The effect of dopamine and its agonist pramipexole on oligodendrocytes in culture
and in the cuprizone mouse model

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Bremen

Göttingen 2013

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Chapter 1

Introduction

Oligodendrocytes of the central nervous system (CNS) belong to the family of glial cells, as which they are responsible for the maintenance of parenchymal homeostasis and formation of myelin (Figure 1.1). They are of fundamental importance for the development and integrity of the CNS as well as to the functionality of signal conduction (McTigue and Tripathi 2008). Their close interaction with neuronal cells is a topic of intense scientific discussion as there is evidence not only for the importance of neuronal signals to oligodendrocyte differentiation, but also for the support of neuronal development and synaptic stability and plasticity by oligodendrocytic signaling (Simons and Trajkovic 2006).
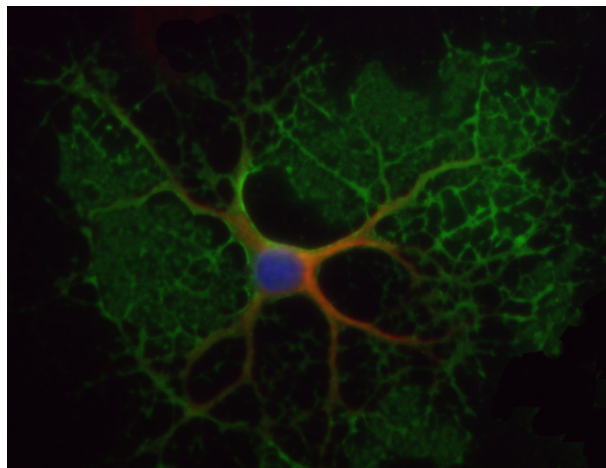
Figure 1.1: Rat primary oligodendrocyte in a mixed glial cell culture, fluorescence-stained with antibodies to myelin basic protein (MBP, green), tubulin (tub, red) and 4',6-diamidino-2-phenylindole (DAPI, blue), staining the nucleus.

1.1 Oligodendrocytes in the central nervous system

1.1.1 The origin and development of oligodendrocytes

Oligodendrocytes originate from a population of so-called oligodendrocyte precursor cells (OPC) that are located in distinct regions of the neural tube (Miller 2002). The development of spinal cord oligodendrocytes, for instance, emanates from ventral mid-line progenitors, as precursors of more rostral areas of the CNS are believed to migrate from the subventricular zone to their region of differentiation in white matter tracts (Kakita and Goldman 1999). Oligodendrocyte differentiation occurs in a temporal and spatial sequence some days prior to myelination and it involves the formation of distinguishable intermediate cell-types, such as the premyelinating oligodendrocyte (see Figure 1.2). It is also interesting to note that further development includes a 50 per cent chance of programmed cell death (apoptosis) for these cells, or, dependent on specific intercellular communication, the eminence of actually myelinating oligodendrocytes (Lazzarini 2004a).

For quite some time it has been known that OPCs have the potential for long-distance movement (Lachapelle et al. 1983) and it is understood that mature oligodendrocytes are not significantly mobile, which underlines the importance of early migration and directionality.

It has been suggested, that the population of oligodendrocytes in the CNS continues to increase in size during maturation and adulthood by ongoing cell divisions(Peters and Sethares 2004, Dawson et al. 2003, Nuñez et al. 2000) and OPCs react in a surprisingly flexible manner as a result to homeostatic changes, hormonal influence (Gregg et al. 2007) as well as diverse insults, such as oxidative stress (French et al. 2009). The ratio of OPCs to mature oligodendrocytes was determined to be 1:4 in the spinal cord tissue and 1:1 in the cerebral cortex and the hippocampus (Dawson et al. 2003), which implies multiple and asymmetrical divisions in case of tissue damage. Cerghet et al. (2006) propose a gender-dependent turnover of oligodendrocytes with a significant advance in females, whereas higher amounts of white matter and myelinated axons are detectable in male brains (Nuñez et al. 2000). The proliferation and migration of progenitors to replace lost cells in the adult organism is
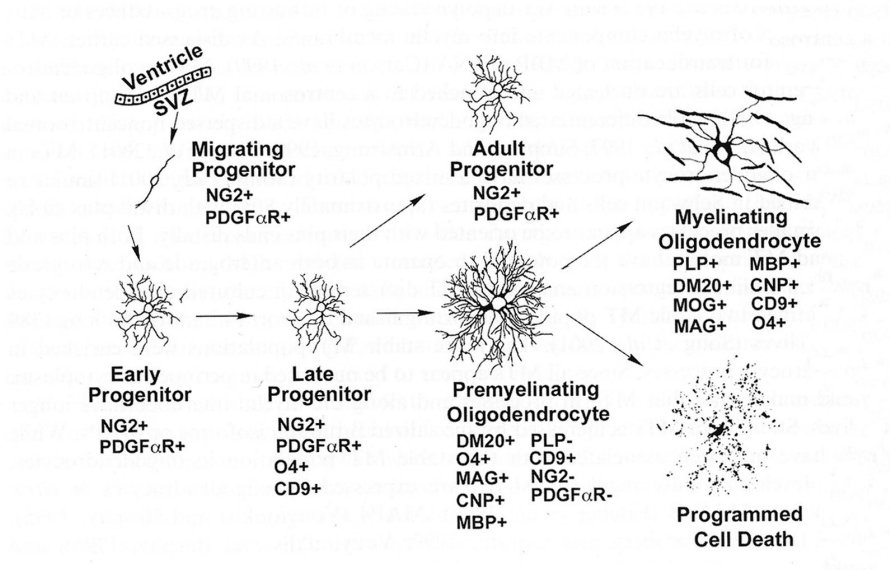
Figure 1.2: Development, maturation and differentiation of oligodendrocytes in the CNS. Oligodendrocytes migrate from distinct regions, i.e. the subventricular zone, to developing white matter areas where they differentiate and myelinate axons or die a programmed cell death. There are typical markers (receptors and membrane proteins) at each state of differentiation. Image from Lazzarini (2004a, p. 42).

facilitated by complex intercellular communication via chemoattractants and chemorepellants (McTigue and Tripathi 2008) and constitutes one of the major regenerative processes and an important form of plasticity of the central nervous system.

1.1.2 The function of oligodendrocytes

Myelination of axonal tracts in the CNS is undertaken by oligodendrocytes (Peters 1960). Cellular extensions ensheath axons to allow a fast, saltatory impulse propagation. Oligodendrocytes can enwrap up to 60 segments of axons. An integral part of their function is to optimize nerve cell signaling in this manner and furthermore lies in their active promotion of ion channel clustering at the nodes of Ranvier which are located at the interspace between two segments of myelin (Dupree et al. 2005).

It is now also widely accepted that oligodendrocyte functions by far exceeds conduction-insulation and facilitation of the development of membrane micro-environments. Evidence has accumulated that there is close interaction between neuronal and glial cells and that oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system

(PNS) are primarily responsible for axonal integrity (Nave and Trapp 2008, Kassmann and Nave 2008). Several pathologies with primary glial damage also exhibit profound axonal damage. Amongst these are hereditary spastic paraplegia (HSP/SPG, Edgar and Nave (2009)) and multiple sclerosis (MS, Irvine and Blakemore (2008)).

1.2 Myelin in health and disease

When Rudolf Virchow discovered myelin as a substance with abundant presence in the nerve tissues of mammals in 1854, he could not have foreseen the effect his discovery would have on the understanding of nervous system function. Since the publication of his descriptions (Virchow 1854), 150 years have passed and myelin today is one of the most researched substances of the nervous system. At the same time it is posing countless questions as to its development and reactivity, to its function and especially to its numerous related pathologies. The most well-known pathology, thought to be primarily caused by the damage of myelin, is multiple sclerosis (MS), which was as such described by Jean-Martin Charcot, only fourteen years after the description of myelin (Charcot 1868), although there have been earlier clinical reports (Landtblom et al. 2009).

1.2.1 Myelin in the central nervous system

Classical neuroanatomy morphologically distinguishes between the white and gray matter. While the gray matter is mainly comprised of neuronal cells and their dendrites, white matter contains myelinated axons, glial cells and blood vessels (Siegel et al. 1999). About 50% of white matter dry weight is provided by myelin itself, which consists of up to 70% of lipids and 30% of protein.

The oligodendrocytic membrane extensions construct central myelin sheaths in a series of complex branching, extensions and wrappings around axons (figure 1.3). Myelin contains high quantities of cholesterol and phospholipids. Myelin formation is achieved by the extrusion of excess cytoplasm to form a dense and spirally arranged compaction of the opposed membranes. This so-called compact myelin microscopically yields a characteristic

periodicity of major dense lines and intraperiod lines formed by fusion of cytoplasmic and extracellular leaflets of oligodendrocytic membranes. Specifically oriented myelin proteins are the myelin basic protein (MBP), the proteolipid protein (PLP), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), the myelin-associated glycoprotein (MAG) and the myelin-oligodendroglial glycoprotein (MOG), all of which seem to play substantial roles for the development and integrity of myelin. They also are associated with several pathologies (Lazzarini 2004b).

Myelination in the growing CNS is controlled by a large number of factors amongst which the direct neuron-glia contact has a significant role. It has been postulated that the myelin sheath protects the growing and mature axon, and it is known that the axon itself determines the extent of its myelination in a direct proportionality to its own thickness. The so-called g-ratio (the ratio of axon plus myelin sheath diameter to axon diameter) usually has a value between 0.6 and 0.7. Thicker axons therefore have thicker myelin sheaths. In the PNS a specific signaling molecule of the neuron, neuregulin, has a considerable influence on the regularity of this ratio (Michailov et al. 2004). Thus, there are characteristic communication mechanisms in the development of myelin.

In mice, myelination has been studied extensively. First sheaths appear at postnatal day 11 and a peak of myelination velocity is reached before the age of seven weeks. However, there is evidence for active myelin formation even after eight months (Sturrock 1980). As will be described later (see section 1.2.2), an important feature of oligodendrocytes and Schwann cells is their high turnover of myelin and their capability of regenerative potential during later stages of the organism's development and adult life.

1.2.2 Myelin pathology - multiple sclerosis, a neurological disorder of great socioeconomic importance and subject of intense scientific research

Multiple sclerosis (MS) is a complex and heterogenous disease affecting the CNS by inflammation of white matter, demyelination and axonal damage.

It is considered to be an inflammatory autoimmune disease affecting the central nervous
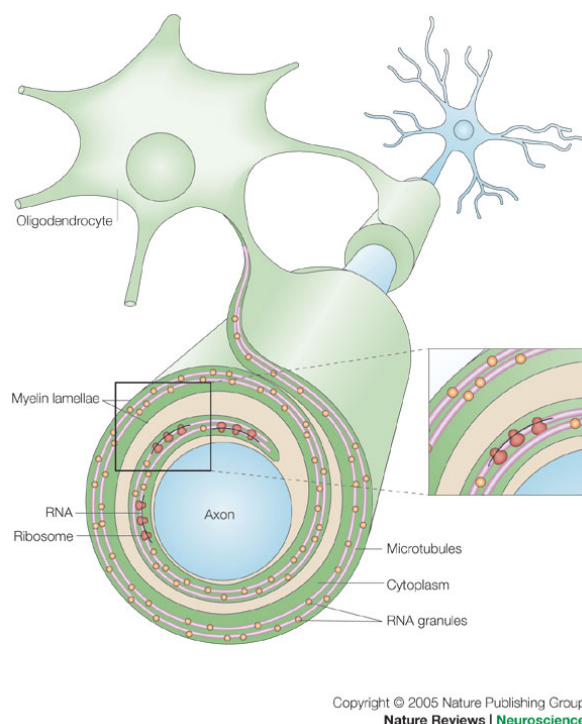
Figure 1.3: Axon ensheathment with myelin by an oligodendrocyte. Intense neuron-glia communication determines the extent and localization of axon myelination. The myelin itself has protective function for the axon. Note that for demonstration only one axon is ensheathed by the oligodendrocyte, while up to 60 segments of different axons can be served by one cell. From Sherman and Brophy (2005, p. 685).

system (CNS) by formation of multifocal demyelinated lesions accompanied by axonal damage (Kornek et al. 2000). Epidemiological studies reveal that it is the most common chronic neurological disorder leading to disability in early to middle adulthood in western societies (Hafler et al. 2005). The disease has major sociologic impacts through promotion of disabilities of the motor-, sensory- and autonomic nervous system and its socioeconomic impact can not be neglected – recent studies estimate 400,000 MS patients in Europe (Rotstein et al. 2006).

Clinical appearance and diagnosis

There are at least three clinical courses, that have been described for MS: relapsing-remitting (RR) MS, which is the most common form (85–90%), characterized by acute episodes of neu-

rological symptoms followed by complete or partial recovery (Sospedra and Martin 2005). Most of the RR-MS patients develop a secondary-progressive (SP) form of MS with steadily decreasing neurological abilities. 10 – 15 per cent of MS patients initially present with a primary-progressive (PP) subtype of MS at disease onset (Friese et al. 2006).

Typical predilection sites of the demyelinating inflammatory lesions are the periventricular corpus callosum, the optic nerve and the spinal cord. Nevertheless, practically any symptom in relation to CNS damage may occur, because lesions can also appear in atypical regions of the nervous system. The first clinical manifestations are typically associated with blurred vision due to optic neuritis and ophthalmoplegia. Other symptoms include diverse sorts of sensory and motor function impairment, as well as dysarthrophonia and cognition. Plegia and spasticity often cause a disability to walk and diffuse pain may induce intense suffering in attained patients (Poeck and Hacke 2007).

There are detailed accounts of many of these symptoms, occuring presumably in correlation with the disease, from as early as the beginning of the nineteenth century by Sir Augustus Frederick d'Este, a decendent of the British King, George III (Firth 1941, Landtblom et al. 2009).

Clinical findings continue to be an important pillar for the diagnosis of MS. It furthermore relies on the proof of a dissemination of lesions in both space and time, which can be achieved by modern magnetic resonance-based neuro-imaging (MRI), in which active and older lesions are easily identified. Generally accepted and internationally adapted criteria for the diagnosis of MS are now based on the McDonald recommendations of 2001 and revisions of 2005 and 2010 (McDonald et al. 2001, Polman et al. 2005, Polman et al. 2011). Accessory findings in the spinal fluid analysis with proof of intrathecal synthesis of high amounts of immunoglobulins and early pathological alterations of the visual evoked potential (VEP) are included in these criteria (Lazzarini 2004c).

Histopathology and pathophysiology

Histopathologically, MS can be categorized into four subgroups according to the immuno-pathology of lesions (Merkler et al. 2006). Pattern 1 plaques contain high densites of

macrophages and T-cells, apparently mediating demyelination. MS plaques pattern 2 are characterized by immunoglobulin and complement deposition, whereas pattern 3 lesions show dysmorph and apoptotic oligodendrocytes. Pattern 4 plaques present oligodendrocyte degeneration in a small rim of periplaque white matter. These subgroups can be distinguished inter-, but not intra-individually.

The etiology of MS remains a topic of intense discussion within the scientific community. It is believed that genetic risk factors as well as environmental effects contribute to disease development. Genetic susceptibility has been linked early on to specific genes on chromosome 6p21 (Sospedra and Martin 2005) and more recently to an even larger variety of familial variations within the proximity of the region of the major histocompatibility complex (International Multiple Sclerosis Genetics Consortium et al. 2011).

Although focal inflammatory lesions are typical for MS, a diffuse inflammation affects all of a patient's white matter and even grey matter regions. Tissue surrounding plaques is not completely "healthy", but rather displays only a quantitative difference in inflammatory extent. When infiltrates develop in perivascular spaces, they primarily consist of mononuclear leukocytes, i.e. lymphocytes and macrophages. Activation of macrophages and local microglia causes a demyelinating process which completely destroys myelin sheaths and subsequently causes a variable degree of axonal injury. Lost tissue may be substituted by glial scars.

It is generally accepted that $CD4^+$ T helper 1 cells mediate the inflammatory reaction specifically targeted against myelin (Friese et al. 2006, Hafler et al. 2005) and in particular the specific auto-antigens, MBP (Hohlfeld and Wekerle 2001), MAG (Salzer et al. 1987) and MOG (Lindert et al. 1999). At the same time, an important involvement of $CD8^+$ T cells has been shown (Booss et al. 1983), as they represent even the largest fraction of the total T-cell population within plaques (Babbe et al. 2000). They may exert a direct cytotoxicity, however, their role in pathogenesis or even in a potential down-regulation of the inflammatory response has not been finally clarified (Lazzarini 2004d). The presence of B cells and activation of the innate immune system have been shown to be involved in pathogenesis of MS (Sospedra and Martin 2005), while their function within lesions is not

resolved. By far the most abundant cells in active lesions are phagocytic macrophages that contain remnants of myelin and execute the actual tissue damage. The obvious heterogeneity of clinical manifestations and pathological processes in MS recently has lead to the statement that MS should be regarded as a syndrome (Gold et al. 2006).

Since myelinating cells are damaged during these events, oligodendrocyte abundance is altered in demyelinating lesions. Interestingly, marked inter- and intra-individual differences concerning the presence of oligodendrocytes could be observed. While there is often an initial loss of cellular density, fast regeneration and increase of their quantity could be found in most lesions. This is understood to be the result of an activation of progenitor cells to initiate regenerative processes (Lazzarini 2004d).

Remyelination in multiple sclerosis lesions

Early in the 20th century, the spontaneous regeneration of damaged myelin was observed in acute multiple sclerosis (Marburg 1906). This remyelination is now a well-known and studied feature of demyelinating processes in human diseases (Kornek et al. 2000) and animal models, such as the cuprizone model (see section 1.4 and Matsushima and Morell (2001)). A characteristic feature of remyelination is that the renewed myelin sheaths are thinner, such that the above mentioned g-ratio no longer applies. Additionally it has shorter internodes, which, interestingly, does not inhibit the reconstitution of conduction velocity and almost complete functional repair. These alterations are the reason for paler staining in the typical luxol fast blue histological stain that renders a clear visibility of the concerned areas.

Remyelination occurs predominantly in active lesions. Although very few OPCs and close to no oligodendrocytes are present in old lesions, they do not actively remyelinate and are understood to be inactive. This lack of remyelination in chronic lesions is surprising. An explanation for the inactivity of the local oligodendrocytes may lie in missing signals from damaged axons. It also appears to be beneficial for their activation to have a limited extent of active inflammation in presence of macrophages and their secreted trophic factors. Generally, there is a much higher efficacy of remyelination in early stages of demyelination.

Early repair in turn provides a protective process to limit demyelination-associated axon degeneration (Irvine and Blakemore 2008). Repeated demyelinating activity in the same brain area can impair effective remyelination.

The extent of remyelination differs clearly between individuals, with almost complete remyelination in some and almost no effective remyelination in other patients (Stadelmann and Brück 2008).

1.3 Dopamine receptors and agonists

Dopamine (DA) and its receptors constitute one of the most polyvalent neurotransmitter and hormone system in human and other vertebrate and invertebrate organisms. Its modulatory activities include influences on indispensable neurological functions, such as cognition, locomotion and emotion as well as endocrine regulation. The integrity of these is of elementary importance to general health. Direct links between high dopaminergic transmission and psychotic diseases, such as schizophrenia, have been known for a long time. Reduced activity and abnormal death of dopaminergic cells in the pars compacta of the substantia nigra is the typical pathophysiological feature of Parkinson's disease (PD), the most prevalent neurodegenerative disorder of locomotion. Specific dopaminergic systems of the CNS have been the focus of interest of many researchers, as in recent years a more general and widespread function of DA, its agonists and receptors has continued to emerge. Dopamine receptors have been identified on a large variety of cell types, especially in the nervous system.

1.3.1 Classical dopaminergic systems

The classical dopaminergic systems comprise four pathways that originate in the pars compacta of the substantia nigra, the ventral tegmental area (VTA) and the hypothalamus. The nigrostriatal pathway transmits signals from dopaminergic neurons of the substantia nigra to the striatum. It plays an important role in motor function and is the main target of damage in PD. Mesocortical and mesolimbic DA transmission from the VTA to the frontal cortex and nucleus accumbens, respectively, has an impact on higher brain functions, such as

emotion, cognition and personality. It is often involved in the development of schizophrenia and depression. The fourth, tuberoinfundibular dopaminergic pathway descends from the hypothalamus to the pituitary gland where it regulates the secretion of secondary hormones, i.e. prolactin. Hyperprolactinemia and pituitary tumors can be a result of its misregulation (Kandel et al. 2000).
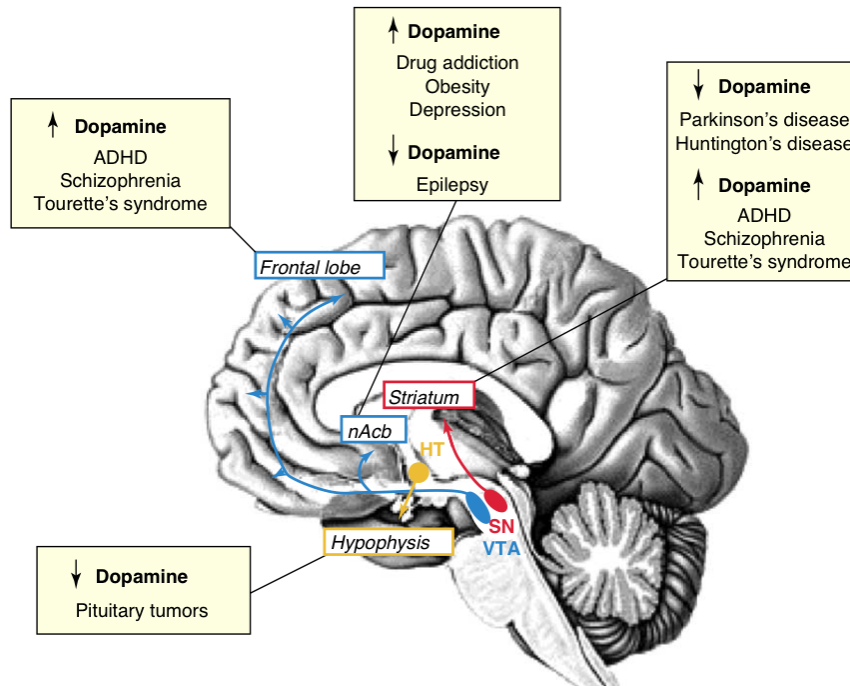
Figure 1.4: Classical dopaminergic systems and their DA-dependent alterations. Dopamine pathways are color-coded (red, nigorostriatal; blue, mesocortical and mesolimbic; orange, tuberoinfundibular). Abbreviations: ADHD, attention-deficit-hyperactivity disorder; HT, hypothalamus; nAcb, nucleus accumbens; SN, substantia nigra; VTA, ventral tegmental area. Image from Bozzi and Borrelli (2006, p. 168).

1.3.2 Dopamine as a neurotransmitter and neurotrophic substance

DA is a catecholamine neurotransmitter that is synthesized mainly by specific neurons. The hydroxylation of the amino acid tyrosine by the enzyme tyrosine hydroxylase yields L-DOPA which in turn is decarboxylated by L-amino decarboxylase to DA (Kandel et al. 2000). DA is then loaded into vesicles that are released upon presynaptic stimulation via action potentials from synaptic release sites.

In addition to being synaptically transferred DA and other neurotransmitters can act as

neurotrophic substances (Mattson and Hauser 1991, Meier et al. 1991). The alteration of intracellular cyclic adenosine monophosphate (cAMP) levels can have adverse effects on cell differentiation and proliferation. Whereas an increase in intracellular cAMP stimulates the first, it inhibits the latter as reviewed by Lauder (1993).

Bozzi et al. (2000) furthermore specify a direct neuroprotective function of DA via a sub-group of DA receptors, the D2-receptor (see section 1.3.3), in a model of excitotoxicity in epileptic seizures. This neuroprotection is mediated by a reduced excitability of D2-receptor expressing cells and possibly a downregulated susceptibility to enter apoptotic cell death pathways.

1.3.3   Dopamine receptors

DA receptors are G-protein coupled membrane receptors that are widely expressed in the CNS. Their existence in nervous tissues is known since the early seventies of the last century and light has been shed on many of their implications in basic CNS function, as described above.

In 1979, Kebabian and Calne proposed a classification of DA receptors into two groups, according to their effect on adenylyl cyclase (AC), which still persists today. The five different receptors are named D_1 to D_5 (table 1.1).

As membrane receptors with seven transmembrane domains and direct coupling to G-proteins (see Figure 1.5), DA receptors can have a stimulatory or inhibitory effect on AC to increase and respectively decrease intracellular cAMP levels. Structurally, the DA receptors are basically similar, except for the primary structure at their C-terminus and on their third intracellular loop. There is also variation in the number of glycosylation sites at their N-terminus (Missale et al. 1998).

The common distinction is that of D_1-like receptors (D_1 and D_5), that activate AC by the stimulating Gαs-protein and D_2-like receptors (D_2, D_3, D_4), that inhibit AC by the inhibitory Gαi-protein. Receptors of the D_2-family additionally rapidly increase inositol-1,4,5-triphophate (IP_3) formation upon stimulation and they can decrease cytosolic calcium levels independent of IP_3 change (Kebabian 1992).
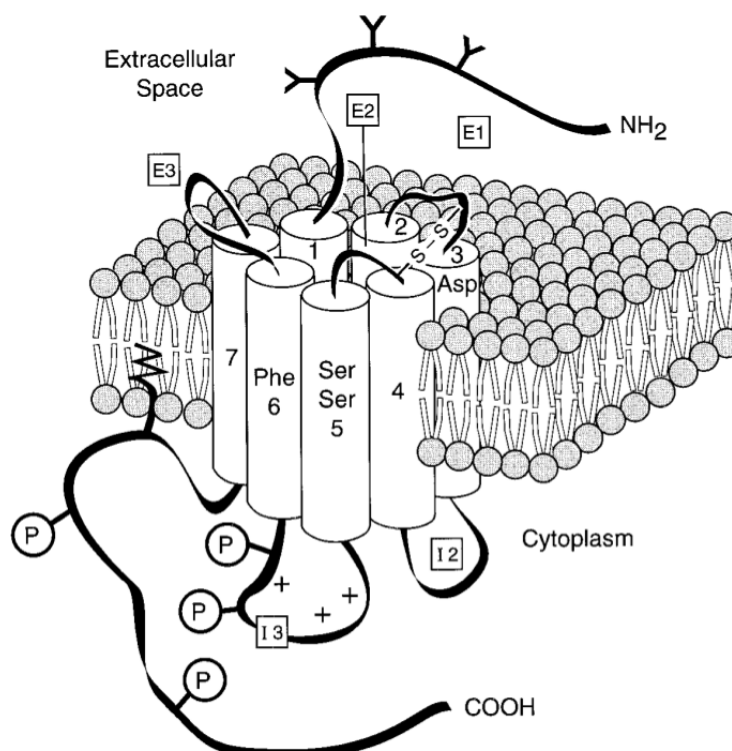
Figure 1.5: The general structure of a dopamine receptor. Seven transmembrane domains, three extra- and two intra-cellular domains characterize all subtypes. Image from Missale et al. (1998, p. 192).

Occurence of dopamine receptors

The D_1 receptor appears mainly on GABAergic neurons of the substantia nigra pars reticulata. It has also been detected in the striatum and the olfactory tubercle and is generally the most widely expressed DA receptor. Concordantly with the D_5 receptor, which is more restrictively expressed in some rostral forebrain regions, the hippocampus, the lateral mamillary nucleus and the parafascicular nucleus of the thalamus, the D_1-like receptors are expressed both pre- and, more frequently, postsynaptically (Vallone et al. 2000, Missale et al. 1998). They are both to be found on dendritic spines. Although both receptors' reaction to pharmacological stimulation is quite similar, their distinct subcellular localization hints as to some functional disparity (Missale et al. 1998).

D_2 receptors cannot typically be colocalized with D_1 receptors. They are found in high concentration on GABAergic cells of the striatum, the olfactory tubercle and in the nu-

cleus accumbens (Le Moine and Bloch 1995). These neurons co-express enkephalins or neurotensin. mRNA of the D_2 receptor has also been detected in the prefrontal, cingulate, temporal and enthorinal cortex and several subcortical areas: the hippocampus, the amygdala and the septal region (Missale et al. 1998). Dopaminergic cells of the hypothalamus, the substantia nigra pars compacta and of the VTA equally express this receptor subtype. The D_3 receptor is mainly associated with the limbic system, more specifically the nucleus accumbens and the island of Calleja. There is only low expression in the striatal areas and clearly less than the D_2 receptor in the substantia nigra pars compacta and the VTA.

D_4 receptors have been detected in the frontal cortex, the amygdala, the hippocampus, the hypothalamus and the mesencephalon. They are also found in the retina and some parts of the limbic system. Lower concentrations were observed in the basal ganglia, in pyramidal and non-pyramidal neurons of the cortex in the substantia nigra pars reticulata and the reticular nucleus of the thalamus (Missale et al. 1998).

Clearly, DA receptors have a functional importance for neurons and their interaction in a number of systems, as described above. Interestingly, both D_2 and D_3 receptors could be localized in oligodendrocytes (Howard et al. 1998, Bongarzone et al. 1998), which raises the question of their function in these types of cells. Early on, the supposition of an implication in the regulation of the development and maintenance of myelin sheaths was proposed.

Functions of dopamine receptors

It is important to note that the exact effect a DA receptor has, depends on the type of G-protein that is expressed in the respective target tissues. Although their most prominent effects are mediated by G-proteins, DA receptors are also capable of gene activation through the supported expression of different chemo- and cytokines as well as certain mitogens and even proto-oncogenes, such as c-Fos or c-Jun. D_1-type receptors directly reduce cellular Ca^{2+} currents and increase the ion's intracellular concentration. The same effect has been observed for D_2-like receptors in pituitary cells. Moreover, some neural populations, such as striatal cells, regulate their intracellular K^+ concentration by activation of D_2 receptors (Vallone et al. 2000). By antagonism of the D_2 receptor, Mitchell et al. (2002) could indirectly show their possibly crucial function in neuroprotection (see section 1.3.4 for more

details).

DA receptors centrally mediate both changes in motor activity and behavioral responses
(table 1.1). In animal experiments, the administration of D_1 and D_2 receptor antagonists
causes a reduction of locomotor function. At the same time, D_2 receptor deficient mice
display a marked motor deficiency, while D_3 deficient animals present with hyperlocomotion
(Vallone et al. 2000, Sealfon and Olanow 2000, Kitamura et al. 2003). This simple example
depicts the numerous and complex interactions of these receptors and their ligands. A large
number of studies have been conducted to clarify the specific functions of each receptor type
by creating specific receptor-deficient mice for each of them (Glickstein and Schmauss 2001).
On a behavioral level, DA receptors of the mesolimbical system (see section 1.3.1) are
implied in reward and mechanisms of reinforcement as well as stress situations which reflects
in increased transmission of the transmitter during such functional events (Le Moal and
Simon 1991). For instance, in experiments of drug self-administration and intracranial self-
stimulation with rats, it has been shown that D_1 and D_2 receptors themselves mediate
reinforcement and alter drug seeking behavior (Phillips et al. 1994).

D_2 and D_3 receptors and their potential role in glial cells

As aforementioned, DA receptors of the type D_2 and D_3 could be identified in non-neuronal
cells of the rat forebrain as early as 1998. Experiments employing a non-radioactive in-situ
hybridization protocol in postnatal rats first showed D_2 receptors to be present on ependy-
mal cells of the neostriatum and subsequently in scattered oligodendritic cells of fiber tracts
adjacent to the neostriatum (Howard et al. 1998). The authors emphasized the fact that
only interfascicular oligodendroglia where labeled and no satellite or perivascular oligoden-
droglia reacted to the stains. The same research group then proceded to demonstrate the
presence of D_3 receptors in oligodendrocytes of primary glial cell cultures in contrast to
their absence in astrocytes (Bongarzone et al. 1998). Moreover, levels of the protein, de-
tected by Western blot analysis, proposed an increase of expression levels proportional to
the culture's age with a late and continuous decrease following day 14 *in vitro*. Immunocy-
tochemical experiments of the same publication also indicate the complete absence of the

Table 1.1: Dopamine receptors: overview of effects on gross locomotion and higher brain function (summarized from Missale et al. (1998)).

Receptor Type	Functions
D_1	- little to no direct effect on locomotion - synergistic function in locomotion with D_2 receptors - inhibition of drug-seeking behavior
D_2	- neuroprotection - decrease or increase of locomotion dependent on localisation - synergistic function in locomotion with D_1 receptors - mesolimbicactivation of drug-seeking behavior - cortical receptors mediate behavioral changes
D_3	- inhibitory effect on locomotion - increased expression of neurotensin (effect on prolactine release) - activation of learning and memory - inhibition of drug-seeking behavior
D_4	- activation of learning and memory
D_5	- activation of learning and memory

receptor in mature cells. Taken together with *in vivo* experiments, linking the expression strongly to the level of myelinating activity in oligodendrocytes, both studies conclude in the proposition of probable regulatory functions of DA receptors in myelinating processes by stimulation of outgrowth formation and other, unknown, mechanisms. D_2 and D_3 receptors may even play complementary roles, which is indicated by their chronologic expression patterns.

It has been known for some time that DA, its agonists and DA receptors through stimulation of the expression of radical scavenging proteins can have protective effects on neuronal cells under oxidative stress (see the following subsection 1.3.4). It therefore did not come as much of a surprise when, in 2005, data was presented that suggested the protective role of these same factors in oligodendrocytes (Rosin et al. 2005). The results of this study showed both expression of D_2 and D_3 receptors in primary oligodendrocyte cultures, while the application of the DA agonists bromocriptine and quinpirole under oxygen and glucose deprivation injury clearly had rescuing effects, as demonstrated in MTT assays. Parallely, the expression of the receptors themselves was upregulated. The exact mechanisms by

which this effect could be mediated, through direct radical scavenging effects of the applied substances or through additional receptor-mediated cascades, remained to be clarified.

1.3.4 Dopamine and its agonists - neurotoxicity of catecholamines and potentials in neuroprotection

A marked toxicity of DA and its derivatives to cellular systems by the production of free radicals has been characterized early on (Graham et al. 1978). Its specifically toxic effect on neural cells of the striatum was demonstrated in turn by Filloux and Townsend (1993). As a possible mediating mechanism, the authors suspected, in addition to the known autooxidative effects described earlier, an excitotoxic effect believed to be generated by the artificial surplus of transmitter in extracellular space and by the resulting postsynaptic over-excitation. Ziv et al. (1994) were able to demonstrate the initiation of dose-dependent, apoptose-like cell death in chick-embryo sympathetic neurons under physiological, but chronic exposure to DA. This effect constitutes the third mechanism of *in vivo* damage. It can therefore be deduced that DA has both direct toxic effects as well as receptor-mediated toxic effects which have been discussed widely (Bozzi and Borrelli 2006).

In contrast to these neurotoxic effects, members of the monoamine family, such as DA and norepinephrine (NE) were discovered to exert antioxidative function by scavenging of free radicals (Liu and Mori 1993). The same could be found in striatal dopaminergic neurons that undergo damage in the Parkinson's disease model that is induced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Lange et al. (1994)). The toxic function of this compound could be relieved to some extent by the application of dopamine agonists. Noh et al. (1999) found catecholamines in physiological concentrations to support neurotrophic factors in their protective functions, while high doses rather induced apoptosis and Sofic et al. (2001) clearly demonstrated both oxidant and antioxidant effects in pheochromocytoma cells.

Dopamine agonists continue to be credited for their numerous neuroprotective properties (see Table 1.2 and Schapira (2002), Schapira (2009)).

Table 1.2: Dopamine agonists' neuroprotective functions, adapted from Schapira (2002).

- Decrease of dopamine metabolism
- Action as free radical scavenger *in vitro* and *in vivo*
- Protection against cerebral ischemia
- Increase of cell survival *in vitro* in response to L-dopa and dopamine toxicity
- Increase of dopaminergic cell survival in response to MPTP toxicity
- Increase of dopaminergic cell survival in response to H_2O_2 toxicity

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
L-dopa = L-3,4-dihydroxyphenylalanine

As mentioned in section 1.3.3, the neuroprotective function of DA agonists is believed to be mediated in major parts by the D_2 receptor (Bozzi and Borrelli 2006). It has been proposed that this is achieved by a feedback mechanism through stimulation of presynaptic autoreceptors that limit the extracellular dopamine levels which in turn decreases free-radical production. Bellucci et al. (2008) find that the stimulation of both D_2 and D_3 autoreceptors with DA agonists increases the membrane incorporation of DA transporter proteins (DAT) and thus the recuperation of DA from the synaptic, extracellular space. In this case they enhance the reduction of DA's autooxidative and excitotoxic functions. Activation of the D_2 receptor also triggers the activation of anti-apoptotic pathways (Bozzi and Borrelli 2006).

Pramipexole

Pramipexole (Schneider and Mierau 1987) is a selective DA agonist that is more selective to D_3 receptors, when compared to other, widely used, agonists, such as Bromocriptine or Quinpirole. The latter are non-selective and D_2/D_4-preferring, respectively (Mierau et al. 1995). Studies propose, however, that D_3 receptors are not responsible for the neuroprotective functions of these substances, as Bromocriptine shows similar efficacy (Sethy et al. 1997).

Dopamine agonists, such as pramipexole (Mirapex® or Sifrol®), are established substances in the symptomatic treatment of Parkinson's disease (Bennett and Piercey 1999). In addition to their postsynaptic effect to stimulate DA receptors in compensation for DA loss, significant antioxidant properties of pramipexole were postulated by Hall et al. (1996), who demonstrated dose-dependent protection of DA neurons in the substantia nigra to ischemic damage in gerbil forebrains. Moreover, they conducted analyses of DA metabolite concentrations following ischemia and reperfusion to demonstrate that this protective effect was most probably not due to DA turnover-suppression by autoreceptor activation but rather to reductive potential of the substance itself. The antioxidant capability of pramipexole was measured at 50 per cent oxidation for a potential of approximately 350mV (Hall et al. 1996).
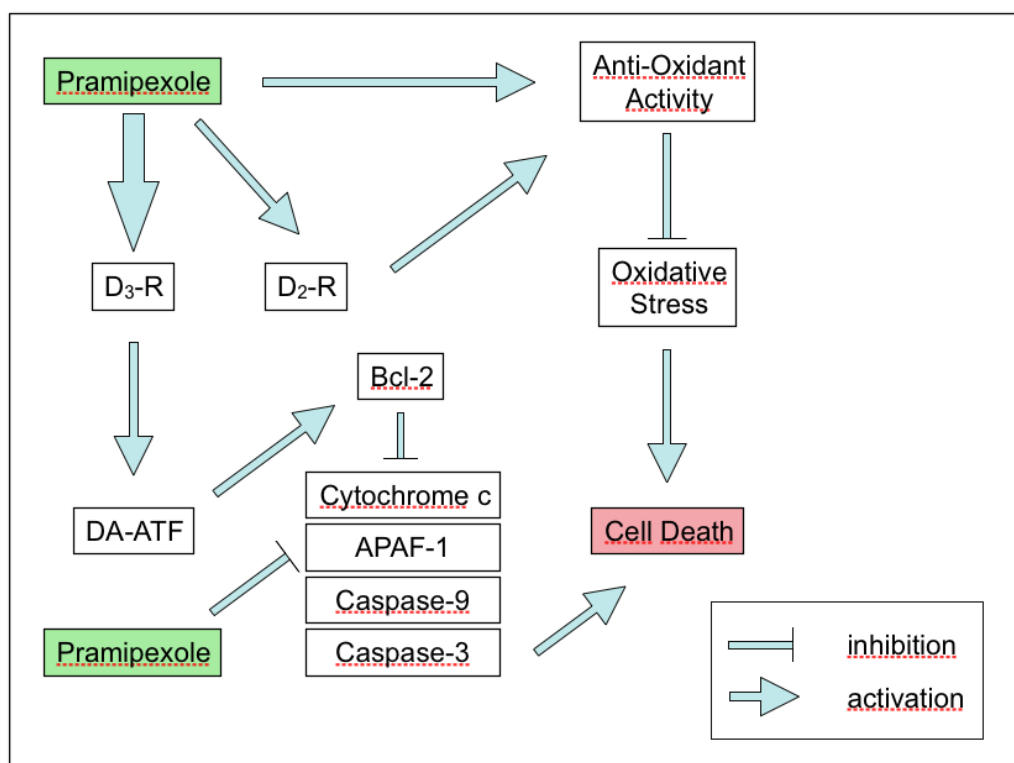
Figure 1.6: Illustration of the possible neuroprotective mechanisms of pramipexole. DA-ATF, 35-kDa dopaminergic autotrophic factor; APAF-1, apoptotic protease activating factor 1; Bcl-2, B-cell lymphoma 2 protein. Adapted from Kitamura et al. (2003).

There have been several studies that concluded the neuroprotective effect of pramipex-

ole to be independent of DA receptors and merely due to its free radical scavenging (Le et al. 2000, Zou et al. 1999). Evidence has accumulated since then that the mechanisms of neuroprotection following pramipexole's application are indeed more complicated than this. Ling et al. (1999) addressed this issue and found an involvement of the D_3 receptor that was more important than that of the D_2 receptor in protection of tyrosine hydroxylase-containing neurons. They concludingly proposed a combined action involving D_3 receptor activation and antioxidation capacity.

Kakimura et al. (2001) investigated DA agonists' direct effects on cellular apoptosis and found pramipexole to inhibit the release of cytochrome c from mitochondria and the activation of caspases 3 and 9, initiators and effectors, respectively, of the apoptotic pathway. This may be achieved by the induction of the B-cell lymphoma 2 (Bcl-2) protein which regulates apoptosis and has a role in neuronal regeneration (Kitamura et al. 1998, Kihara et al. 2002). At higher concentrations pramipexole also inhibited the apoptotic protease activating factor 1, also known as APAF-1. It has also been reported that pramipexole induced a 35-kDa DA autotrophic factor that possibly enhances Bcl-2 expression (Ling et al. 1998). Figure 1.6 gives an overview of pramipexole's possible neuroprotective functions.

1.4 Oligodendrocyte injury and animal models for demyelination

1.4.1 Oxidative stress in oligodendrocytes

The oligodendrocyte's importance in the maintenance of CNS integrity has been addressed in chapter 1. Oligodendrocytes, as metabolically highly active cells are especially vulnerable to oxidative stress to the CNS, which is characterized by imbalance of the concentration of reactive oxygen species, also called oxygen free radicals, and antioxidative systems to counteract their accumulation. Free radicals may accumulate as a result to ischemic insult, hypoxia, inflammation and as a normal byproduct of biological metabolism. Oligodendrocytes can be compromised in their myelinating function by oxidative species, such as H_2O_2 by their induction of apoptosis (Richter-Landsberg and Vollgraf 1998).

As part of the stress response oligodendrocytes express so-called heat-shock-proteins (HSP), comprising a large number of proteins (Goldbaum and Richter-Landsberg 2001). They have been shown to act as molecular chaperones and as supportive proteins in translocation and transportation processes. As such, they were brought into context with neurodegenerative diseases, specifically MS, by several authors (Birnbaum 1995, Brosnan et al. 1996), which in turn linked the oxidative damage of oligodendrocytes to MS. Especially in remyelinating processes, oxidative stress can have profound effects, as it is particularly harmful to oligodendrocyte progenitors in differentiation (French et al. 2009).

1.4.2 Animal models for multiple sclerosis - demyelination in the cuprizone model

Most of the scientific discoveries and hypotheses in research concerning MS have been revealed and studied in the animal model (Steinman and Zamvil 2006, Friese et al. 2006). Although most of these studies employ the well-known model of experimental autoimmune encephalomyelitis (EAE), others have been established. Cuprizone, a copper chelator and known neurotoxicant, leads to massive demyelination in large areas of mouse brains. Upon termination of application in the diet of treated animals, myelin regenerates almost completely within weeks. Oligodendroglia are directly harmed by considerable stress and the induction of extensive apoptotic cell death. Therefore, the cuprizone model has been employed in simulation of de- and remyelinating processes (Matsushima and Morell 2001). Especially the corpus callosum, where up to 90% of axons can be demyelinated under cuprizone influence, is a favored location for histochemical studies. Remyelination is believed to occur upon proliferation and differentiation of local and recruited OPCs under the influence of astrocytes (Skripuletz et al. 2012). Brück et al. (2012) show in a recent paper that the downregulation of pro-inflammatory factors in astrocytes has a beneficial effect on demyelination.

In contrast to EAE, cuprizone's effects rely more on a direct toxicity and less on complex immunological reactions. It can therefore be considered as a somewhat reduced model for de- and remyelination, less dependent on the immune system. Taking into account that the direct toxicity implies a reversibility only to a certain extent of damage over time, complete

reversal of functional conduction deficits is only possible in animals that have been fed cuprizone for up to three weeks. Longer influence of cuprizone leads to conductivity losses that only partially recover (Crawford et al. 2009). Prolonged feeding of cuprizone leads to so-called chronic demyelination, as opposed to acute demyelination (Kipp et al. 2009, Liebetanz and Merkler 2006, Manrique-Hoyos et al. 2012). The cuprizone model, although questionable in it's resemblance to human MS pathology, constitutes a highly reproducible and predictable model for de- and remyelination. It may therefore deliver a valuable source for the discovery of important pathological features and therapeutic strategies for demyelinating diseases of the CNS (Pott et al. 2009), such as the highly complex syndrome that presents itself under the term MS.

1.5 Aims of this study

The aims of this thesis were to analyze the effect of dopamine and its agonist pramipexole on oligodendrocytes in culture and in the cuprizone mouse model system.

The following issues were addressed:

1. *Can the DA agonist pramipexole act as a protective agent for oligodendrocytes exposed to oxidative stress?*

   The model to study the antioxidant and protective capacity of pramipexole was the stable OLN-93 cell-line, analyzed morphologically under oxidative stress and metabolically in the MTT-assay. Furthermore, oligodendrocytes in primary mixed glial cultures were set under oxidative stress and microscopically evaluated.

2. *Do the D_2 and D_3 receptors play a role in oligodendrocyte differentiation and de- as well as remyelination?*

   The neurotoxicant cuprizone was fed to D_2- and D_3-receptor-deficient mice to study the receptors' implication in de- and remyelination in this murine model. It was evaluated by the quantitative determination of demyelination and oligodendrocytic composition of the corpus callosum.

3. *Is there a protective effect of pramipexole in oligodendrocyte differentiation and de-*

as well as remyelination in the cuprizone model?

The cuprizone model was exerted under parallel administration of the DA agonist pramipexole. It was then evaluated by the quantitative determination of demyelination and oligodendrocytic composition of the corpus callosum.

Chapter 2

Material and Methods

2.1 Cell culture

All cell cultures were kept at $37\,°C$ and $10\,\%\,CO_2$. Culture dishes were coated with poly-l-lysine (PLL) to enhance cell-adhesion to ϵ-amino acids of this polypeptide. For coating, dishes were covered in PLL-solution for 30 minutes at room-temperature and subsequently washed once with phosphate buffered saline (PBS).

2.1.1 OLN-93 cells

For cytotoxicity assays, OLN-93 cells were maintained as described by Richter-Landsberg and Heinrich (1996). In maintenance, they were passaged weekly by washing twice with phosphate buffered saline (PBS) and incubation for 5 min with trypsin/EDTA at $37\,°C$. Trypsination was stopped by transfer into $10\,ml$ of DMEM/$10\,\%$ FCS in which the cells were centrifuged for 5-10 minutes at $800\,g$.
The cells were then resuspended in DMEM/$10\,\%$ FCS and counted in a Neubauer chamber to be replated on 10 cm plastic dishes and kept in DMEM/$10\,\%$ FCS.

Table 2.1: Material used in cell cultures. [1]Heraeus Holding GmbH, Hanau, Germany; [2]Olympus Deutschland GmbH, Hamburg, Germany; [3]American Optical, Horsham, PA, U.S.; [4]Hettich Instruments, Beverly, MA, US; [5]Bachhofer, Reutlingen, Germany;[6]Nunc GmbH & Co. KG, Langenselbold, Germany; [7]Invitrogen Corporation, Carlsbad, CA, U.S.; [8]Biochrom AG, Berlin, Deutschland; [9]Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim am Rhein, Germany; [10]Merck KGaA, Darmstadt, Deutschland; [11]Sigma-Genosys, Pampisford, U.K.

Incubator	Heraeus[1]	
Microscope	Olympus[2]	
Neubauer chamber	American Opt.[3]	
Table top centrifuge	Hettich[4]	Hettich Universal II
Vacuum pump	Bachhofer[5]	Vacumat 100
Cell culture plastic	Nunc[6]	
Dulbecco's Modified Eagle's Medium	Gibco[7]	DMEM #52100, 70 g DMEM, 18.5 g NaHCO$_3$ in 5 l aqua bidest, pH 7.2-7.4, sterile filtered
DMEM 10 % FCS	Biochrom[8]	450 ml Medium, 50 ml FCS (inactivated),50 U/ml Penicillin, 50 U/ml Streptomycin
DMEM 0.5 % FCS	Biochrom	450 ml Medium, 50 ml FCS (inactivated),50 U/ml Penicillin, 50 U/ml Streptomycin
Fetal calf serum	Boehringer9	30 minutes at 56 °C
NaHCO$_3$	Merck[10]	
Poly-L-Lysine (PLL)	Sigma[11]	#P-2636, stock-solution: 1 mg/ml in aqua bidest, used: 50 μg/ml in aqua bidest, autoclaved
Penicillin/Streptomycin	Boehringer	#0210404
Phosphate Buffered Saline (PBS)		8 g NaCl; 0.2 g KH$_2$PO$_4$; 1.15 g Na$_2$HPO$_4$ in 1 l aqua bidest, pH 7.4
Trypsin/EDTA	Gibco	#043-05400; 0.05 % trypsin; 0.2 % EDTA, stem solution: 1 ml/10 ml aqua bidest

2.1.2 Primary mixed glial cultures

Primary cultures of glial cells for immunocytochemistry were prepared from the brains of 1-2-day-old Wistar rats. Cerebral hemispheres were freed of the meninges and mechanically disrupted using a pasteur pipette. Single cell suspensions were transferred to culture flasks (1 brain/75 cm^2) and kept for 6–8 days in DMEM supplemented with 10 % heat-inactivated FCS, 50 U/ml penicillin, and 50 µg/ml streptomycin. After 8–10 days cultured cells were removed from the flasks and replated on polylysine-coated plastic dishes and incubated for another 5–6 days. These cultures contained astrocytes and oligodendrocytes.

2.1.3 Administration of pramipexole and H$_2$O$_2$

Pramipexole was directly administered to the cell cultures and MTT-assay with prepared media at time points described during maintenance and experimental conduction. The final concentration was consistently adjusted to 100 mM. H$_2$O$_2$ was diluted and directly added to predefined volumes of cell culture medium to yield the required concentrations of 50-500 µM, according to the experiment.

2.1.4 Morphologic analysis

For morphologic analysis a microscope type IX 70-88F with Hofmann modulation contrast (Olympus model EP 40[1]) was employed. Cell images were digitally documented with a CF 8/4 camera[2] with fluorescence imaging via the Olympus U-RFL-T module. Images were analyzed with the software analySIS®[3].

2.1.5 Indirect immunocytochemistry

Cellular proteins and structures, such as tubulin, tau, αB-crystallin and α-synuclein were visualized by using specific antibodies in indirect immunocytochemistry (see tables 2.2 and 2.3).

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[1]Olympus Deutschland GmbH, Hamburg, Germany
[2]Kappa optronics GmbH, Gleichen, Germany
[3]Soft Imaging System GmbH, Münster, Germany

Cells were cultured in 3.5 cm plastic-dishes, containing 4 PLL-coated coverslips. For immunocytochemistry, dishes were washed twice for 5 minutes with PBS and fixed either with 3% paraformaldehyde (PFA)-solution at room temperature for 15 minutes (αB-crystallin and α-synuclein) or with methanol at -20 °C for 15 minutes (tubulin and tau). PFA was then removed by repeated washings with PBS. PFA-fixed cells were permeabilized with Triton X-100 (30 minutes), which was in turn removed by washing three times (10 minutes) with PBS.

Cells were incubated with primary antibodies in a moist chamber at the desired concentration overnight. After washing with PBS (3 x 10 minutes), incubation with secondary antibodies was carried out for 60 minutes. Finally, coverslips were washed three times with PBS and then mounted in 2-3 μl mounting medium on glass slides.

Table 2.2: Material for indirect immunocytochemistry. [1]Carl Zeiss AG, Oberkochen, Germany; [2]Gerhard Menzel, Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany; [3]Fisher Scientific GmbH, Schwerte, Germany; [4]Sigma Aldrich Chemie GmbH, Steinheim, Germany; [5]Serva Electrophoresis GmbH, Heidelberg, Germany; [6]Vector Laboratories, Inc., Burlingame, CA, U.S.

Microscope	Zeiss[1]	Axiophot
Camera	Zeiss[1]	AxioCam
Software	Zeiss[1]	AxioVision 3.3
Glass slides	Menzel[2]	
Cover slips	Fisher [3]	Fisherbrand® cover glass, autoclaved
Triton X-100	Sigma[4]	0.1% Triton X-100 in PBS
Phosphate Buffered Saline (PBS)		see Table 2.1
Paraformaldehyde (PFA)	Serva[5]	#31628, 750 mg PFA in 3.5 ml aqua bidest, 3-4 drops 1N NaOH, dissolved in warm waterbath, filled to 25 ml with PBS
Mounting medium	Vector[6]	Vectashield H-1200 with DAPI

2.1.6 MTT assay

To measure viability, OLN-93 cells were plated on PLL-coated 96-microwell cell culture plates at a density of 10,000 cells per well. 24 h of incubation in DMEM/0.5 % FCS were

Table 2.3: Antibodies (AB) used in immunocytochemistry

	Origin	Isotype	Conc.
Primary			
MBP	McMorris et al. (1981)	polyclonal rabbit IgG	1:200
tau	V. Lee, Philadelphia, PA, U.S.	polyclonal rabbit IgG	1:500
tubulin	Sigma	monoclonal mouse IgG	1:400
Secondary			
TexasRed	Jackson ImmunoResearch	anti-rabbit	1:50
TexasRed	Jackson ImmunoResearch	anti-mouse	1:50
FITC	Jackson ImmunoResearch	anti-rabbit	1:100
FITC	Jackson ImmunoResearch	anti-mouse	1:100

followed by exchange of the medium ($100\,\mu$l/well). Half of the samples were then treated and pre-incubated for 30 minutes with pramipexole at a concentration of $100\,\mu$M, followed by the addition of hydrogen peroxide ($200\,\mu$M to $500\,\mu$M). The cells were incubated overnight and analyzed 24 hours later.

The colorimetric MTT assay (Mosmann 1983) was conducted according to protocol using $10\,\mu$l MTT solution ($5\,\mu$g/ml in PBS) in each of the 96 wells. After $4\,$h of incubation, $100\,\mu$l of solubilization solution ($10\,\%$ sodium dodecyl sulfate[4] in $0.01\,$mol/l HCl) were added and incubated overnight to dissolve the built-up formazan salt. Quantification was then carried out with a microplate reader (model 3550 Bio-Rad[5]) at 595 nm. Data are expressed as percentage of the untreated controls, and values represent the means +/- SD of 8 microwells each of at least two independent experiments (n = 16).

2.2   Animal experiments - cuprizone model

2.2.1   Animals

For cuprizone experiments, male and female C57BL/6J mice were purchased from Charles River Laboratories Inc.[6] and used as controls. Mice with a disrupted D_2 dopamine receptor gene (Kelly et al. 1997) with the strain name B6.129S2-Drd2tm1Low/J were purchased from

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[4]SERVA Electrophoresis GmbH, Heidelberg, Germany
[5]Bio-Rad Laboratories GmbH, München, Germany
[6]Charles River Laboratories Inc., Sulzfeld, Germany

The Jackson Laboratories[7], D_3 dopamine receptor deficient animals (strain B6.129S4-Drd3) likewise.

All experiments and animal husbandry were undertaken and maintained according to rules and regulations of the animal facilities of the University Medical Center Göttingen. Experiments were accredited by the regional government (Bezirksregierung Braunschweig).

EAE animals were allowed to adjust to the temperature-controlled environment of the experimental facilities in automated circadian rhythm for seven days before launching of the experiments. For the entire time of the experiment they were provided with pressed granulated food pellets and water *ad libitum*.

2.2.2 Feeding of cuprizone

Cuprizone[8] was mixed with ground food pellets in a concentration of $0.2\,\%$ and fed to the animals for six weeks, as specified in Chapter 3. For the analysis of demyelination, animals were sacrificed immediately after this time period. Spontaneous remyelination, however, was examined after one week of normal diet prior to sacrifice. Non-treated control animals were fed with normal pellet food at the same time.

2.2.3 Application of pramipexole

$200\,\mu$l of pramipexole were applied intraperitoneally on a daily basis at a concentration of $0.5\,$mg/ml, corresponding to $0.1\,$mg per mouse or 4mg/kg body weight. Controls were injected similarly with PBS solution.

2.2.4 Determination of demyelination in the corpus callosum

LFB/PAS stained slices of the CC were assessed at a magnification of 100-fold magnification of the complete corpus callosum, with a semi-quantitative score for demyelination (see table 2.4) according to Hiremath et al. (1998).

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[7]Jackson Laboratories, Bar Harbor, ME, U.S.A.
[8]Sigma Aldrich Chemie GmbH, Steinheim, Germany

Table 2.4: Semi-quantitative score for demyelination of the corpus callosum

Score	Degree of demyelination of the corpus callosum
0	no demyelination
1	less than $\frac{1}{3}$ demyelination
2	$\frac{1}{3}$ to $\frac{2}{3}$ demyelination
3	more than $\frac{2}{3}$ demyelination

2.3   Neuropathology and immunohistochemistry

2.3.1   Animal preparation

Animals were anesthetized by injection of 300 μl chloral hydrate[9] 14 % i.p. and then perfused transcardially with 1x PBS, followed by 4 % paraformaldehyde[9](PFA). Tissues were post-fixed in 4 % PFA at 4°C overnight and then dissected. The brain was cut into 5 coronal sections by hand. The 2 to 3 mm thick slabs were embedded in paraffin (Paraffin Paraplast Plus[10]) to be cut into 1 μm-thick sections with a microtome type SM 2000R[11]. These sections were subsequently utilized for histological and immunohistochemical stainings.

2.3.2   Tissue preparation

All sections were deparaffinized with xylene[9] and isoxylene[9] and then serially diluted and rehydrated in alcohol[9] and deionized water. Each animal was evaluated on sections stained with Hematoxylin (Mayers Hämalaunlösung[9])-Eosin (from 2 g Eosin-G Certistain[9] in 40 ml distilled water and 160 ml ethanol 95 %), Luxol-Fast-Blue (solution prepared from 1 g LFB[12] in 1000 ml Ethanol and 5 ml acetic acid 10 %)/1 % Periodic acid Schiff (LFB/PAS) and Bielschowsky silver impregnation.

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[9]Merck, GKaA, Darmstadt, Germany
[10]Tyco Healthcare GmbH, Neustadt a. d. Donau, Germany
[11]Leica Microsystems, Bensheim, Germany
[12]BDH Laboratory Supplies, VWR Intern. Ltd., Poole, UK

Hematoxylin-eosin stain

The hematoxylin-eosin staining, used to evaluate morphology, was performed after deparaffination and rehydration, by a coloration of 8 minutes in Mayer's hematoxylin bath, acidic differentiation in HCl-alcohol and blueing for 10 minutes under tap water. The sections were counterstained with Eosin, yielding sections with blue nuclei and red cytoplasm (for histological HE stain protocol, see Table A.1).

LFB-PAS

LFB-PAS, producing a strong coloration of myelin (blue), Nissl substance and nuclei (dark blue) was needed for quantitative determination of de- and remyelination. The sections were only deparaffinized, not rehydrated, and then stained overnight in alcoholic LFB-solution. On the following day, differentiation was achieved by short, alternating dipping in 0.05 % lithiumcarbonate[9], 70 % alcohol and distilled water. Further staining involved placement in periodic acid for 5 minutes, blueing under running tap water for 5 minutes and 20 minutes of reaction in Schiff's reagent. Finally, sections were rinsed and counterstained in Mayer's hematoxylin bath, differentiated in HCl and rehydrated (for exact histological LFB-PAS stain protocol, see Table A.2).

Bielschowsky silver impregnation

To evaluate axonal loss within the samples, we additionally performed a Bielschowsky silver impregnation, in which axons appear black. Following the deparaffination, slices were prestained with $AgNO_3$-solution[8] for 20 minutes and then stained for another 15 minutes after addition of a 3 drops of 25 % ammonia solution to the same cuvette. 1ml of developer (from 20ml 37 % Formalin[8], 100ml distilled water, 0.5g citric acid, 1 drop of concentrated nitric acid) was added to the solution for 2–3 minutes, after which a series of washing and placement in 3 % Thiosulfate solution finalized the coloration. Dehydration was followed by mounting in DePex[4], as in all the other staining protocols.

Immunohistochemistry

Mouse sections were stained with anti-NogoA (NogoA/11C7 from Oertle et al. (2003), 1:10,000), a monoclonal mouse antibody to highlight oligodendrocytes and anti-Olig2[13] (1:200), a rabbit polyclonal antibody to mark OPCs.

Immunohistochemical staining was performed after antigen-demasking with boiling $10\,mM$ citrate buffer (2.1014g Citric acid-monohydrate[6] in 1000ml distilled water, pH 6) for 5 x 3 minutes (microwave treatment). Coverglass slides were placed in $3\,\%H_2O_2/PBS$ for 10 minutes to quench endogenous peroxidase activity. Slides were transferred to a coverplate system and treated for 10 minutes with $10\,\%$ FCS in PBS to block unspecific binding. The primary antibody was added and incubated at 4^oC overnight. For both stainings we used biotinylated sheep anti-mouse IgG antibody[14] (1:200) as secondary antibody. They were added on the second day for 60 minutes after PBS-rinsing. The secondary antibody was then labelled with Avidin-Peroxidase,[10] which has a high affinity to Biotin. It was applied for 45 minutes in a dilution of 1:1000. The Peroxidase develops a brown coloration in reaction with a diamino-benzidine developer[10] (DAB D5627, 1 cuvette with 1ml in 50ml PBS and $20\mu l$ $30\,\%H_2O_2$)). To reach an enhancement of the DAB chromogenic reaction, the sections were rinsed with distilled water and incubated with $2\,\%CuSO_4$/physiological NaCl solution for 10 minutes (Boenisch et al. (2003)). Counterstaining was achieved with 30 seconds in Mayer's Hematoxylin bath, HCl-differentiation and blueing under running tap water for 7 minutes. Sections were then dehydrated and mounted in DePex as described previously (for exact immunohistochemical protocol, see Tables A.3, A.4).

Genotyping

Both mouse strains were genotyped by taking tissue from the ears of mice and digesting them with $10\mu l$ protein kinase K, $350\,\mu l$ prepared tail lysis buffer (prepared from $100\,mM$ tris-HCl (pH 8,5), $5\,mM$ EDTA (0.731g), $200\,mM$ NaCl (5.844 g) and $0.2\,\%SDS$) at $56\,^oC$ overnight in a thermomixer (Thermomixer comfort[15]). The samples were then vortexed and

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[13]IBL, Gunma, Japan
[14]Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany
[15]Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany

subsequently centrifuged (centrifuge model 5415R[15]) for 5 minutes at maximal speed (13200 rpm). The supernatant was mixed with 350 μl isopropanol and centrifuged again at maximal speed for 5 minutes. The pellet was washed once with 500 μl ethanol, again centrifuged at maximal speed for five minutes and then dried after discarding the supernatant. Finally the sample was diluted in 100 μl distilled water.

A Polymerase chain reaction (PCR) mastermix was prepared with 2 μl of previously isolated DNA (see Table A.5). PCR ran for 2-3 hours and PCR products were then loaded to a 2 % agarose (2 g agarose in 100 ml TBS) gel for gel-electrophoresis at 100 V, 60 minutes.

Microscopy

For all histological analyses we used the Olympus BX 51 microscope[1] with the following objectives (all Olympus): 10x/0.25, 20x/0.40, 40x/0.65, 100x/1.25 oil. The oculars used were all 10x, for cell density determination with an integrated, standardized grid. Digital pictures were recorded on a Windows PC system with a 3.3 megapixel CCD-camera and processed with the microscopy software analySIS[®][3].

2.4 Statistical analysis

2.4.1 Data acquisition

Data were acquired with the software Microsoft Excel 2000[17] on a personal computer.

2.4.2 Data analysis

Data were organized and analyzed with the statistical analysis program GraphPad PRISM 4.00[18]. The data obtained from morphometric analysis of different experimental groups were evaluated for possible statistically significant differences. For comparison of de- and remyelination in the corpus callosum of our experimental animals, mean values as well as standard deviation of the aforementioned semi-quantitative score (see table 2.4) were determined for each experimental group. The density of NogoA- and Olig2-positive oligodendrocytes was

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[17]Microsoft Deutschland GmbH, Unterschleißheim, Germany
[18]GraphPad Software, San Diego, USA

determined (cells/mm2) and equally averaged for each subgroup of animals. Comparisons of these data for two experimental groups were performed by unpaired t-test. To assess the effect of two independent variables, such as treatment and gender on de- and re-myelination we used a two-way factorial ANOVA. We applied a significance level of $p < 0.05$ with Bonferroni's posttest.

Chapter 3

Results

3.1 The effect of pramipexole as a neuroprotectant in vitro

3.1.1 Immunocytochemical assessment of protective properties of pramipexole in OLN-93 cells under oxidative stress

The cell line that was used in this work to monitor the *in vitro* effects of pramipexole under conditions of oxidative stress, were OLN-93 cells. These have originally been derived as spontaneously transformed cells from primary rat oligodendrocytes (Richter-Landsberg and Heinrich 1996). They provide a cell culture system, representing immature oligodendroglial cells which can easily be maintained. Although OLN-93 cells do not form myelin sheaths, they do have peripheral cellular extensions, similar to those of primary oligodendrocyte precursors, in part with antigenic properties of mature oligodendrocytes. In our study, they were used to assess whether pramipexole exerts a protective role against oxidative stress in oligodendroglial cells.

Microtubuli together with intermediate filaments and actin filaments (microfilaments) are the major constituents of the cytoskeletal network (Alberts et al. 2005). However, oligodendrocytes and also OLN-93 cells are devoid of intermediate filaments. Oligodendrocytes also contain the microtubulus-associated protein tau (Gorath et al. 2001), and OLN-93 cells used in these studies were stably transfected with plasmids encoding tau (Goldbaum et al. 2003). Hence, antibodies against tubulin and tau were used to evaluate cellullar morphology and

cytoskeletal integrity.

Pramipexole-treated OLN-93 cells show no morphological differences when compared to non-treated controls

In a first *in vitro* analysis of pramipexole's potential protective effects, untreated OLN-93 cells were morphologically compared to pramipexole-treated OLN-93 cells as seen in Figure 3.1. The 24 h-incubation of OLN-93 cells with pramipexole (100 mM)-supplemented cell culture medium did not show any morphological alteration, nor toxic effects or changes in cell densities. Cellular processes were as well-developed as in non-treated controls.
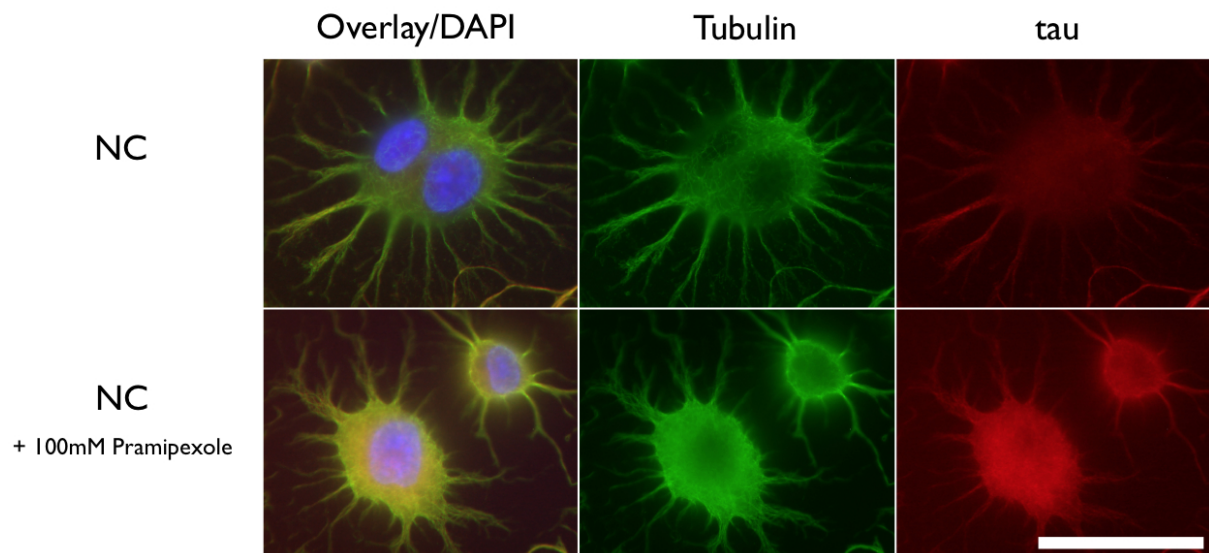
Figure 3.1: Indirect immunofluorescence of OLN-93 cells with antibodies against tubulin (green) and tau (red). Nuclei were stained with DAPI (blue). NC, negative control, untreated. Below, non-stressed OLN-93 cells with pramipexole pre-incubation. Scale bar 20 μm.

Pramipexole-treated OLN-93 cells under oxidative stress show less profound morphological damage when compared to non-treated controls

In a next step OLN-93 cells were exposed to oxidative stress, induced by different concentrations of H_2O_2 (300 μM and 500 μM).

To assess the possible protective effect of pramipexole, cells were either untreated or pre-

incubated with pramipexole (100 mM; 30 minutes), followed by treatment with H_2O_2 as indicated and left in the incubator for 24 hours.

Figure 3.2 depicts representative microscopic images of two independent experiments. H_2O_2 in concentrations of $300\,\mu$M and $500\,\mu$M caused severe morphological changes in a concentration-dependent manner. Cellular processes were retracted and cytoskeletal organization was disturbed.
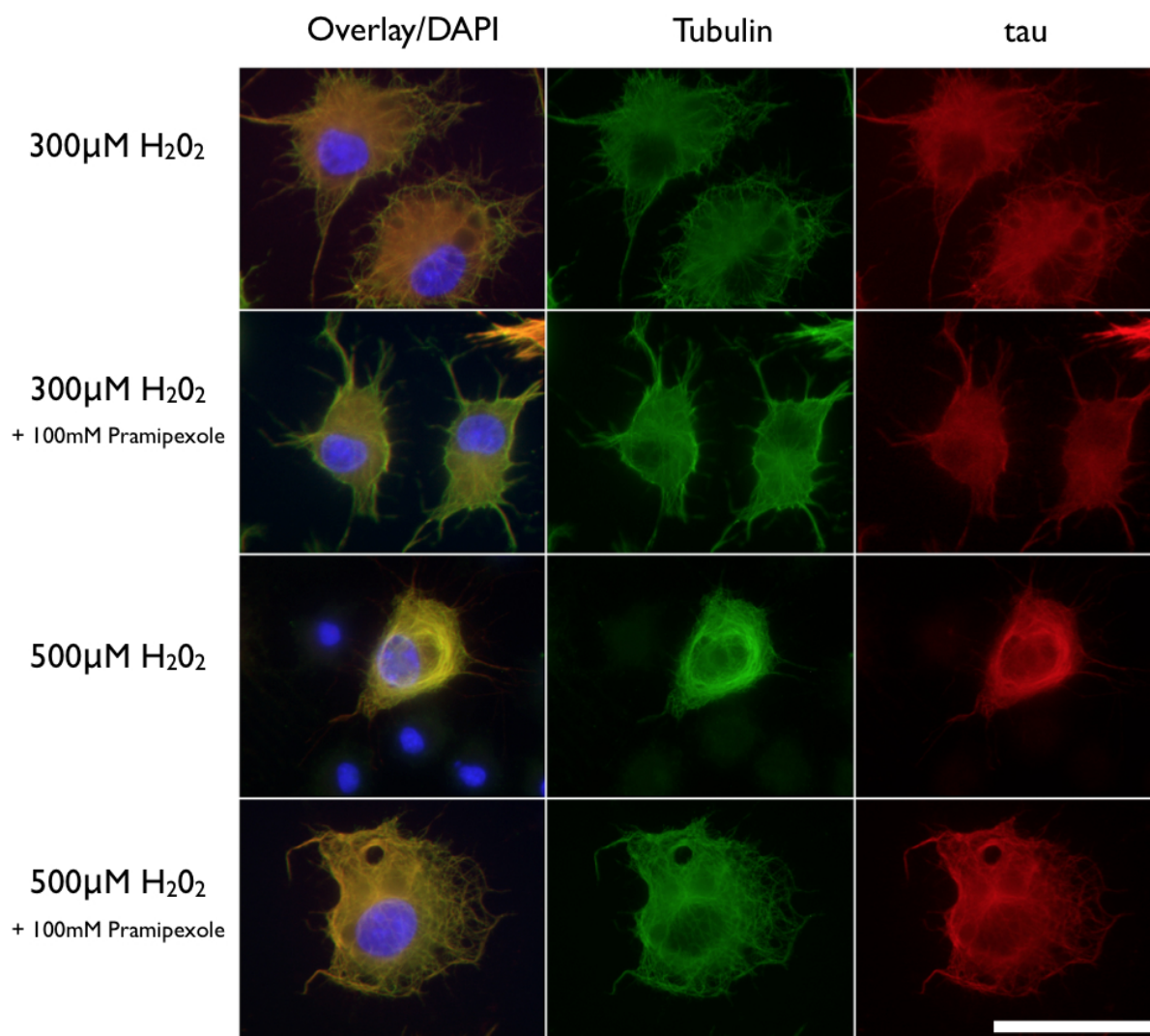
Figure 3.2: Indirect immunofluorescence of OLN-93 cells with antibodies against tubulin (green) and tau (red). Nuclei were stained with DAPI (blue). Cells were treated with indicated concentrations of H_2O_2, while either being pre-incubated with pramipexole, or not. Scale bar $20\,\mu$m.

When pramipexole pre-treated cells, incubated with H_2O_2 in the same manner, were compared to these findings, it could be seen that there was a less severe loss of peripheral processes, especially with the lower, $300\,\mu M$, H_2O_2 concentration. Processes seemed to retract less markedly, while the perinuclear density is likewise less evident.

3.1.2 Metabolic assessment of protective properties of pramipexole in the MTT assay

To assess whether pramipexole also prevents cytotoxic effects of H_2O_2, MTT assay was carried out. The MTT assay is a quantitative evaluation of the metabolic activity of the cell. 96-well-plates were loaded at a density of 10,000 OLN-93 cells per well and treated with four different concentrations of H_2O_2, without or with pre-incubation with pramipexole.
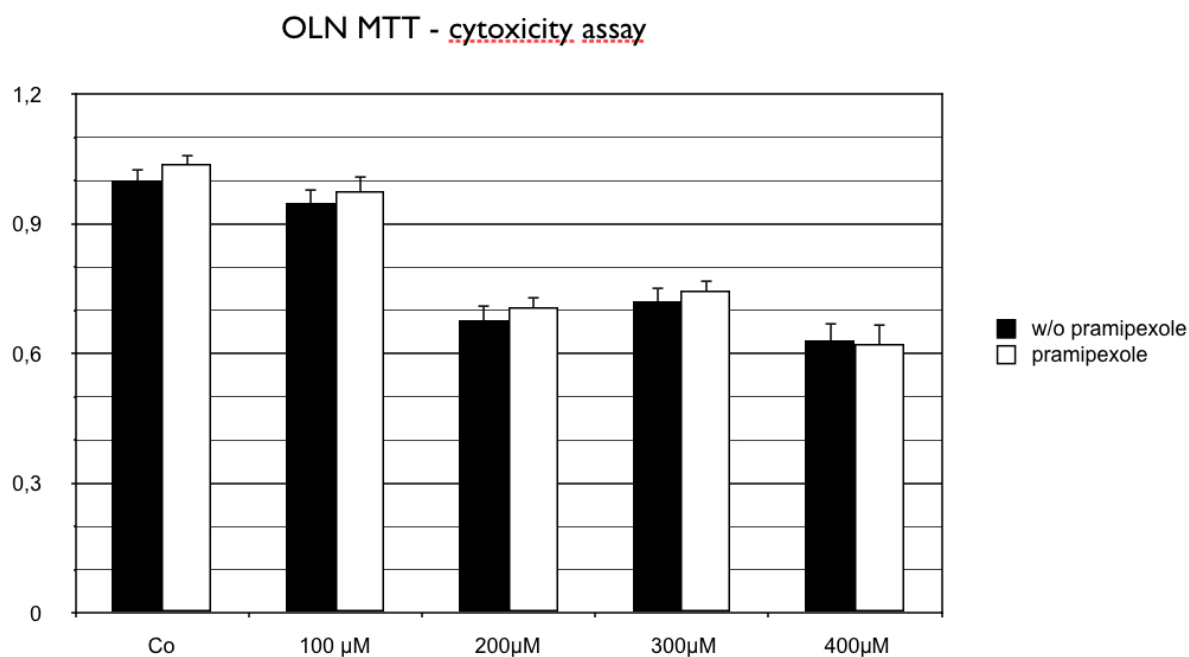
Figure 3.3: MTT-assay showing metabolic activity of OLN-93 cells after 16 hours of incubation with indicated concentrations of H_2O_2. Pramipexole-treated cells are compared to non-treated controls. Metabolic activity was normalized to the untreated control. Data are expressed as percentage of untreated controls and values represent the means +/- SD of 8 microwells each of two independent experiments (n = 16)

Although overall-toxicity clearly increased with higher concentrations of H_2O_2, there

were no significant differences between the pramipexole-pre-treated and non-treated groups. Marginally better survival could be measured up to $300\,\mu$M for the pramipexole-pre-treated groups of OLN-93 cells, while higher concentrations of H_2O_2 virtually induced the same toxicity-levels in both groups. Thus, pramipexole preserved cell morphology, it could not prevent cytotoxic effects of H_2O_2 on cell metabolism.

3.1.3   The protective effect of pramipexole in primary mixed glial cultures

It has been demonstrated before that oligodendrocytes in culture are specifically sensitive to oxidative stress exerted by H_2O_2 (Richter-Landsberg and Vollgraf 1998). To assess the protective function of pramipexole further, rat oligodendrocytes in primary mixed glial cultures were used. In the presence of astrocytes, these cells differentiate and express flat membranous sheets.

Pramipexole-treated oligodendrocytes in primary mixed glial cultures show no morphological differences when compared to non-treated controls

Figure 3.4 shows primary oligodendrocytes of a mixed glial culture that were stained with antibodies against myelin basic protein (MBP) and tubulin.

Untreated controls of oligodendrocytes, as represented in Figure 3.4, show no difference between pramipexole-treated and non-treated specimens.

Pramipexole protects oligodendrocytes morphologically in primary mixed glial cultures from oxidative stress

Oligodendrocytes are more vulnerable to oxidative stress induced by H_2O_2-administration when compared to OLN-93 cells. Experiments were therefore conducted with lower concentrations of the compound. At $100\,\mu$M, oligodendrocytes already show a clear loss of myelin integrity and cytoskeletal structure, as seen in Figure 3.5. When comparing these cultures to similarly stressed, but pramipexole-pre-treated cells, there are several differences to be noted.

At a concentration of $100\,\mu$M H_2O_2, pramipexole-treated cells clearly remain in a more intact condition, both in regard to flat membranous sheets as well as in regard to their

| Overlay/DAPI | MBP | Tubulin |

Figure 3.4: Indirect immunofluorescence of primary oligodendrocytes in a mixed glial culture with antibodies against MBP (green) and tubulin (red). Nuclei were stained with DAPI (blue). NC, negative control, untreated. Lower panel, oligodendrocytes incubated with pramipexole (100 mM, 24 h). Scale bar 20 μm.

cytoskeletal organization. Non-treated oligodendrocytes in turn, seem to suffer more profoundly in the periphery, where processes are exposed and form developing myelin. Patches of myelin-like sheets that are isolated peripherally only seem to be remnants of intact cellular structures. Perikarya are, at this stage, not patently impaired.

When oxidative stress is induced with an increased concentration of 150 μM H_2O_2, oligodendrocyte morphology is completely destroyed. The nucleus, however, remains intact. At this level of oxidative stress, pramipexole has pronounced protective effects: cell morphology is preserved and MBP-positive membranous sheets are observable. Striking as the depicted image in Figure 3.5 is, it has to be noted, that not all oligodendrocytes in this culture were saved to a similar degree. While the control showed no oligodendrocyte survivors, however, pramipexole was able to provide protection for at least 50 per cent of the cells.

After the treatment with 200 μM H_2O_2 no surviving oligodendrocytes and only completely shattered cellular remnants could be found. Pre-incubation with pramipexole (100 mM), partly rescued cells and patches of MBP-positive membranes were observable. Cellular nuclei with remnants of microtubuli were seen. Hence, pramipexole was capable of protecting

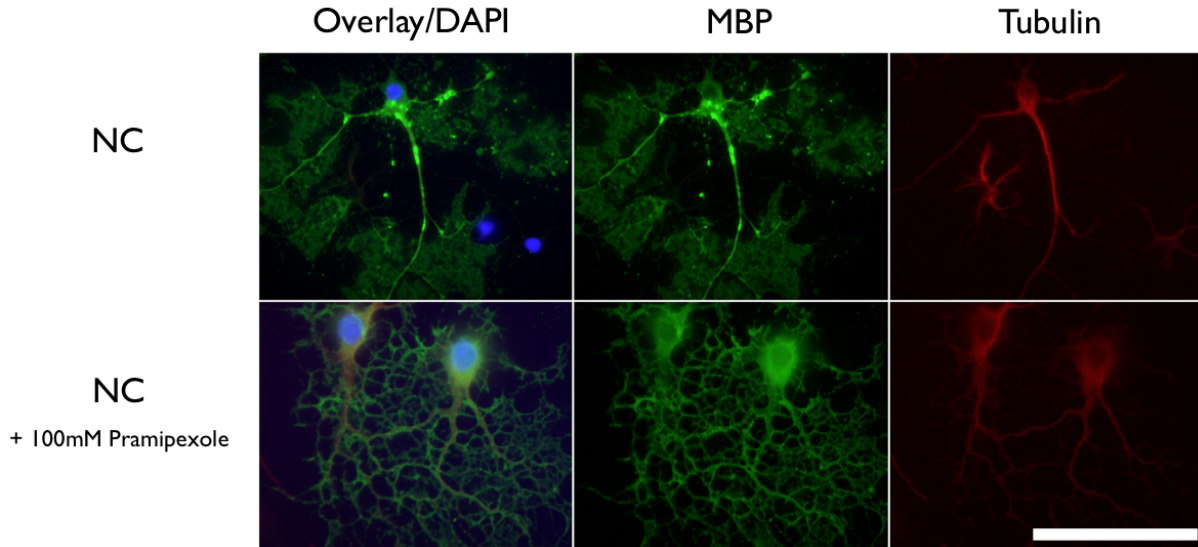oligodendrocytes from oxidative stress exerted by H_2O_2.
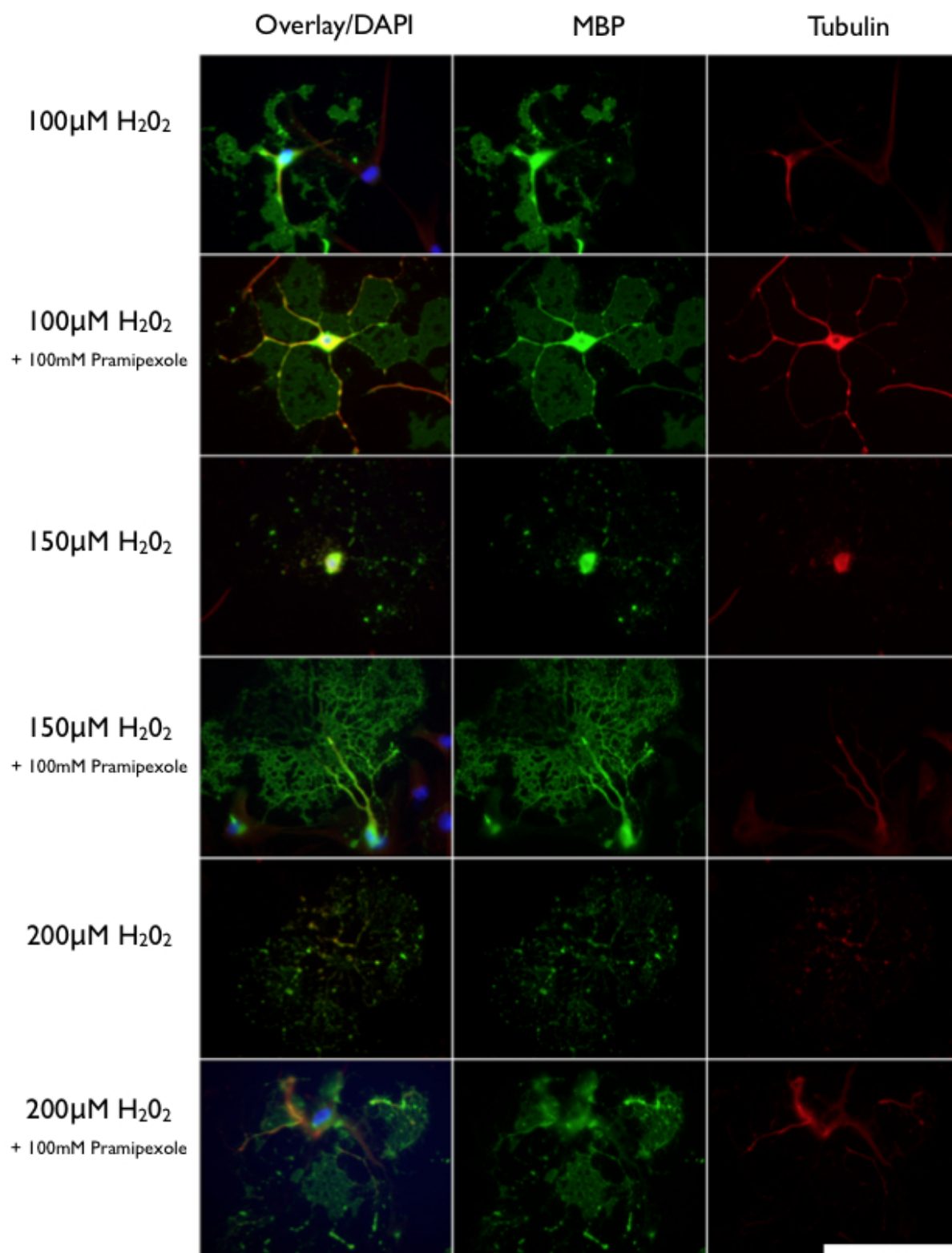
Figure 3.5: Indirect immunofluorescence of primary oligodendrocytes in a mixed glial culture with antibodies against MBP (green) and tubulin (red). Nuclei were stained with DAPI (blue). Cells were treated with indicated concentrations of H_2O_2, while either being pre-incubated with pramipexole or not. Scale bar $20\,\mu$m.

3.2 The endogenous effect of dopamine via D2- and D3-receptors

3.2.1 Demyelination in D_2-receptor deficient mice - no significant effect of genotype on the vulnerability to cuprizone treatment; females demyelinate less severely

To study demyelination in D_2-receptor deficient mice compared to wildtype animals, male and female mice were treated with cuprizone diet for six consecutive weeks, as described in chapter 2.2.2, after which they were sacrificed and analyzed for the degree of induced damage. Figure 3.6 shows the results of this analysis. Using two-way factorial ANOVA with Bonferroni posttest, no significant difference regarding the extent of demyelination in the medial corpus callosum (CC) between wildtype and D_2-receptor deficient mice was noted, neither in male nor in female mice ($F(1,48)=0.20$; $p=0.6592$). However, the overall extent of demyelination proved to be significantly different depending on the sex, with female mice showing less extensive demyelination ($F(1,48)=23.64$; $p<0.0001$). This effect was also observed in the lateral CC, although less pronounced ($F(1,48)=7.126$; $p=0.0103$). Again, no difference between the genotypes regarding the extent of demyelination was observed ($F(1,48)=2.43$; $p=0.1252$).

3.2.2 Remyelination in D_2-receptor deficient mice - D_2-receptor deficient mice and wildtype controls remyelinate similarly

In human demyelinating pathologies such as MS, phases of acute demyelination are typically followed by recovery periods in which inflammation cedes and remyelination begins (see chapter 1.2.2). The cuprizone model can be employed to imitate this sequence of events, when feeding periods, in our case six weeks, are succeeded by one week of normal diet to relieve the toxic effect of cuprizone on the oligodendrocyte metabolism. We addressed the question whether the genotype of D_2-receptor deficiency can itself affect remyelination and subsequently applied pramipexole treatment to analyze if the dopamine agonist had an effect, possibly receptor-mediated on remyelination in knockout animals and wildtype mice.

Figure 3.6: Demyelination in the medial and lateral corpus callosum of cuprizone-treated male and female mice with D_2-receptor deficiency versus wildtype controls. Values represent the mean semiquantitative myelination score +/- SD of each animal subgroup. Medial CC: genotype ($F_{(1,48)}=0.20$; p=0.6592); lateral CC: genotype ($F_{(1,48)}=2.43$; P=0.1252); medial CC: gender ($F_{(1,48)}=23.64$; p<0.0001); lateral CC: gender ($F_{(1,48)}=7.126$; p=0.0103). (CC, corpus callosum; DRD2-/-, D_2-receptor deficient animals; wt, wildtype; ns, no significant difference; *, p-value<0.05; ***, p-value<0.0001.)

After demyelination during six weeks of cuprizone diet, remyelination was initiated in D_2-receptor deficient mice and yielded the results graphed in figure 3.7 compared to wildtype animals. The medial and lateral CC were again evaluated separately. There were no significant statistical differences between knockout animals and the wildtype controls (genotype: $F_{(1,14)}=0.014$; p=0.91; treatment: $F_{(1,14)}=0.693$; p=0.41 in the medial CC – genotype: $F_{(1,14)}=0.007$; p=0.94; treatment: $F_{(1,14)}=0.172$; p=0.69 in the lateral CC).

3.2.3 Pramipexole treatment has no measurable effect on remyelination in the D_2-receptor knockout and in the wildtype

Under the applied conditions, pramipexole, as a potential neuroprotective agent, had no effect on remyelination (figure 3.7). Demyelination-scores of treated and non-treated animals were similar in all groups (treatment: $F_{(1,14)}=0.693$; p=0.41 in the medial CC - treatment: $F_{(1,14)}=0.172$; p=0.69 in the lateral CC).
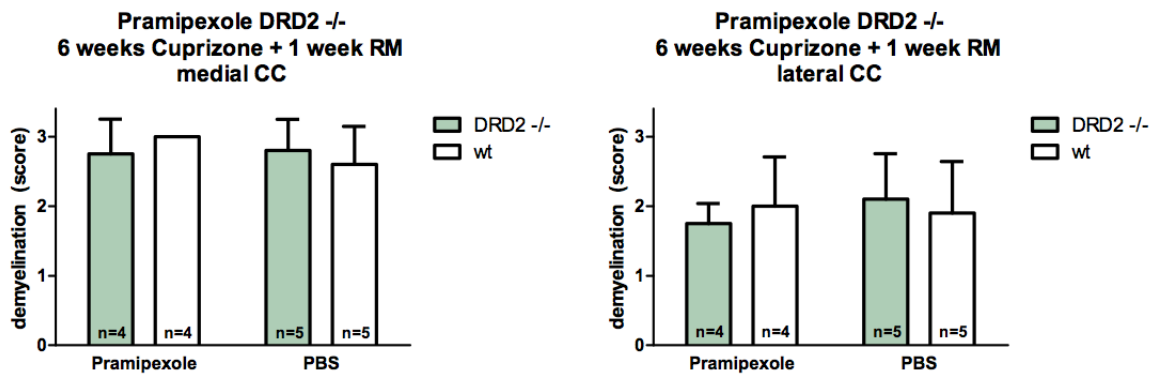
Figure 3.7: Remyelination after one week of regeneration in D_2-receptor deficient mice in the medial and lateral corpus callosum of cuprizone-treated male mice with D_2-receptor deficiency versus wildtype controls. Pramipexole-treated and PBS-treated animals in each group. Values represent the mean semiquantitative demyelination-score +/- SD in each animal subgroup. Medial CC: genotype: $F(1,14)=0.014$; $p=0.91$; treatment: $F(1,14)=0.693$; $p=0.41$ – lateral CC: genotype: $F(1,14)=0.007$; $p=0.94$; treatment: $F(1,14)=0.172$; $p=0.69$ (CC, corpus callosum; DRD2 -/-, D_2-receptor deficient animals; RM, remyelination; wt, wildtype).

3.2.4 Mature oligodendrocytes are negatively affected by the absence of the D_2-receptor as by treatment with pramipexole, while oligodendrocyte precursor cells are not

Immunohistochemical stains with primary antibodies directed at NogoA and Olig2 were then performed on brain slices of the same experimental series. Since remyelination is dependent on the metabolic functionality of oligodendrocytes, one of the leading interests was to see the reaction of this cell population to the toxic insult of cuprizone and their subsequent recovery. NogoA, staining mature oligodendrocytes (figure 3.8, left) revealed an extremely significant effect of genotype ($F(1,13)=26.27$; $p=0.002$) with higher amounts of positively stained cells in wildtype animals in both the pramipexole- ($p<0.05$, Bonferroni posttest) and PBS-treated groups ($p<0.01$, Bonferroni posttest). Furthermore, treatment with pramipexole significantly reduced NogoA-positive mature cells ($F(1,13)=28.84$; $p=0.0001$) in the wildtype ($p<0.01$, Bonferroni posttest) and in the knockout animals ($p<0.05$, Bonferroni posttest). Using two-way factorial ANOVA, OPCs targeted by the primary antibody Olig2 (figure 3.8, right) were shown to be significantly more numerous in the PBS-treated groups ($F(1,13)=8.34$; $p=0.018$) while genotype had no statistically significant effect ($F(1,9)=0.174$;
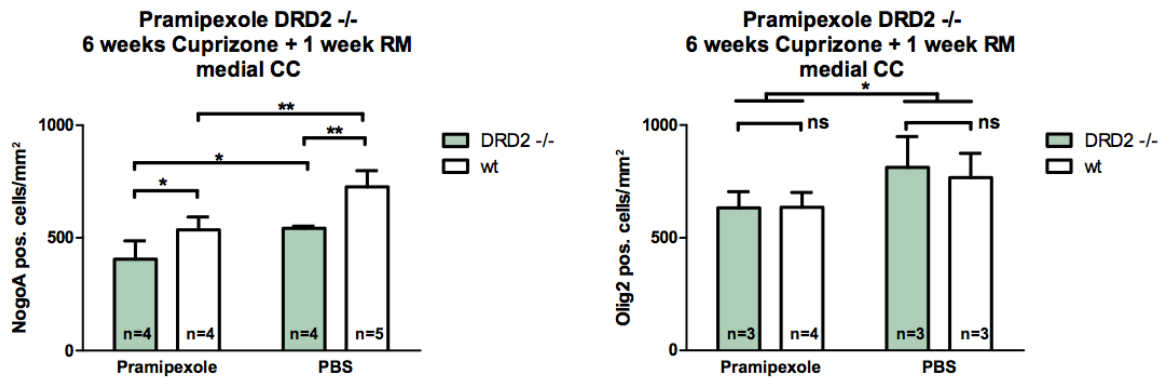
Figure 3.8: NogoA- and Olig2-positive oligodendrocytes after one week of regeneration in the medial corpus callosum of cuprizone-treated male mice with D_2-receptor deficiency versus wild-type controls. Pramipexole-treated and PBS-treated animals in each group. NogoA-positive cells: genotype: $F_{(1,13)}=26.27$; p=0.002, treatment: $F_{(1,13)}=28.84$; p= 0.0001 – Olig2-positive cells: genotype: $F_{(1,9)}=0.174$; p=0.69, treatment: $F_{(1,13)}=8.34$; p=0.018. Values represent the mean density of NogoA- and Olig2-positive cells +/- SD in each animal subgroup (CC, corpus callosum; DRD2 -/-, D_2-receptor deficient animals; RM, remyelination; wt, wildtype; *, p<0.05; **, p<0.01).

p=0.69).

In summary, cuprizone-experiments concerning D_2-receptor deficient mice reveal a receptor-independent effect of pramipexole the density of mature oligodendrocytes (NogoA-positive cells) and on oligodendrocyte precursor cells (Olig2-positive cells), reducing these significantly in both genotypes. At the same time, there is a reduction in mature oligodendrocytes when the D_2-receptor is not present in both treatment groups, suggesting a beneficial receptor-mediated, endogenous effect of dopamine via this pathway. This may imply a negative effect of pramipexole and a positive effect of the D_2-receptor's presence on oligodendrocyte maturation. However, our experiments do not show a significant effect of pramipexole treatment or D_2-receptor-deficiency on de- or remyelination in the disease model.

3.2.5 Demyelination in D_3-receptor deficient mice - no significant effect of genotype on the vulnerability to cuprizone treatment

The examination of demyelination in the medial and lateral CC of cuprizone-treated D_3-receptor deficient mice, when compared to control animals with the wildtype receptor gene,

as seen in Figure 3.9, showed no significant effect of genotype on demyelination (F(1,44); p=0.6464). Both groups demyelinated similarly in the medial as well as in the lateral corpus callosum.
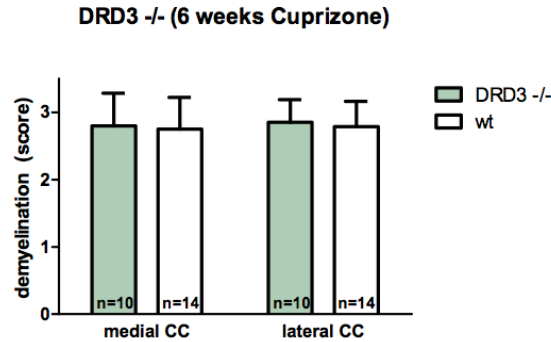
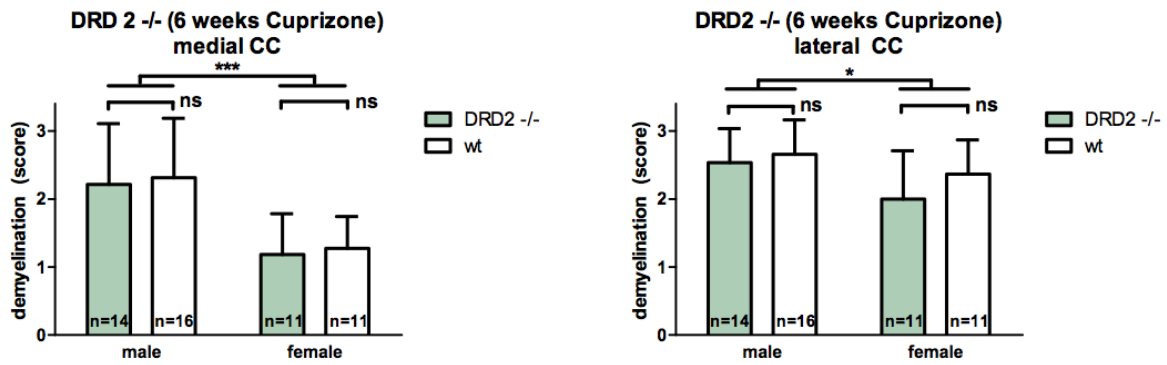Figure 3.9: Demyelination in the medial and lateral corpus callosum of cuprizone-treated male mice with D_3-receptor deficiency versus wildtype controls. Values represent the mean semiquantitative demyelination-score +/- SD in each animal subgroup. Genotype: (F(1,44); p=0.6464). (CC, corpus callosum; DRD3-/-, D_3-receptor deficient animals; wt, wildtype.)

3.2.6   Remyelination in D_3-receptor deficient mice - D_3-receptor deficient mice remyelinate less when compared to wildtype controls

Experiments to examine remyelination after six weeks of demyelination were conducted in D_3-receptor deficient mice. In comparison to wildtype controls they showed significant differences with better remyelination in case of expression of the D_3-receptor in the medial CC (genotype: F(1,28)=11.32; p=0.002) in the pramipexole- (p<0.05 Bonferroni posttest), but not in the PBS-treated group (p>0.05, Bonferroni posttest).

For verification, the experiment was repeated. However, because of a slightly less severe, nonetheless homogenous overall quantity of demyelination within the second experiment, the two independent, representative experiments were not pooled and are separately shown in figure 3.10. In the second experiment, in the medial CC, wildtype animals consistently remyelinated better when they expressed the D_3-receptor (F(1,33)=36.26;p<0.0001) in both the pramipexole- (p<0.01 Bonferroni posttest) and PBS-treated groups (p<0.001, Bonferroni posttests). Laterally the same results were reproduced in the second experiment

(F(1,33)=41,74, p<0.0001) with significantly better remyelination in presence of the receptor under pramipexole treatment (p<0.001, Bonferroni posttest) and under PBS (p<0.001, Bonferroni posttest).

3.2.7 Pramipexole treatment has no measurable effect on remyelination in the D$_3$-receptor knockout and in the wildtype

There was no reproducible significant effect of treatment on remyelination medially (F(1,28)=5.90; p=0.022 in experiment 1, F(1,33)=2.32; p=0.14 in experiment 2) or laterally (F(1,28)=0.45; p=0.51 in experiment 1, F(1,33)=0.23; p=0.64 in experiment 2).
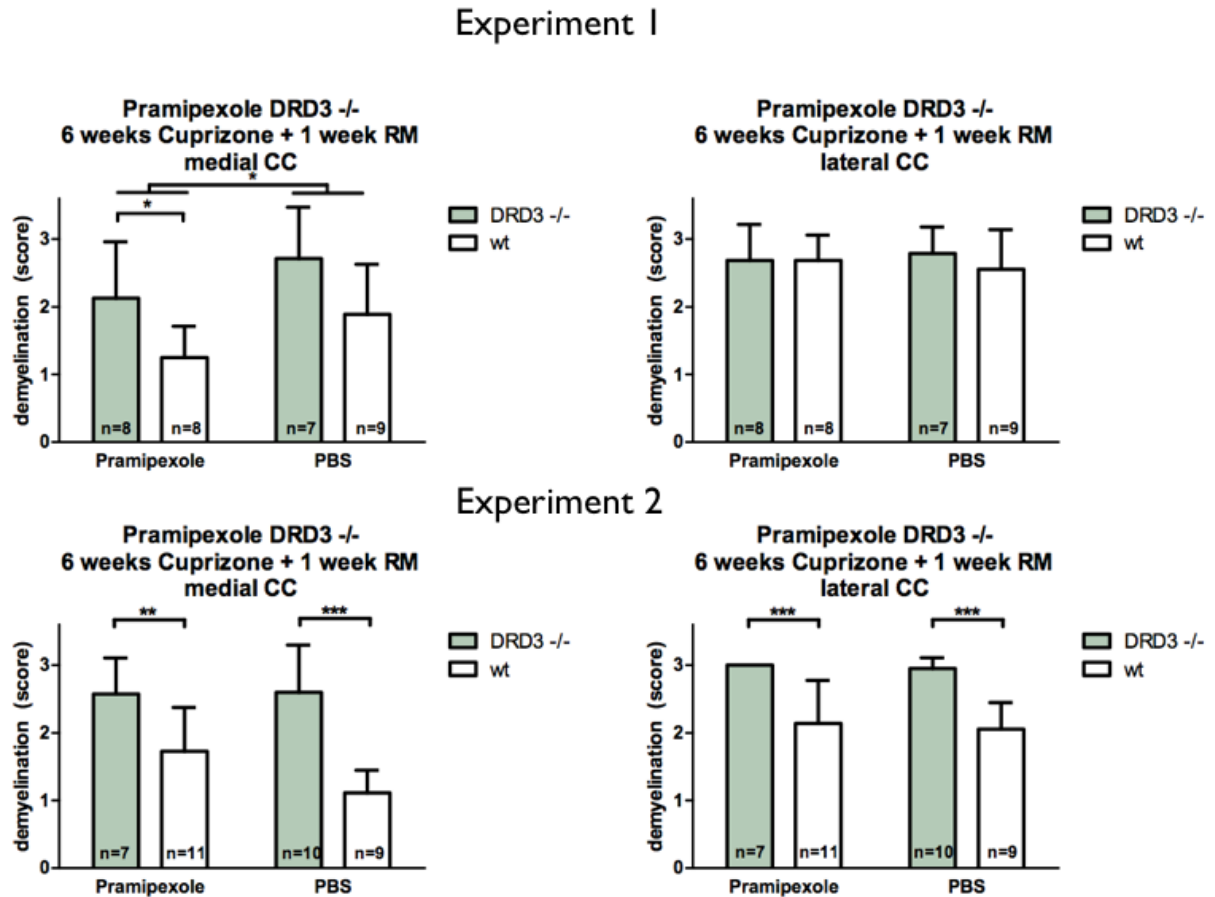
Figure 3.10: Remyelination after one week of regeneration in D_3-receptor deficient mice in the medial and lateral corpus callosum of cuprizone-treated male mice with D_3-receptor deficiency versus wildtype controls. Values represent the mean semiquantitative demyelination-score +/- SD in each animal subgroup. Pramipexole-treated and PBS-treated animals in each group. Two independent experiments are shown. Experiment 1: genotype: $F(1,28)=11.32$; $p=0.002$, medially; $F(1,28)$; $p=0.5094$), laterally. Experiment 2: genotype $F(1,33)=36.26$;$p<0.0001$, medially; $(F(1,33)=41,74$, $p<0.0001)$, laterally. (CC, corpus callosum; DRD3 -/-, D_3-receptor deficient animals; RM, remyelination; wt, wildtype; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.)

3.2.8 Mature oligodendrocyes increase in D_3-receptor deficient mice while oligodendrocyte precursor cells are reduced in a receptor-dependent manner by pramipexole treatment

Mature oligodendrocytes were significantly more numerous in the PBS-treated group of D_3-receptor deficient mice (F(1,26); p=0.0019). Olig2-positive OPCs were significantly reduced in their density by pramipexole treatment (F(1,25)=24.74; p<0.0001). Wildtype animals showed notably less Olig2-positive cells upon pramipexole treatment (p<0.001) while there was no difference in PBS-treated mice (p>0.05), which points to a receptor-dependent mechanism (see figure 3.11).
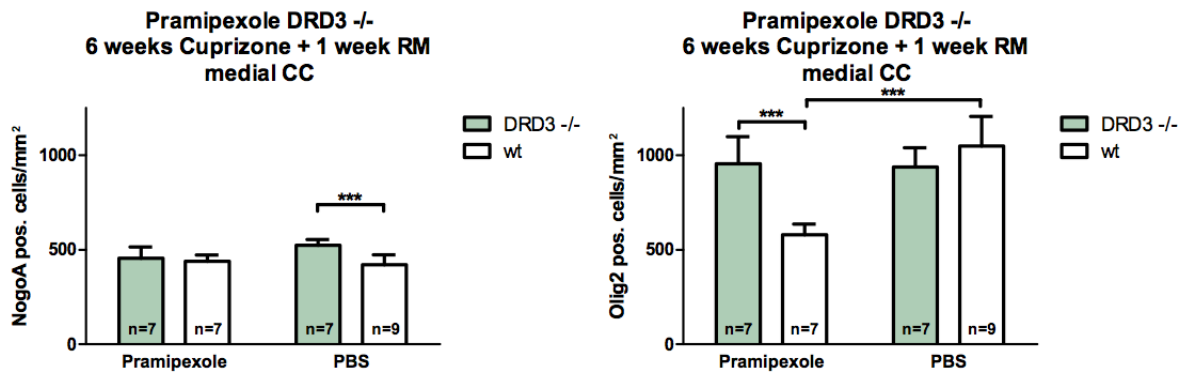
Figure 3.11: Counts of NogoA- and Olig2-positive oligodendrocytes in remyelination after one week of regeneration in D_3-receptor deficient mice in the medial corpus callosum of cuprizone-treated male mice with D_3-receptor deficiency versus wildtype controls. Pramipexole-treated and PBS-treated animals in each group. Values represent the mean density of NogoA- and Olig2-positive cells +/- SD in each animal subgroup (CC, corpus callosum; DRD3-/-, D_3-receptor deficient animals; RM, remyelination; wt, wildtype; ***, p < 0.001).

In summary, cuprizone-experiments in D_3-receptor deficient mice indicate a detrimental effect on remyelination in absence of this receptor. As documented by immunocytochemistry, pramipexole exerts a negative effect on oligodendrocyte precursor cell density in a receptor-dependent mechanism. Non-treated mice show more mature oligodendrocytes in the absence of the D_3-receptor. The negative effect of pramipexole on the density of NogoA-positive cells in wildtype mice was not reproduced.

Chapter 4

Discussion

Remyelination, following demyelination, can be regarded as means of neural plasticity, since they compose some of the most long-lasting and active processes of the central nervous system (Emery 2010). Although terminally differentiated oligodendrocytes lose their capacity to myelinate (Watkins et al. 2008), mature oligodendrocytes can be enabled to myelinate actively by intercellular communication (Rivers et al. 2008). The continuous activity of oligodendrocytes in the CNS and Schwann cells in the PNS, to enwrap neural axons throughout adulthood, then is of essential importance to physiological processes, such as learning (Fields 2005, Young et al. 2013). This significance is even more evident in the recuperation of functional losses due to pathologies, such as MS.

At the same time, mechanisms that regulate oligodendrocytes and OPCs are numerous and complex. Amongst them are a variety of extracellular ligands, such as the growth factors like neuregulin-1 or platelet-derived growth factors, secreted molecules and neurotransmitters, thus neural activity (Nave and Trapp 2008, Emery 2010). OPCs that have been found to build synapse-like junctions with glutamatergic neurons in the hippocampus (Bergles et al. 2000) furthermore mediate the intense communication between these two cellular families in the CNS, as has been also delineated in chapter 1.1.2.

As important as this interaction may be for myelin development, as important is myelin's own stabilizing function for neurons and neuritic owtgrowths, axons. Its decomposition has severe and diverse impacts on CNS functionality (Edgar and Nave 2009, Kassmann and Nave 2008).

Ever since glial cells were identified as the source of myelin, their regulation and behavior, development and reactivity to damage have been at the attention of neuroscientists. As many regulatory molecules and mechanisms have been identified, more and more detailed questions, as to their influence, have arisen. One group of regulatory systems that is least understood, is that of intrinsic neurotransmitters (Emery 2010). Dopamine (DA), one of the most abundant and wide-spread neurotransmitters (see also chapter 1.3), its agonists and receptors, have repeatedly been brought into context with neurotoxicity, neuroprotection and oligodendrocytes (Bongarzone et al. 1998, Bozzi and Borrelli 2006). In this manner, pramipexole, a selective DA-, especially D_3-receptor-agonist, became of interest in the exploration of myelination.

In cell cultures, oxidative stress is used in controlled models, to imitate limited nervous system injury. Oligodendrocytes are typically vulnerable to these sorts of insults and react with different defense mechanisms (French et al. 2009, Goldbaum and Richter-Landsberg 2001), many of which have also been reported in the analysis of MS patients' tissues (Birnbaum 1995). Despite this sensitivity, oxidative stress will not entirely correspond with the induction of demyelinating processes *in vivo*, which is the reason for the utilization of animal models, such as the cuprizone model (Matsushima and Morell 2001). The application of the toxic substance practically induces demyelination that ceases with its deprivation, leaving space for regeneration and remyelination by remaining and newly recruited oligodendrocytes.

This work targeted the understanding of oligodendrocytic behavior and regulation under damaging conditions. It was specifically oriented towards the analysis of the cell's reaction to oxidative stress, as a model for pathological insults, and the neurotransmitter DA, more precisely its target, the DA receptor as well as its agonist, pramipexole. Particularly, the interest of studying D_2- and D_3-receptor deficient animals was to get an insight into the *in vivo* regulation of de- and remyelination, employing the cuprizone-model of demyelination. To this day, there are only few published prior studies, addressing the DA receptors' and

its ligands' functions concerning myelination.

4.1 Oxidative stress in oligodendrocytic cells and its modulation by pramipexole

The effect of oxidative stress in oligodendrocytes and oligodendrocytic cell lines can be devastating, as apoptosis is activated and extensive alterations of protein expression arise in an attempt to rescue the cell (Richter-Landsberg and Vollgraf 1998, Goldbaum and Richter-Landsberg 2001). Detailed descriptions and experiences in the accurate dosing of the induction of oxidative stress in *in vitro* systems allow for the experimental use of substances, like H_2O_2, to study cellular responses to it.

Since there are several reports of the different ways by which pramipexole acts as a neuroprotectant (overview in figure 1.6), one of the experimental approaches in this study was to investigate the drug's effects in cell culture models of oxidative stress.

4.1.1 OLN-93-cells react with morphological destruction and cell death to oxidative stress and are partially rescued by pramipexole

The morphologic study of our H_2O_2-stressed OLN-93-cell cultures shows the typically severe impediment to cellular integrity and morphology. While the condensation of cellular processes and compaction of perinuclear tubulin was clearly reduced by the pre-treatment with pramipexole, cell survival itself was not significantly increased. Further studies are required to measure in detail the effects of pramipexole on oligodendrocytes undergoing oxidative stress.

While this protective effect is in accordance with the direct antioxidant capacity of pramipexole that has been repeatedly shown (Hall et al. 1996) and antioxidants are known to be capable of rescuing function in OLN-93-cells (Ernst et al. 2004), it has also been postulated, that DA receptors, as targets of the molecule pramipexole, are involved in the substance's protective effects (Ling et al. 1999). This may function through regulatory influences on apoptotic pathways (Kakimura et al. 2001). It remains to be clarified whether these mechanisms can actually be implied in the effects that were qualitatively seen in the our cell

culture experiments. The expression of D_2- and D_3-receptors has still to be shown on the OLN-93-cell's outer membrane, although studies have shown that haloperidol, a known blocker of DA receptors, primarily of the type D_2, has specific protective effects in OLN-93-cells (Steiner et al. 2010). In accordance with our findings this study reported a protection from energy deprivation in this cell population. Additionally, haloperidol exerted protective effects in nutrient deprivation models (Steiner et al. 2011). Although this may seem surprising, the neurotoxic effects of DA itself are in known contrast to the DA-receptor's potential neuroprotective effects (Filloux and Townsend 1993, Ziv et al. 1994). This toxicity, a result of DA's autooxidative and excitotoxic abilities, may in part be counteracted by the agonist-induced function of DA receptors, which protects neural and glial cells.

4.1.2 Oligodendrocytes in primary mixed glial cultures are less damaged by oxidative stress after pramipexole pre-treatment

Primary oligodendrocytes in mixed glial cultures affirm the observations made with OLN-93-cells. These native cells are much more vulnerable to oxidative stress, which is the reason for a reduction of concentrations of the applied H_2O_2 to less than half, while pramipexole was added consistently at a concentration $100\,mM$ concentration to the cultures. Under these conditions the protective potential of pramipexole was quite striking, as denoted in chapter 3.1.3 and figure 3.5. Cellular foot processes and spread-out myelin sheets were better preserved in cultures preincubated with pramipexole.

The timepoint of the application of pramipexole in respect to the application of H_2O_2 leaves room for alteration. While the incubation 30 minutes prior to application of H_2O_2 was consistently adhered to in order to leave time for possible receptor activation and intracellular cascades to be activated, it may not have been sufficient to provide a full range of intracellular protective mechanisms. We could assume the D_2- and D_3-receptors' presence in our model systems as publications of Howard et al. (1998) and Bongarzone et al. (1998) claim these during development on membranes of oligodendrocytes. More importantly, Rosin et al. (2005) detected mRNA for both receptor types in rat oligodendrocytes and successfully stained both proteins directly with primary antibodies in immunocytochemistry. There

are, however, no published studies so far that characterize the mode of neuroprotection that is activated by DA's or DA-agonists.

4.2 Influence of D_2- and D_3-receptors on de- and remyelination in the cuprizone model

A recent publication addressed the link between oligodendrocyte malfunction and myelination to dopamine circuits (Takahashi et al. 2011). The authors reflect on the fact that induction of demyelination in the cuprizone model induced a higher concentration of DA in the prefrontal cortex (Xu et al. 2009) and the earlier discovery that DA itself may initiate and promote myelination and be beneficial to cell survival (Belachew et al. 1999). This would not be in line with the protective effects postulated by Bellucci et al. (2008), who believe the transfer of DA to the intracellular compartment to be protective by a decrease of extracellular DA-toxicity. It does, however, raise the interesting point that both toxicity and protection may be part of a delicate equilibrium. It is, at any rate, evident that the dopaminergic system on the one hand and oligodendrocytes and myelin on the other are closely linked and interdependent.

In order to study the interplay of dopamine receptors and their role in myelinating and remyelinating processes, this study further investigated their impact in the cuprizone model for de- and remyelination.

4.2.1 Dopamine receptors of the type D_2- and D_3 do not influence demyelination in the cuprizone model – demyelination shows gender-differences

The feeding of cuprizone as an additive to normal animal diet leads to demyelination processes that are controllable by the duration and concentration of this addition (Matsushima and Morell 2001). These demyelinating processes primarily attain the CC and white matter tracts, although it has been shown that cerebral cortex reacts in a comparable manner that is delayed and qualitatively distinguishable by the inflammatory composition (Gudi et al. 2009). Prolonged administration of cuprizone may lead to what is commonly referred

to as "chronic demyelination", as opposed to "acute demyelination", which is considered to be a result of five to six-week intervals of feeding of the neurotoxicant. Even during an interval of this relatively short period of time, however, it is known that oligodendrocytes react with the down-regulation of genes encoding proteins that are essential in the formation of myelin, such as MAG and MBP (Kipp et al. 2009). It therefore cannot be regarded merely as an acute reaction to a toxic insult. Oligodendrocytes will begin with apoptotic degradation after one to two weeks of treatment.

The extent of demyelination after six weeks of cuprizone-feeding showed neither a difference between the wildtype controls and D_2-receptor deficient animals, nor between wildtype animals and D_3-receptor deficient knockouts. This may not come as a great surprise, considering that, despite the fast gene-regulating mechanisms mentioned above, the cuprizone insult is rather acute and directly targeting oligodendrocytes. There has not been so far an indication as to how the acute demyelination could be dependent on neurotransmitter action (other than the mentioned transfer of DA into cells and consecutive reduction of its extracellular cytotoxicity) or receptor activation, which is why our *in vivo* experiments were targeted mainly at differences in recuperation after acute demyelination and oligodendrocyte differentiation.

An interesting finding of our study was a difference in the extent of demyelination in male and female mice in the medial CC. In both experimental groups, the D_2-receptor knockout and the wildtype controls, females demyelinated less under the toxic influence of cuprizone (see chapter 3.2.1). A study by Cerghet et al. (2006) demonstrates gender differences in the regulation of oligodendrocyte and myelin survival, stating a larger number of oligodendrocytes in the male CC than in the female's, which is incongruent with our results. Gender-dependent differential characteristics of myelination have been known for some time. Cuprizone could have a more severe impact in males, as more myelinated fibers are present in the CC of male animals (Mack et al. 1995, Nuñez et al. 2000). However, it is not clear how a higher number of target cells for the toxin should lead to more pronounced damage.

4.2.2 Presence and activation of the D_2-receptor does not have an effect on remyelination

The D_2-receptor has been isolated from many cortical and subcortical brain regions (see chapter 1.3.3). *In vitro* studies show the receptor's potential to protect neurons from glutamate excitotoxicity (Kihara et al. 2002) and to ease glutamate-induced oxidative stress in mesencephalic neurons (Sawada et al. 1998). Additionally, other authors have demonstrated the neuroprotective potential of ligands to this receptor (O'Neill et al. 1998, Kitamura et al. 2003, Bozzi and Borrelli 2006). Dijkstra et al. (1994) for instance showed that the treatment with the D_2-agonist bromocriptine had a beneficial effect on clinical signs in experimental models of multiple sclerosis. At the same time they do not specify histologically what the morphological correlate of this benefit is. In our study, we did not observe a difference in remyelination levels in D_2-receptor deficient mice and wildtype controls, as quantified in LFB/PAS stains, after a demyelinating insult. Although this is surprising in light of the previously published data concerning the D_2-receptor, this negative result may in our case be partially to the experimental conditions, which imply a toxic insult and delay of histological analysis thereafter, where there is possibility of variation. At the same time, it may be necessary to conclude that the D_2-receptor does not have the significant impact on remyelination that one would have expected.

4.2.3 Presence and activation of the D_3-receptor has a beneficial effect on remyelination

The absence of the D_3-receptor showed a negative effect on remyelination efficacy, which implies a positive receptor-mediated effect on remyelination. Taking into account data showing the D_3-receptor's expression to be increased in young oligodendrocytic cultures, but subsequently decreased to complete absence in mature cells (Bongarzone et al. 1998), this may support an interesting point. If D_3-receptor levels and their activation correlate negatively with the maturity of oligodendrocytes, wildtype mice could benefit more from the myelinating and proliferating activity of immature, differentiating cells as stated by Peters and Sethares (2004). Later, as mature oligodendrocytes have developed, they would

stabilize intact myelin (Islam et al. 2009).

4.2.4 Presence and activation of dopamine receptors has an influence on oligodendrocyte differentiation

A proposition by Howard et al. (1998) and Bongarzone et al. (1998) stating possible complementary roles for the D_2- and D_3-receptors, may deliver a plausible model to explain the DA receptors' influence in oligodendrocytic differentiation and thus in myelination (compare chapter 4.2.3). By regulatory action through the receptor, especially D_3, OPCs could be induced to increase in number, or rather be delayed in their maturation to oligodendrocytes and continue to form large membranous sheets, thus being beneficial to myelinating processes.

Pramipexole, which predominantly acts on D_3-type receptors, qualitatively did not show any effect on myelin sheet formation in our *in vitro* models (see 3.1), but it had a stabilizing effect on oxidatively stressed cells. This effect was not differentiated further to a receptor-mediated or direct effect of the substance with the conducted cell-culture experiments.

However, the *in vivo* experiments of this study support the notion that the dopamine-receptors have an effect on oligodendrocyte differentiation (compare chapters 3.2.4 and 3.2.8). They showed opposite effects of the two receptors examined on the presence of mature oligodendrocytes. NogoA-positive cells were reduced in D_2-deficient animals and increased in D_3-deficient mice. The latter result concurs with the aforementioned theory of a link between oligodendrocyte maturity and D_3-receptor expression in these cells (compare chapter 4.2.3) and with the postulated positive effect on (re-)myelination of the receptor which may be a direct consequence. The D_2-receptor's effect on presence of mature oligodendrocytes, namely an increase, has no apparent effect on myelination, as we found no difference in receptor-deficient animals. Concluding from our data, the myelin-directed effect of dopamine receptors seems to be limited to D_3-type receptors, which would again be in line with the scarce published indications of links of D_2-receptors to myelination (see chapter 4.2.2), even if there is clinical evidence for protection from demyelinating disease in

animals (Dijkstra et al. 1994).

It is not too far-fetched to assume that complex regulatory changes occur under the toxic influence of cuprizone and numerous intercellular reactions follow. Of course, the deficiency of dopamine receptors in the knockouts is not reduced to glial cells or even oligodendrocytes, making it difficult to judge the missing receptor's effect independently of the other, large dopamine-dependent cell populations. They could in turn reflect on the differentiation of oligodendrocytes, as their function is notably dependent on axonal activity (Miller 2002). A logical approach to answer some of these questions could be to alter the time points of histological and immunohistochemical analyses in the different experimental groups of mice. One could expect typical expression- and development-patterns in oligodendrocytic populations characterized chronologically. To study more specifically the role of DA receptors on oligodendrocytes and their relation to the development of glial cells, it may be interesting to study cell-specific knockouts for the different receptors.

4.3   Influence of pramipexole on de- and remyelination as well as on oligodendrocyte differentiation

Pramipexole's discovery as not only an antioxidant of high capability (Hall et al. 1996) which could be expected to have an effect on oligodendrocytic cells (Ernst et al. 2004), but also as a selective DA agonist and preferred ligand to D_3-receptors, nurtured the interest in its capacity to affect damage and regeneration of myelin. As mentioned earlier, dopamine circuits were repeatedly brought into context with oligodendrocyte function and malfunction (Takahashi et al. 2011). Pramipexole's extensive application in Parkinson's disease, where it compensates DA loss, makes pramipexole and its side-effects well-known to the medical community, which facilitates assessments concerning its application *in vivo*.

4.3.1 Pramipexole-treatment in cuprizone mice does influence remyelination but expression of mature oligodendrocytes and their precursors

Even though pramipexole's effects in the cell culture experiments of this study were hinting to potentially promising effects on oligodendrocytic damage under external impairment, no data in support of this theory could be collected in course of the cuprizone experiments. In the conducted experiments involving D_2-receptor-deficient animals, presented in chapter 3.2.2, no effect on the extent of de- and remyelination could be recorded.

Conversely, immunohistochemical cell-counts in D_2-receptor-experiments revealed a negative effect of pramipexole on the density of mature oligodendrocytes (see figure 3.8, left, compare wildtypes), which could not be reproduced in D_3-receptor-experiments (see figure 3.11, left, compare wildtypes). Interestingly, pramipexole proved to negatively affect, by a D_3-receptor-mediated mechanism, the presence of OPCs (figure 3.11, right). This comes somewhat as a surprise, given the proposition that the D_3-receptor was expected to up-regulate myelinating precursors in accordance with a positive effect on myelination, but it importantly shows a direct effect of this DA agonist on oligodendroglia density that has not been reported previously. The account of DA's toxic effects by activation of intracellular apoptotic processes (Hemdan and Almazan 2008) raises the question whether its agonist has similar capabilities. Mi et al. (2009) propose an effect of oligodendrocyte differentiation on remyelination in the cuprizone model which they tested for by the application of other reagents.

4.3.2 Does pramipexole promote OPC differentiation?

In spite of other reports and earlier belief in the potential of pramipexole to reduce the extent of demyelination, this study does not provide any data to support these. Pramipexole did not trigger any detectable protective mechanisms that would show as significant readout-parameters in our model system of de- and remyelination. It is evident, however, that pramipexole has an effect on oligodendrocytic differentiation. It may well be that a potential effect, with respect to the chronologic expression of D_2- and D_3-receptors as proposed by Bongarzone et al. (1998), so far remains hidden. The positive effects on oxidative toxicity

in cell cultures are concordant with results presented by Rosin et al. (2005), but they do not seem to be transferable in a direct line to the cuprizone animal model for de- and remyelination.

Chapter 5

Summary

Oligodendrocytes are of crucial importance for the integrity and function of the central nervous system (CNS) as well as for efficacy of signal conduction and thus for brain function itself. They are responsible for axonal integrity in the CNS (Nave and Trapp 2008) and a target for pathological changes in diseases of myelin, such as multiple sclerosis (MS), the most common chronic neurological disorder in western societies (Hafler et al. 2005).

Receptors of the catecholamine neurotransmitter Dopamine (DA) have been reported to exert neuroprotective function (Bongarzone et al. 1998, Missale et al. 1998). The DA agonist pramipexole, in addition to having direct antioxidative effects (Le et al. 2000, Zou et al. 1999), is known to stimulate these receptor-mediated neuroprotective effects (Ling et al. 1999).

This study addresses the protective effects of pramipexole in cell cultures of the OLN-93 cell-line and primary oligodendrocytes in mixed glial cultures as well as in the cuprizone mouse model for central demyelination. It furthermore analyzes receptor-mediated influence on demyelination and remyelination in the cuprizone model by use of D_2- and D_3-receptor-deficient mice (D_2-/-, D_3-/-).

Both OLN-93 cells and oligodendrocytes in primary mixed glial cell cultures were morphologically preserved when treated with pramipexole before being exposed to oxidative stress. There was, however, no significant difference in survival of pramipexole-treated

OLN-93 cells in the MTT-assay.

There was no difference in de- and remyelination or oligodendrocyte differentiation in D_2-/-animals compared to the wildtype. Demyelination in D_3-/- mice was not different from that in the wildtype. However, remyelination was less efficient in D_3-/- mice. Treatment with pramipexole did not influence the extent of remyelination. Less oligodendrocyte precursor cells were observed in pramipexole treated wiltypes but not in D_3-/- mice, suggesting a receptor-mediated effect.

This work constitutes the first study to approach the role of the DA system in the cuprizone mouse model. It shows that pramipexole partially rescues oligodendrocytic cells from oxidative stress. While the lack of D_2- and D_3-receptors does not influence demyelination, remyelination is hampered in D_3-/- mice. However, pramipexole-treatment does not alter the extent of de- and remyelination in cuprizone-treated mice, although it leads to a decrease in the number of oligodendrocyte precursor cells.

Appendix A

Lab Protocols

A.1   Histology - Protocols

Table A.1: Hematoxylin-Eosin staining protocol - modified from lab protocols

	Step	Time	
1.	Deparaffination	3 x 4 min	Place sections in xylene bath
2.		4 min	Place sections in iso-xylene bath
3.	Hydration	2 x 3 min	Hydrate sections in alcohol (100%)
4.		3 x 2 min	Hydrate sections in serially diluted alcohol (90,70,50%)
5.		2 min	Place sections in Aqua dest
6.	Coloration	8 min	Place sections in Mayer's Hematoxylin bath
7.			Dip sections in Aqua dest
8.	Differentiation		Dip sections in HCl-Alcohol
9.	Blueing	10 min	Rinse sections under running tap water
10.	Coloration	5 min	Place sections in Eosin bath
11.		2 min	Place sections in Aqua dest
12.	Dehydration	3 x 2 min	Dehydrate sections in serially diluted alcohol (90,70,50%)
13.		2 x 3 min	Dehydrate sections in alcohol (100%)
14.		4 min	Place sections in iso-xylene bath
15.		3 x 4 min	Place sections in xylene bath
16.	Mounting		Mount sections with DePex

Table A.2: Luxol Fast Blue-PAS staining protocol - modified from lab protocols

	Step	Time	
1.	Deparaffination	3 x 4 min	Place sections in xylene bath
2.		4 min	Place sections in iso-xylene bath
3.	Hydration	2 x 3 min	Place sections in alcohol (100%)
4.		2 min	Place sections in alcohol (90%)
5.	Myelin Coloration	Overnight	Place sections in LFB-Solution
6.			Place sections in alcohol (90%)
7.	Differentiation		Dip sections in 0.05% Lithiumcarbonate solution
8.			Dip sections in alcohol (70)%
9.			Rinse sections in Aqua dest
10.			Repeat steps 9-7, 7-9 as many times as needed
11.	PAS Coloration	5 min	Place sections in periodic acid
12.	Blueing	5 min	Rinse sections under running tap water
13.			Rinse sections in Aqua dest
14.		20 min	Place sections in Schiff's reagent
15.		5 min	Rinse sections under running tap water
16.	Counterstaining	2 min	Place sections in Mayer's Hematoxylin bath
17.			Dip sections in Aqua dest
18.			Dip sections in HCl-Alcohol
19.		5 min	Rinse sections under running tap water
20.		2 min	Place sections in Aqua dest
21.	Dehydration	3 x 2 min	Dehydrate sections in serially diluted alcohol (90,70,50%)
22.		2 x 3 min	Dehydrate sections in alcohol (100%)
23.		4 min	Place sections in iso-xylene bath
24.		3 x 4 min	Place sections in xylene bath
25.	Mounting		Mount sections with DePex

Table A.3: Immunohistochemistry for single antigen labelling staining protocol - modified from lab protocols. Day 1

	Step	Time	
1.	Deparaffination	3 x 4 min	Place sections in xylene bath
2.		4 min	Place sections in iso-xylene bath
3.	Hydration	2 x 3 min	Hydrate sections in alcohol (100%)
4.		3 x 2 min	Hydrate sections in serially diluted alc. (90,70,50%)
5.		2 min	Place sections in Aqua dest
6.			Rinse sections with Aqua dest until foam disappears
7.	Pre-treatment	5 x 3 min	Place sections in 10 mMol Citrate buffer (pH 6) and heat in microwave
8.			Place sections in cuvette with phospate buffer (1x)
9.	Block endogenous peroxidase	10 min	Place sections in 3% H_2O_2/PBS
10.			Rinse several times with PBS
11.	Coverplate		Transfer to Coverplate system Check tight fit with PBS twice
12.	Block unspecific binding	10 min	Add $120\mu l$ 10% FCS/PBS to each plate Do NOT rinse afterwards!
13.	Primary antibody (AB)		Add $120\mu l$ of appropriate dilution of primary AB Incubate overnight at 4ºC

Table A.4: Immunohistochemistry for single antigen labelling staining protocol - modified from lab protocols. Day 2

	Step	Time	
14.			Rinse with PBS
15.	Secondary AB	60 min	Add $120\mu l$ of appropriate dilution of biotinylated, secondary AB - Incubate at room temperature
16.			Rinse twice with PBS
17.	Peroxidase	45 min	Add $120\mu l$ of 1:1000 Avidin-Peroxidas (Sigma) Incubate at room temperature
18.			Rinse with PBS
19.			Place sections in upright cuvette
20.	Developing	Under sight	Develop in upright cuvette with:
			1 ml DAB stem solution,
			50 ml PBS,
			$20\mu l$ $3\%H_2O_2$
21.			Rinse sections with Aqua dest
22.	Amplification	10 min	Place sections in $CuSO_4$-solution
23.			Rinse sections with Aqua dest
24.	Counterstaining	30 sec	Place sections in Mayer's Hematoxylin bath
25.			Dip sections in Aqua dest
26.	Differentiation		Dip sections in HCl-Alcohol
27.	Blueing	7 min	Rinse sections under running tap water
27.	Dehydration	3 x 2 min	Dehydrate in serially diluted alc. (90,70,50%)
28.		2 x 3 min	Dehydrate in serially diluted alcohol (100%)
30.		4 min	Place in iso-xylene bath
31.		3 x 4 min	Place in xylene bath
32.	Mounting		Mount sections with DePex

A.2 PCR

Table A.5: PCR - Drd2 knockout genotyping

	Volume for 1 mouse	Step	PCR temperature	Time
Buffer	2.4μl	1.	94°C	3min
dNTPs (1:4)	0.96μl	2.	94°C	20sec
oIMR013 (1:10)	0.3μl	3.	64°C	30sec
oIMR014 (1:10)	0.3μl	4.	72°C	35sec
oIMR991 (1:10)	0.3μl		Repeat 2–3 x12	
oIMR992 (1:10)	0.3μl	5.	94°C	20sec
Tag-Polymerase	0.06μl	6.	58°C	30sec
DMSO	0.12μl	7.	72°C	35sec
Aqua bidest.	5.26μl		Repeat 5–7 x25	
Tissue lysate	2μl	8.	72°C	2 min
		10.	4°C	Stop
Total	10μl	Duration		1:51:01h

PCR for Drd2 knockouts: PCR machines operated according to a program, similar to the 10 step cycle depicted on the right.

Appendix B

List of abbreviations

AB	Antibody
AC	Adenylyl cyclase
APAF 1	Apoptotic protease activating factor 1
Bcl-2	B-cell lymphoma 2
cAMP	Cyclic adenosine monophosphate
CC	Corpus callosum
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
DA	Dopamine
DAT	Dopamine transporter proteins
DMEM	Dulbecco's Modified Eagle's Medium
DRD2	Dopamine receptor type 2
DRD3	Dopamine receptor type 3
D_2-/-	Dopamine receptor type 2 deficient
D_3-/-	Dopamine receptor type 3 deficient
EAE	Experimental autoimmune encephalomyelitis
FCS	Fetal calf serum
HSP	Heat shock protein
HSP/SPG	Hereditary spastic paraplegia
IP_3	Inositol-1,4,5-triphophate
MAG	Myelin-associated glycoprotein
MOG	Myelin-oligodendroglial glycoprotein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NE	Norepinephrine = Adrenaline
OPC	Oligodendrocyte precursor cell
PD	Parkinson's disease
PBS	Phospate buffered saline
PLL	Poly-L-Lysine
PLP	Proteolipid protein

PNS	Peripheral nervous system
PP	Primary-progressive
PLL	Poly-L-Lysine
PLP	Proteolipid protein
PNS	Peripheral nervous system
PP	Primary-progressive
RM	Remyelination
RR	Relapsing-remitting
SP	Secondary progressive
VEP	Visual evoked potential
VTA	Ventral tegmental area
wt	Wildtype

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Acknowledgments

In order to finish the experimental work and to succeed in the writing of this thesis, I relied on the invaluable help and unconditional support of distinguished characters and institutions. I would like to deeply thank them:

Prof. Dr. med. C. Stadelmann-Nessler, my supervisor and guide throughout the development of this work, whom I have known now for ten years and who is to me an achieved scientist and friend. In my eyes, amongst many other qualities, you know how to prioritize in the struggle of family and career and it is for this that you have become a role model.

Prof. Dr. rer. nat. C. Richter-Landsberg, who honored me with the possibility to perform the cell culture experiments of this thesis in her laboratory as well as with many comments and improvements to my work as a whole. You have not only been of scientific guidance throughout my professional life, but you have simply always been there.

Prof. Dr. med. W. Brück, in his kind and professional manner allowed for my early experiences in the field of Neurosciences and the possibility to perform many of this work's experiments in his department.

Members of both the labs of the Department of Neuropathology at the University of Göttingen and of the Department of Molecular Neurobiology at the University of Oldenburg, especially the technical staff, introduced me to modern microbiological and biochemical methods and helped with the data aquisition.

The IMPRS for Neurosciences in Göttingen in which I was able to intensify my scientific experience in a manner that I could otherwise not have hoped for. It was an honor to participate in the program.