*, 1974; Zaprianova *et al.*, 1998). The proportion of GD3 in the ganglioside-fraction of white substance from human healthy adult brain is 4 % (Svennerholm *et al.*, 1994). In contrast, GD3 makes up about 30 % of the total ganglioside amount in CSF of selected patients suffering from MS (Miyatani *et al.*, 1990). It might be asked, if GD3 detected in CSF is brain-derived or if it increases in liquor because of leakage from the plasma through the blood-brain-barrier. Inflammatory conditions were shown to open the bbb, facilitating the passage of immune cells and mediators (Kermode *et al.*, 1990; Merrill and Murphy, 1997). GD3 was reported to be one of the most abundant gangliosides in human plasma (Kundu *et al.*, 1985) and might accumulate in CSF due to the existing concentration gradient. This possibility cannot be excluded, but strongly increased amounts of GD3 have been detected in tissue samples of sclerotic plaques (Yu *et al.*, 1974), indicating that GD3 in CSF is probably of tissue-origin and not blood-derived.

The substantial amounts of GD3 detected in CSF of MS victims might be produced by cells activated or damaged during inflammatory demyelination. Candidate cells that will be discussed below are: (i) lymphocytes undergoing apoptosis; (ii) regenerating oligodendrocytes; (iii) degenerating neurons; (iv) activated microglia cells.

(i) In lymphocytic cells, GD3 was described for the first time to accumulate in response to stimulation of CD95 (De Maria *et al.*, 1997). Following reports demonstrated, that GD3 is detectable in a variety of cells undergoing apoptosis, such as infiltrating T-lymphocytes during Hashimoto's thyroiditis and apoptotic colonocytes in Farber disease (Farina *et al.*, 2000; Stassi *et al.*, 1999). In experimental allergic encephalitis (EAE), massive apoptosis of brain-invading

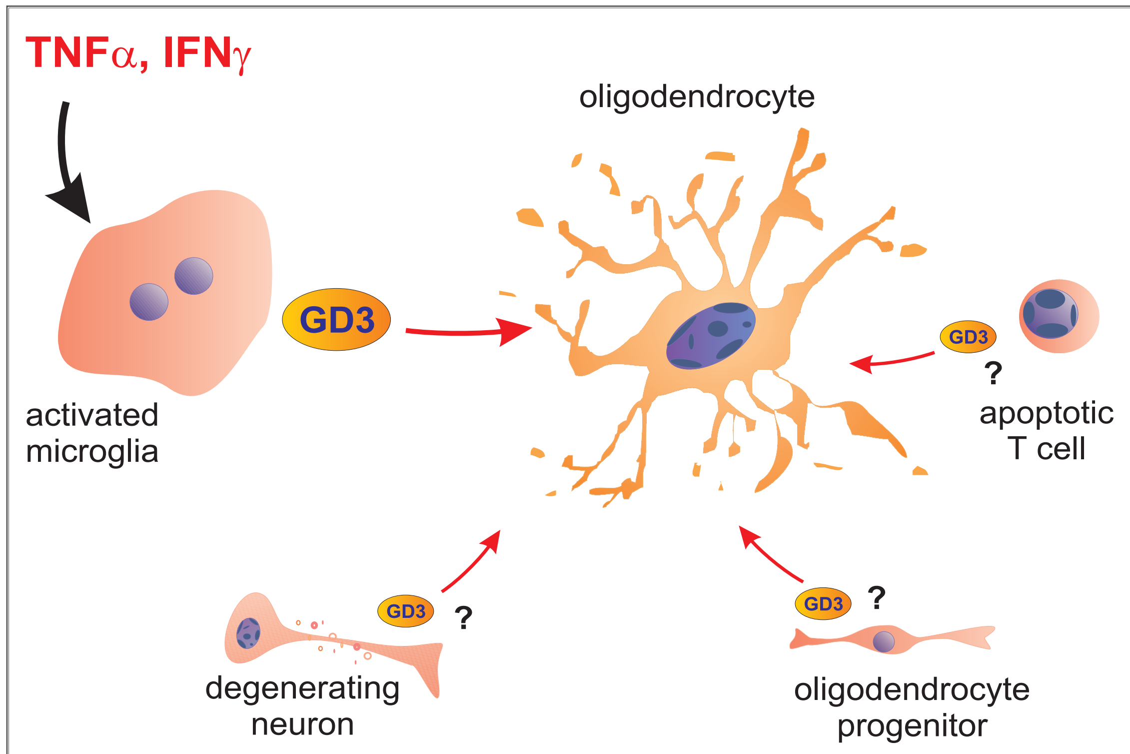

Figure 26: GD3 as mediator for demyelination.
GD3 is known to increase in Multiple Sclerosis. Possible sources for elevated GD3:
(i) Brain invasive lymphocytes that undergo apoptosis in EAE and MS in large scale. (ii) Progenitors of oligodendrocytes during phases of remyelination. (iii) Neurons degenerating in consequence to demyelination might release apoptotic bodies that contain GD3. (iv) Microglia cells in conditions of brain inflammation might be activated by inflammatory mediators to release GD3.

lymphocytes has been elucidated (Bonetti *et al.*, 1997; White *et al.*, 1998). CD95-dependence of cell death induced in these lymphocytes, was proposed (Sabelko *et al.*, 1997; White *et al.*, 1998), implicating the possibility of GD3 to accumulate in dying lymphocytes. Apoptosis is commonly believed to occur rapidly and by fast elimination of apoptotic cells by phagocytosis (Savill, 1997). Nevertheless, conditions of fulminant apoptosis, like in EAE, might hinder timely elimination of dying cells, leading to loss of membrane integrity and to liberation of cell contents probably containing GD3 (Savill and Fadok, 2000).

(ii) Episodes of demyelination are followed by regenerative phases in relapsing-remitting MS and in monophasic EAE (Brown *et al.*, 1982; Miller *et al.*, 1995; Scolding and Lassmann, 1996). Remyelination was shown to start from oligodendrocyte progenitors differentiating to mature cells (Wolswijk, 2000). Oligodendrocyte precursors were already described before to contain GD3 (see 1.1.). They were identified by positive GD3-staining in remyelinating white matter lesion (Reynolds and Wilkin, 1993). GD3 was detected in CSF of patients in stationary progressive and active progressive MS. Unfortunately, no data exists from patients with relapsing-remitting MS in the recovery phase. If GD3 should be derived from regenerating oligodendrocytes, GD3 content in CSF should be expected to be higher during remyelination, than in conditions of active demyelination, as mature oligodendrocytes do not contain GD3.

Investigations of the GD3-content in CSF and tissues of patients in acute and remitting phases of MS have to be performed to rule out a possible correlation of remyelination and elevated GD3-levels.

(iii) Neuronal plasma membranes are rich in gangliosides as gray matter contains gangliosides in a higher amount than white matter (Kracun *et al*., 1984). GD3 is found in some neuronal populations, like Purkinje cells, granule cells and basket cells of the cerebellum (Kawai *et al*., 1994; Molander *et al*., 2000), and retina cells (Dreyfus *et al*., 1997). During demyelination, initially oligodendrocytes are damaged, but with progressing disease also neurons are affected and degenerate (Trapp *et al*., 1998). If demyelinating plaques should occur in the cerebellum, GD3 might be liberated from damaged neurons. Cells dying apoptotically were described to release plasma membrane vesicles, the so-called "blebs". However, apoptotic debris is usually not just released but supposed to be phagocytosed quickly in order to avoid the release of cell contents and subsequent inflammation (Ren and Savill, 1998). For this reason, GD3 contained in apoptotic blebs of neuronal origin is not very likely to contribute to elevated GD3 levels in CSF and MS plaques.

(iv) Microglia cells have been discussed already in detail above as important components of the pathogenetic mechanism in demyelination. We have demonstrated the ability of microglia cells to synthesise GD3 *in vitro* after activation by inflammatory stimuli. Other studies found GD3-staining in activated microglia during demyelination. GD3 might also be the "missing link" explaining findings about microglia-mediated oligodendrocyte death *in vitro* (Vartanian *et al*., 1995). In the latter study, microglia cells were pretreated with IFNγ for 24 h, washed thoroughly and cocultured with oligodendrocytes for 48 h. After this time, 30%-40% of oligodendrocytes were killed. This effect was reversed by an anti-IFNγ-antibody, but not by an inhibitor of NO-synthase. In this way, NO was excluded as mediator of oligodendrocyte death. The authors argued, that IFNγ-pretreatment either induced further IFNγ-synthesis by microglia or that IFNγ adhering to cells might be released during the time of coculture. In this way, IFNγ would have killed oligodendrocytes directly. The possibility of IFNγ-production by microglia is not very realistic, because IFNγ is known to be produced by lymphocytes, such as T-cells and natural killer cells, but not by cells of the monocytic lineage (Billiau *et al*., 1998). Rather, we propose GD3-release by microglia cells stimulated with IFNγ. Such a mechanism would explain the relatively slow induction of oligodendrocyte death in this system. Also we observed that accumulation of GD3 in the SN was only reaching significant amounts after 24-72 h of incubation.

Concluding the findings about microglia *in vitro* and *in vivo*, we suggest GD3 as a mediator of oligodendrocyte damage in inflammatory demyelination (Fig. 26). GD3 is one of the gangliosides with the lowest representation in healthy brain. Pathologic GD3-elevation in

MS-plaques, CSF of MS-patients and EAE-brain has not yet been addressed to a defined source. Microglia cells stimulated by one of the numerous immune mediators present in inflammatory demyelination have the potential and the cellular machinery to synthesise and release GD3 to the extracellular space. In demyelination, microglia have the function to clear apoptotic debris and myelin remnants, getting into close vicinity of oligodendrocytes. GD3 release in this situation might trigger or deteriorate damage of oligodendroglia. In this way, GD3 might be essentially involved into the pathogenesis of inflammatory white matter diseases. Future investigations will have to elucidate sources of GD3 in pathology *in vivo*. By more profound study of model systems for demyelination, the role of GD3 for oligodendrocyte demise will have to be investigated. Disialoganglioside GD3 might be another piece of the mosaic presenting the complex pathomechanisms of multiple sclerosis.

5. SUMMARY

Oligodendrocytes are target of an autoimmune attack during demyelinating diseases, e.g. multiple sclerosis. Until now, the crucial mediator causing oligodendrocyte loss and myelin degradation has not yet been defined.

In order to investigate the degeneration and death of murine oligodendrocytes *in vitro*, a culture system enriched in oligodendrocytes was established, where the presence of other neural cells might resemble closely the cellular environment *in vivo.* An average of 30 % of all cells developed to mature oligodendrocytes, expressing myelin oligodendrocyte glycoprotein, the molecule known to be expressed in terminally differentiated oligodendrocytes.

In this culture, no sensitivity of oligodendrocytes to inflammatory cytokines or to stimulation of CD95, a receptor known to trigger apoptosis, was observed. Inhibition of m-RNA transcription (actinomycin D) or protein translation (cycloheximide) did not sensitise oligodendrocytes for the effect of cytokines or CD95-ligation. This result finds correlates and contrary examples in the literature and indicates that the mentioned stimuli are not the only ones involved in oligodendrocyte damage *in vivo*.

Althoug ganglioside levels are alterated in demyelination, the molecule class of ganglioside has not been studied before with regard to demyelination. Disialoganglioside GD3, but none of the structurally similar gangliosides, damaged oligodendrocytes with high cell type specificity. The effect was carefully studied with regard to possible contamination responsible for oligodendrocytotoxicity. Parallel evaluation of chemically synthesised and bovine brain-derived GD3 further ensured the purity of the effect.

GD3 was shown to induce apoptotic cell death in oligodendrocytes, causing typical changes like chromatin condensation, phosphatidylserine exposure, cytochrome *c* release from mitochondria, activation of caspase-3 and prolonged maintenance of cellular integrity. The involvement of caspases was found to be of minor importance in GD3 induced oligodendrocyte death, as their inhibition did not prevent cell death. Mitochondria are damaged during GD3-triggered apoptosis. GD3-caused oligodendrocyte death was inhibited in cells overexpressing a human transcript of *bcl-2*. We conclude that mitochondria play an essential role in GD3 triggered apoptosis.

The ability of microglia to synthesise GD3 *in vivo* was observed before. However, this study presents evidence that microglia cells are capable of releasing GD3 into the culture medium upon activation by inflammatory stimuli (lipopolysaccharide, inactivated *Escherichia coli*). This provides evidence for GD3 as a microglia-derived mediator during inflammation.

ZUSAMMENFASSUNG

Oligodendrozyten sind Ziel einer entzündlichen Autoimmunreaktion in demyelinisierenden Erkrankungen, wie z.B. Multipler Sklerose. Bisher ist unklar, welche Faktoren das Absterben von Oligodendrozyten und den Abbau von Myelin verursachen.

Um die Effekte entzündlicher Mediatoren auf Oligodendrozyten *in vitro* zu untersuchen, wurde ein Zellkultursystem aufgebaut, das mit den enthaltenen Zelltypen die Zellzusammensetzung im Gehirn wiederspiegelt. Durchschnittlich 30 % aller Zellen entwickelten sich in der Kultur zu reifen Oligodendrozyten und exprimierten MOG (ein Myelinprotein, das von terminal differenzierten Oligodendrozyten synthetisiert wird).

Oligodendrozyten wurden in dieser Kultur weder durch entzündliche Zytokine (TNFα, IFNγ) noch durch die Aktivierung des CD95-Rezeptors geschädigt. Die Inhibition von m-RNA- oder Proteinsynthese hatte keine sensitivierende Wirkung auf die Zellen. Ähnliche, aber auch gegensätzliche Ergebnisse finden sich in der Literatur und legen nahe, daß die genannten Faktoren nicht die einzigen sind, die an der Schädigung von Oligodendrozyten *in vivo* beteiligt sind.

Obwohl Gangliosidspiegel in demyelinisierenden Krankheiten verändert sind, wurde die Molekülfamilie der Ganglioside bisher nicht auf mögliche toxische Effekte in degenerativen Erkrankungen des Myelins hin untersucht. Es wurde gezeigt, daß nur GD3, aber nicht strukturell ähnliche Ganglioside, Oligodendrozyten mit hoher Selektivität bezüglich des Zelltyps schädigen. Diese Wirkung von GD3 wurde im Hinblick auf mögliche toxische Verunreinigungen der Substanz gründlich untersucht. Der Vergleich der Effekte von chemisch synthetisiertem mit aus Rinderhirn isoliertem GD3 erlaubte, den zytotoxischen Effekt von GD3 auf die reine Substanz zurückzuführen.

Es wurde ausserdem gezeigt, daß GD3 apoptotischen Zelltod in Oligodendrozyten auslöst. Charakteristika der von GD3 verursachten Zelldegeneration waren die Kondensation von Chromatin, das Erscheinen von Phosphatidylserin auf der Zelloberfläche, die Freisetzung von Cytochrom *c* aus Mitochondrien, die Aktivierung von Caspase-3 und die Erhaltung der Zellintegrität. Da die Inhibition von Caspasen keinen Schutz vor GD3 bewirkte, wird den Caspasen eine untergeordnete Rolle in der GD3-induzierten Apoptose zugeordnet. Mitochondrien wurden im Verlauf der GD3-induzierten Apoptose geschädigt. GD3-vermittelter Zelltod wurde durch die Überexpression eines humanen *bcl-2*-Gens verhindert. Daraus wird deutlich, daß Mitochondrien eine wichtige Rolle im GD3-induzierten Zelltodprogramm einnehmen.

In anderen Studien wurde gezeigt, dass Mikrogliazellen GD3 synthetisieren können. In der vorliegenden Arbeit wurde nachgewiesen, dass Mikroglia *in vitro* produziertes GD3 in das Kulturmedium freisetzen können, wenn sie durch entzündliche Stimuli (Lipopolysaccharid, inaktivierte *Escherichia coli)* aktiviert werden. Dies beweist, daß GD3 als entzündlicher Mediator von Mikrogliazellen in Frage kommt.

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