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DISSERTATION

Progesterone's Activity in the Central Nervous System: A Murine Model Study on Gliotoxin Injured Myelin of the Spinal Cord

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von

Victoria Tüngler

aus Marburg

Gutachter/in:

1. Prof. Dr. rer. nat. Rüdiger Schade
2. Prof. Dr. Alejandro De Nicola
3. Prof. Dr. med. Thomas Meyer

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It's only through refined measurement and careful experimentation that we can get a wider vision. And then we see unexpected things. We see things that are far from what we would guess and imagine. So our imagination is stretched to the utmost, not as in fiction, to imagine things which aren't really there, but just to comprehend those things which are there.
Richard Feynman (1964)

I dedicate this thesis to the mice.

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ABBREVIATIONS

ABC	avidin-biotin-peroxidase
ACTH	adrenocorticotrophic hormone
AF	activation function
Ag	antigen
ALLO	allopregnanolone
BDNF	brain-derived neurotrophic factor
C	cervical
CC1	adenomatous polyposis coli
cDNA	copy deoxyribonucleic acid
CNS	central nervous system
Coc	coccyx
CR3	complement receptor type 3
DEPC	diethylpyrocarbonate
DHEA	dehydroepiandrosterone
DHP	dihydroprogesterone
DNA	deoxyribonucleic acid
E2	estradiol
EAE	experimental autoimmune encephalomyelitis
Erk	extracellular-regulated kinase
FAK	focal adhesion kinase
FD	funiculus dorsalis
FL	funiculus lateralis
FV	funiculus ventralis
FVL	funiculus ventrolateralis
FVM	funiculus ventromedialis
GABA A	g-aminobutyric acid type A
GFAP	glial fibrillary acidic protein
Gi	inhibitory G proteins
GRE	glucocorticoid response element

HE	haematoxylin and eosin
HSD	hydroxysteroid dehydrogenase
i.a.	inter alias
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
kDa	kiloDalton
L	lumbal
LFB	Luxol fast blue
LPC	lysophosphatidylcholine
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAPR	membrane-associated progesterone receptor
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
mPR	progesterone membrane receptors
MS	multiple sclerosis
nAChR	nicotinic acetylcholine receptor
NaCl	sodium chloride
NF-κB	nuclear factor kappa of activated B-cells
NG2	neuron-glial antigen 2
NIH	National Institute of Health
OLG	oligodendrocyte
OPC	oligodendrocyte progenitor cell
OX42	monoclonal complement receptor 3
p.i.	post-injection
P450scc	cytochrome P450 side-chain cleavage
PAQR	progesterone adiponectin Q receptor

PBS	phosphate-buffered saline
PGMRC	progesterone receptor membrane component
pH	potentiometric hydrogen ion concentration
PI	phosphoinositide
PI3K	phosphotidylinositol 3-kinase
PIBF	progesterone-induced blocking factor
PKB	protein kinase B
PLP	proteolipid protein
PNS	peripheral nervous system
PPAR	peroxisome proliferator-activated receptor
PPMS	primary progressive multiple sclerosis
PR	progesterone receptor
PRE	progesterone response element
PROG	progesterone
PT	pertussis toxin
RhoA	ras homolog A
RIA	radioimmunoassay
ROCK	rho-activated kinase
RRMS	relapsing-remitting multiple sclerosis
S	sacral
SEM	standard error of the mean
SH	src homology domain
SPMS	secondary progressive multiple sclerosis
StAR	steroidogenic acute regulatory protein
TBI	traumatic brain injury
TCR	T cell receptor
Th	thoracic
Th1, Th2	T helper cell 1, T helper cell 2
THP	tetrahydroprogesterone
TNF	tumour necrosis factor
TSPO	translocator protein

1 INTRODUCTION

1.1 Anatomy of the Spinal Cord

The spinal cord or medulla spinalis forms the elongated, nearly cylindrical, part of the central nervous system that lies in the vertebral canal. Its approximate average length in humans is 43 cm, and it weighs about 30 g (≈ 5 cm and 0.1 g in mice). It extends from the level of the upper border of the atlas to the lower border of the first lumbar vertebra. Above, it is contiguous with the brain; below, it ends in a conical extremity, the conus medullaris, from which a delicate filament, the filum terminale, descends as far as the first segment of the coccyx (fig. 1) (Gray, 1973).

31 pairs of spinal nerves emanate from the spinal cord, each nerve originates from the ventral and dorsal roots. Each root consists of several bundles of nerve fibers, and at its attachment extends for some distance along the side of the medulla spinalis. The pairs of spinal nerves are designated in groups according to the position of their origins in the spinal cord: 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal in humans. In mice the distribution differs: there are 8 cervical, 13 thoracic, 6 lumbar and 4 sacral spinal nerve pairs (Brichta et al.1985).

1.1.1 Gray and White Matter

The spinal cord is divided into two symmetrical portions by a connective dorsal median septum (septum medianum posterium) and a deep anterior groove (fissura mediana anterior). In a transverse section, two different areas that vary in different regions of the spinal cord can be distinguished: the gray matter (substantia grisea), shaped like a butterfly, and the it surrounding white matter (substantia alba).

As in the cerebrum, gray matter is composed primarily of cell bodies and the white matter of mostly myelinated nerve fibres. Through the middle of the grey substance runs the minute, fluid-filled central canal (canalis centralis). The anterior and dorsal projections of the gray matter are named anterior and dorsal horns (cornua anteriora et posteriora) respectively. In the thoracic region and, to a lesser extent, in the sacral region there is also a lateral horn (cornu laterale) which contains motor cells of the autonomic nervous system. The anterior horn contains motor neurons from which the axons leave the spinal cord

through the anterior root to supply striated muscles. The posterior horn contains sensory neurons receiving afferent fibers coming from the periphery end.

The myelinated nerve fibers of the white matter are imbedded in a spongelike network of neuroglia and are arranged in different funiculi, the funiculus ventralis (FV), the funiculus lateralis (FL) and the funiculus dorsalis (FD) (fig. 2). Furthermore they are classified based on their function in different tracts and fascicles named according to their origin and destination. Between the anterior and posterior columns the gray substance extends as a series of processes into the lateral funiculus, to form a network called the formatio reticularis. The quantity and form of gray substance, portrayed on transverse sections, varies at different levels. In the thoracic region it is small, not only in amount but relatively to the surrounding white substance. In the cervical and lumbar enlargements it is greatly increased: in the latter, and especially in the conus medullaris, its proportion to the white substance is greatest (Fig. 3). In the cervical region its posterior column is comparatively narrow, while its anterior is broad and expanded; in the thoracic region, both columns are attenuated, and the lateral column is evident; in the lumbar enlargement, both are expanded; while in the conus medullaris the gray substance assumes the form of two oval masses, one in each half of the cord, connected together by a broad gray commissure (Faller and Schuenke, 2004).

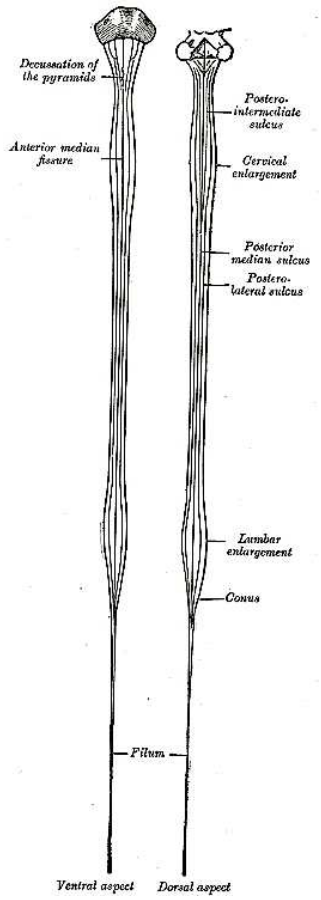


Figure 1

Diagrams of the Medulla Spinalis (Gray, 1918)

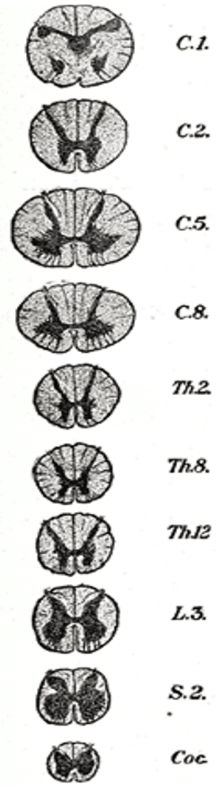


Figure 3
Transverse Sections of the Medulla Spinalis at Different Levels (Gray, 1918)

C = cervical
Th = thoracal
L = lumbal,
S = sacral
Coc. = Coccyx
with numbers indicating the respective heights

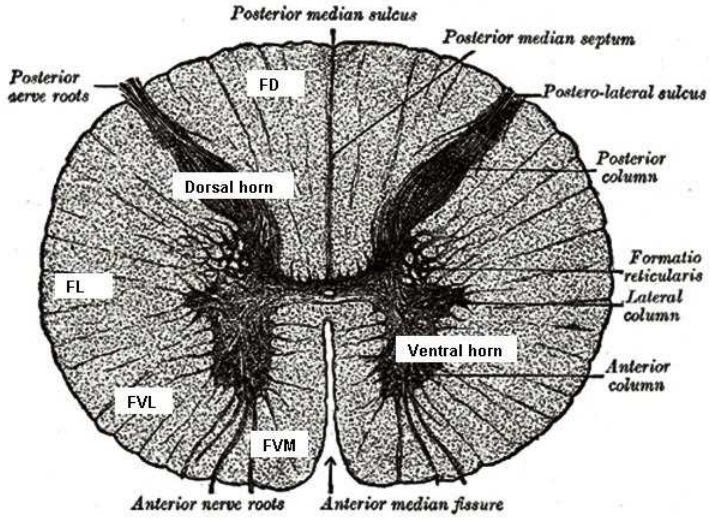


Figure 2
Transverse Section of the Medulla Spinalis in the Mid-thoracic Region (adapted from Gray, 1918)

FVM = Funiculus ventromedialis
FVL = Funiculus ventrolateralis
FL = Funiculus lateralis
FD = Funiculus dorsalis

1.2 Myelin

The myelin ensheathment, material that fills spaces between nerve elements, was first described by the German pathological anatomist Rudolf Virchow in the mid-19th century. His French colleague Louis-Antoine Ranvier later refined histological techniques and he was credited for his observations of both injured and normal nerve fibers. Since then myelin has been subject of extensive studies. We now know that in the central nervous system (CNS) myelin is formed by neuroglial oligodendrocytes (OLGs) which extend processes that concentrically wrap around axons (Ritchie et al., 1981). The synthesized myelin shapes a dynamic multilamellar lipid-rich membrane system which serves as an insulator enabling rapid, saltatory conduction of electrical signals for proper nerve impulse propagation, decreasing metabolic costs of neural activity and reducing space requirements of the nervous system (Sherman and Brophy, 2005). In cross-sections the mature sheath is revealed as a highly regular arrangement of alternating thick, major dense, and thin, intraperiod, lines that represent adhering cytoplasmic and extracellular membrane surfaces, respectively (Colman, 1989).

Myelin is metabolically active in synthesis, processing and metabolic turnover of several of its own components, including degradation, phosphorylation and dephosphorylation of proteins as well as ion transport with respect to not only maintenance of its own structure but also participation in buffering of ion levels in the vicinity of the axons (Brady et al., 1999).

The importance of the myelination process is illustrated by the neurological deficits caused by demyelinating diseases of the CNS, such as multiple sclerosis (MS).

Since the discovery that remyelination may occur within the central nervous system (Bunge et al., 1961) there has been a great interest in clarifying the molecular and cell biology of OLGs, including factors that influence their proliferation, migration, differentiation and ability to remyelinate nerve axons.

1.2.1 Composition of Myelin

Myelin has a water content of about 40%. Biochemically, the dry mass of myelin is characterized by a high proportion of lipid (70 to 85%) and, consequently, a low proportion of protein (15 to 30%). In contrast, most biological membranes have a higher ratio of proteins to lipids.

1.2.1.1 Myelin Lipids

Multiple lipids make up the myelin with major lipids being galactosylceramide (cerebroside and its sulfated derivative sulfatide), cholesterol, ethanolamine-containing plasmalogens and lecithin (phosphatidylcholine) (Norton, 1984). Each, with its own distinct physical properties contributes to the myelin membrane structure; to its adhesive stability as well as its susceptibility.

1.2.1.2 Myelin Proteins

There is a relatively simple array of myelin-specific proteins with proteolipid protein (PLP) and myelin basic proteins (MBP) representing the two predominant proteins comprising approximately 60 to 80% of the total proteins found in CNS myelin in most species. Many other proteins and glycoproteins are present but in lower amounts (Morell, 1994).

1.2.1.3 Proteolipid Protein (PLP)

PLP is the most abundant protein in the CNS. It is highly conserved and one of the most hydrophobic proteins in nature (Schliess and Stoffel, 1991; Weimbs and Stoffel, 1992). PLP seems to contain four transmembrane domains and binds strongly to other copies of itself on the extracellular side of the membrane being therefore essential for the compact apposition and spanning of the oligodendrocyte plasma membrane processes around the axon. It further contains two disulphide bonds which covalently bind lipids (Schliess and Stoffel, 1991; Weimbs and Stoffel, 1992)

1.2.1.4 Myelin Basic Protein (MBP)

MBP is the second most abundant protein in the CNS. As a membrane protein it is primarily located in the cytoplasmic spacing of the myelin sheath and is believed to play an active role in stabilizing the periodic myelin structure via non-specific interactions with the opposing lipid bilayers (Roussel and Nussbaum, 1981; Inouye and Kirschner, 1994). MBP is essential for the CNS myelin sheath formation, eminent in the *Shiverer* autosomal recessive mouse mutant, which due to a deficiency of functional myelin basic protein, results in failure of oligodendrocyte morphogenesis (Chernoff, 1981; Galiano et al., 2006).

1.2.1.5 Myelin Oligodendrocyte Glycoprotein (MOG)

With only 0.1% of total myelin protein, Myelin Oligodendrocyte Glycoprotein (MOG) is a minor constituent of the CNS myelin sheath (Chekhonin et al., 2003). The full-length protein contains 218 amino acids. The MOG gene belongs to the immunoglobulin gene superfamily and is located within the major histocompatibility complex (MHC) both in human and in mice (Pham-Dinh et al., 1993). The physiological function of MOG has not been identified, yet its external location on myelin sheaths and its late appearance during myelinogenesis suggest a role in the completion of myelin and maintenance of its integrity (Pham-Dinh et al., 1995). The surface exposition of its IgV-like domain makes it a target antigen (Ag) accessible to initial autoimmune attack for both humoral and cellular CNS-directed immune responses (von Budingen et al., 2001). Indeed, in both rodent and primate MS models, antibodies against MOG directly induce demyelination, whereas anti-MBP or anti-PLP are not associated with an increase in myelin injury (Schluesener et al., 1987). In a subgroup of MS patients, IgG antibodies pathogenic for native MOG expressing cells are elevated and result in demyelination and axonal damage (Zhou et al., 2006).

1.3 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disorder of the human central nervous system. Already over one and a half century ago Cruveilhier and Carswell described the pathological characteristics of this enigmatic neurological disease (Fig. 4) (Cruveilhier, 1835-42; Carswell, 1838). In 1868 Jean Martin Charcot first noted the association of accumulation of inflammatory cells in a perivascular distribution within the brain and spinal cord white matter of patients with the clinical presentation of intermittent episodes of neurologic dysfunction. He introduced the term *sclérose en plaques disseminées*, in English it is termed *multiple sclerosis* (Charcot, 1877). In 1948 Elvin Kabat found further evidence of an inflammatory nature of the disease with his observations of increases in oligoclonal immunoglobulin in the cerebrospinal fluid of patients with MS (Kabat et al., 1948).

MS is the major cause of non-traumatic neurological disability in young adults affecting an estimated 2.5 million individuals worldwide (Hauser and Oksenberg, 2006; Warren et al., 2008). Studies from several countries demonstrate a gender difference of the disease with a female/male ratio of 2:1 denoting an increase in the incidence of MS particularly in women (Orton et al., 2006; Alonso et al., 2008). The global geographic distribution of MS is uneven with a low prevalence near the equator, which increases the higher the degree of latitude in both hemispheres (Taylor et al., 2010). There is a large body of evidence indicating that, despite genetic influences on immune mechanisms, MS is primarily of environmental origin, which appears to be ordinarily acquired after birth with a prolonged latency period between acquisition and onset of symptoms. Support for this has come largely from studies of migrants to and from different geographic MS risk areas which correlate with many physical, chemical, biological, and social factors (Wallin et al., 2009). Nevertheless a strong environmental determinant could not be identified so far. Actual genomewide association studies suggest a weak genetic influence of susceptibility to multiple sclerosis, with variants most notably within the major histocompatibility complex, but these variants probably account for only a small proportion (Hafler, 2004; Hoppenbrouwers et al., 2008).

The clinical disease course is variable, it usually begins with reversible episodes of neurological disability in the third or fourth decade of life, and transforms into a disease of

continuous and irreversible neurological decline by the sixth or seventh decade (Lublin and Reingold, 1996). While inflammation and demyelination induce neurological deficits, which are in part reversible, destructions of axons, when past the threshold of compensation, are always accompanied by irreversible clinical deficits (Kornek et al., 2000).

Depending on its onset, MS can be clinically categorized as either relapsing-remitting (RRMS), observed in 85-90% of patients, or primary progressive (PPMS) MS. Relapses typically present subacutely, with a rapid onset of symptoms developing over hours to several days, and a short course, persisting for several days or weeks, and then gradually dissipating. An increased frequency of attacks and poor recoveries from attacks from the beginning of disease predict a more rapid deterioration. Ultimately neurological disability occurs in approximately 40% of relapsing-remitting patients and they develop a progressive form of neurodegeneration consequential to the chronic CNS inflammation, known as secondary progressive multiple sclerosis (SPMS) (Kantarci and Weinshenker, 2005).

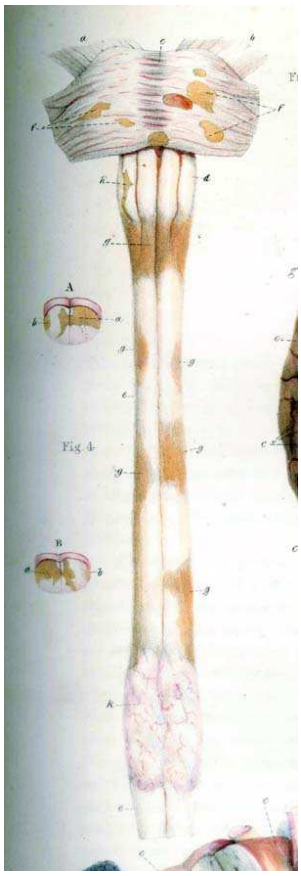


Figure 4. Sclerotic Chord and Pons Varolii (Carswell, 1838)

Carswell was the first to portray the plaques of multiple sclerosis, although he did not identify them as such:
“A peculiar diseased state of the chord and pons Varolii, accompanied with atrophy of the discoloured portions” (Carswell, 1838).

1.3.1 Immunopathology of Demyelination in MS

The pathological hallmark of MS is the presence of focal areas of inflammatory-mediated demyelination with reactive glial scarring. The immunological mechanisms responsible for the destruction of myelin sheaths are, however, diverse and the consequence of a complicated heterogeneous process.

By immunopathological analysis of samples of MS lesions, four general patterns of myelin destruction have been described. All patterns occur on the background of a T-cell, macrophage and microglia dominated inflammatory response. In pattern I, lesions are mainly mediated by the cytotoxic machinery of T-cells and toxic products, produced by activated phagocytes, similar to lesions seen in autoimmune encephalomyelitis. In pattern II, the dissolving myelin sheaths are additionally coated with immunoglobulins and activated complement (Prineas and Graham, 1981; Storch et al., 1998). In these lesions, antibodies against myelin OLG glycoprotein or other surface components of the myelin sheath appear to be involved in the process of demyelination (Genain et al., 1999). The third pattern seems to be more suggestive of a primary oligodendrocyte dystrophy, reminiscent of virus- or toxin-induced demyelination rather than autoimmunity with a less pronounced activation of inflammatory cells within the lesions. The process of demyelination initially affects the most distal oligodendrocyte processes and is followed by destruction through apoptotic cell death similar to lesions found in hypoxic injuries (Itoyama et al., 1980; Lucchinetti et al., 2000). The inflammatory reaction of the fourth pattern leads to an unusually extensive myelin breakdown. There are indications that this pattern is associated with particular deficiencies leading to increasing tissue vulnerability (Linker et al., 2002; Lassmann, 2004).

1.3.2 Current Pharmacological Therapies for Multiple Sclerosis

Since the exact aetiology of multiple sclerosis is unknown, there is no curative treatment. Therapeutic approaches focus therefore on disease-modifying agents that can be grouped into immunomodulatory and immunosuppressive therapies. Exacerbations, meaning the appearance of new, or worsening of old, clinical symptoms in the absence of infection, are presently treated with high doses of intravenous glucocorticosteroids. This treatment has been shown to reduce the intensity and duration of the neurologic disability

during relapses, nevertheless there is no evidence that it has any effect on the ultimate course of the disease (Filippini et al., 2000; Nos et al., 2004). Corticosteroids and corticotrophin (adrenocorticotrophic hormone – ACTH) have been employed for more than 50 years (Jonsson et al., 1954). Corticotrophin acts by stimulating adrenocortical cells and thereby induces synthesis and secretion of corticosteroids, among them cortisol. Because of unpredictable undesired cortisol responses of corticotrophin, studies focused on orally or intravenously administrated corticosteroids. Four randomized placebo-controlled trials of intravenous or oral methylprednisolone have been reported. The studies showed benefit in favour of methylprednisolone compared to placebo on speed of recovery from relapses, but not on degree of recovery, risk of later relapses or long-term effects on disability (Durelli et al., 1986; Milligan et al., 1987; Filipovic et al., 1997; Sellebjerg et al., 1998).

Over the last two decades four classes of immunomodulating drugs demonstrated modest efficacy, altering the progression of multiple sclerosis at least in the early course. The first to be approved were beta interferons (IFN-β-1a and IFN-β-1b), which have pleiotropic effects on cellular functions and may prevent leukocyte migration across the blood brain barrier by altering the expression of adhesion molecules as well as Glatiramer acetate, a MHC-binding protein that engages the T cell receptor (TCR) (Kozovska et al., 1999; Zang et al., 2001; Muraro et al., 2004). These agents are generally well tolerated and moderately effective in the short term in preventing relapses and attenuation of lesion activity visible on magnetic resonance imaging (Galetta et al., 2002; Cohen and Rivera, 2010). Other treatment strategies for multiple sclerosis include immunosuppressants, intravenous immunoglobulins, plasmapheresis and monoclonal antibodies such as Natalizumab. The only immunosuppressant explicitly approved for the treatment of multiple sclerosis is the chemotherapeutic agent Mitoxantrone, whose benefit has been demonstrated in a number of clinical studies (Millefiorini et al., 1997; Hartung et al., 2002). The use of this agent is however restricted to certain high-risk patients because of potentially serious side-effects, notably cardiomyopathy and treatment-related acute leukaemia, events which are assumed to emerge in between 1/800 and 1/250 of patients (Morrissey et al., 2005; Wiendl et al., 2008). Natalizumab, a selective adhesion molecule inhibitor that attenuates inflammation by selectively reducing the transmigration of mononuclear cells into the CNS, was approved in 2004 after convincing results on relapse

rate reduction, but serious adverse effects (severe opportunistic infection) are associated with it (Rudick et al., 2006; Berger, 2009).

The aforementioned therapies are all based on the inhibition of inflammation, which is an important aspect but covers only a fraction of the whole characteristics of MS. While the root cause remains undetermined and the cure remote, therapeutic approaches need to target both inflammation and promotion of myelin repair.

1.4 Biosynthesis and Actions of Steroids in the Central Nervous System

It is common for substances or drugs with biological activity, which have first been found in one particular tissue and in coherence with one particular function, to take many decades until their distinctive additional activities are revealed. Steroid hormones are such an example. Gonadal steroid hormones were for a long time only assigned to reproductive functions and it was not until many decades later that their additional, essential neuromodulatory activity was recognized.

Peripherally synthesized steroids, which derive from the adrenal gland, gonads and placenta are released into the bloodstream, easily cross the blood-brain barrier, and exercise a large array of biological effects on the central nervous system. Once this new information was acknowledged even more surprising evidence emerged in the early 1980s when Baulieu and co-workers (1981) proposed that the brain itself serves as a steroidogenic organ and introduced the term “neurosteroids” referring to their site of synthesis. He discovered that the steroids dehydroepiandrosterone (DHEA), pregnenolone, and their sulphated esters, were present in higher concentration in the brain than in circulation and even more significantly remained high after gonadectomy and adrenalectomy (Corpechot et al., 1981; Jo et al., 1989; Baulieu et al., 1999). Existence of steroidogenesis within the nervous tissue was further substantiated by the immunohistochemical localization and proof of biological activity of the enzyme cytochrome P450 side-chain cleavage (P450scc) in rat oligodendrocytes (Le Goascogne et al., 1987).

The first step of steroidogenesis is the conversion of cholesterol into pregnenolone by the mitochondrial cholesterol P450scc located on the matrix side of the inner mitochondrial membrane (Stocco and Clark, 1996). Whereas there is a slow basic regulation of neurosteroid secretion achieved through trophic hormones which stimulate transcription of

genes encoding steroidogenic enzymes, its concentrations are primarily regulated at the level of steroid hormone synthesis. Since all steroid hormones derive from cholesterol, the regulation of the level of cholesterol flow from the outer to the inner mitochondrial membrane allows a fast induction, with a 10-100-fold increase in synthesis, and fast termination (Miller, 1988). Two proteins located in the mitochondrial membrane, the steroidogenic acute regulatory protein (StAR) and the peripheral-type benzodiazepine receptor, recently renamed as translocator protein (TSPO), are involved in this highly controlled transport (Lavaque et al., 2006; Papadopoulos et al., 2006b).

After its conversion from cholesterol to pregnenolone it passes to the cytosol, where it serves as the precursor of all neurosteroids (fig. 5). Via consecutive enzymatic steps, pregnenolone is converted to progesterone, and the latter is metabolized to other potent neurosteroids such as tetrahydroprogesterone (3α,5α-THP), also known as allopregnanolone (ALLO). Pregnenolone also is a common precursor for dehydroepi-androsterone. Further metabolism of DHEA leads to the production of the androgens dihydrotestosterone DHT and testosterone, which via action of aromatase is converted to estradiol (E2). On the other hand, progesterone is predominantly converted to its highly active metabolites dihydroprogesterone (5α-DHP) and 3α,5α-THP. All enzymes required for the CNS steroid biosynthesis and metabolism are expressed in astrocytes, oligodendrocytes and neurons (Melcangi et al., 2008).

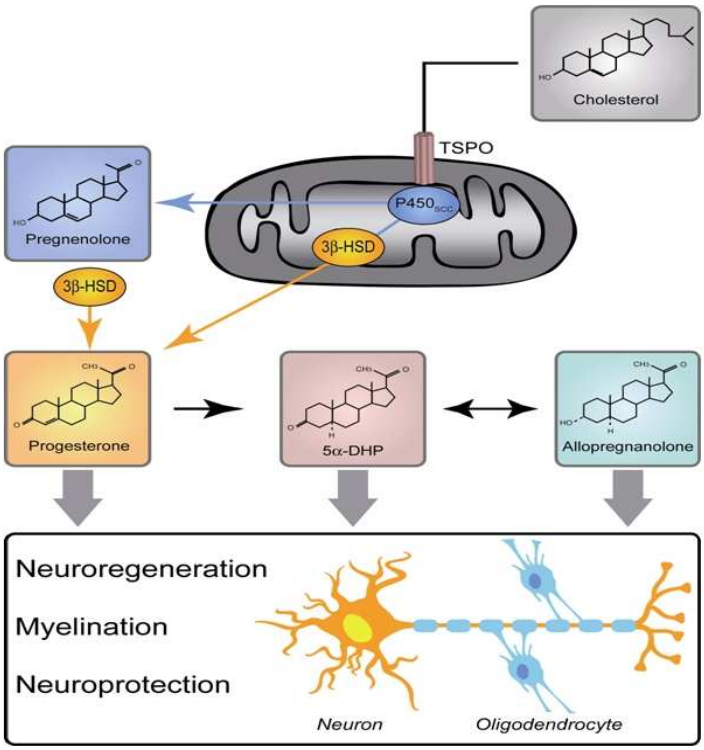


Figure 5
Biosynthesis of Neurosteroids (Schumacher et al., 2008)

Progesterone is synthesized by neurons and glial cells. Ligands of the transport protein TSPO stimulate the passage of cholesterol into the mitochondria and, as a consequence, the synthesis of pregnenolone from cholesterol through cytochrome P450scc inside steroidogenic mitochondria. The conversion of pregnenolone to progesterone by different isoforms of the 3b-hydroxysteroid dehydrogenase (3b-HSD) takes place either inside the mitochondria or within the cytoplasm.

The action of gonadal steroid hormones in the CNS was initially thought to be restricted to a few brain areas related to predominantly endocrine control of reproductive behaviour, ovulation and pregnancy. In the meantime it is well established that gonadal steroids also play a general maturation-stimulating and survival-promoting role on neurons as well as glial cells, in physiological conditions and CNS injury, which could be demonstrated in experimental animals as well as in humans (De Nicola, 1993; Schumacher et al., 1996; Wright et al., 2007). Estrogens for instance, particularly 17β-estradiol, have been widely studied in different experiments using *in vitro* and *in vivo* approaches (Garcia-Segura et al., 1994; Kipp et al., 2006). Interest in estrogens as neuroprotectants was roused by experimental observations in female animals where the stage of the oestrous cycle

influenced the size of tissue damage as well as the functional outcome after neuronal ischemic injury (Hall, 1972). Since then numerous studies added a variety of higher activities for oestrogen including the promotion of neuronal and glial plasticity in the hypothalamus, axonal growth and synaptogenesis in the hippocampus, (McEwen et al., 1995; Toran-Allerand et al., 1999; Compagnone and Mellon, 2000). So far estrogens are the best studied and therefore most evident protective steroid hormones in the CNS, however ample evidence suggests additional roles also for progesterone and glucocorticoids within the nervous system.

1.5 Progesterone

Together with estradiol, progesterone (PROG) is known for its importance as reproductive steroid hormone regulating functions of the reproductive organs, sexual behaviour and the release of gonadotropins. In non-pregnant women, the major source of progesterone synthesis is the corpus luteum, a gland that during a day within the mid luteal phase produces more than ten times its own weight of progesterone (Ottander et al., 2000). During pregnancy progesterone is produced in even larger amounts by the placental syncytotrophoblasts. Based on the notion of its prototypical supportive role in gestation, preparing and maintaining the uterus for pregnancy, it was named progesterone (from lat. *pro* + *gestare*) (Allen et al., 1935). However, the adrenal glands also contribute significant amounts of circulating progesterone (PROG) in humans and rodents, which is why it is not only present in females but also circulates in males (Gutai et al., 1977; Kalra and Kalra, 1977). Elevated levels can furthermore be measured in various brain regions, equally both in men and women (Weill-Engerer et al., 2002; Oren et al., 2004). As in the case of most free steroid hormones (which are neither esterified nor bound to protein), the lipophilia of circulating progesterone accounts for the easy passage through the blood-brain barrier. Via free diffusion it enters the central nervous system at various sites. Additionally, as mentioned above, progesterone is also synthesized locally within the CNS mainly by neurons, either *de novo* from cholesterol or *in situ* from blood-derived pregnenolone (Baulieu et al., 1999). For its catalytic conversion from pregnenolone to progesterone, the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) is extensively distributed both in brain and spinal cord (Guennoun et al., 1995; Coirini et al., 2002).

Microglial cells, including astrocytes and oligodendroglial cells, also have the capacity to synthesize progesterone, but the expression and activity of 3β-HSD in these cells are regulated by cellular interactions responding to neuronal signals. The mechanisms, which regulate the activity of CNS progesterone synthesis, seem to involve the mediation of neurotransmitters and neuropeptides, but require further detailed exploration (Vaudry et al., 2005). In Schwann cells for instance, the myelin producing glial cells of the peripheral nervous system (PNS), progesterone is synthesized in response to diffusible neuronal signals (Robert et al., 2001). Of historical interest is the fundamental discovery of Selye who was the first to show the relatively immediate (within minutes) anaesthetic and anticonvulsant properties of progesterone intraperitoneally administered to rats (Selye, 1941). This provided the first evidence that endogenous steroid hormones could influence neuronal excitability on a time scale inconsistent with the classic genomic mechanisms of steroid action. In keeping with this early observation of Selye, there are more and more experimental data that give fuel to the idea that progesterone exerts further functions within the nervous system in addition to the “classical” endocrine nature.

1.5.1 Neuroprotective Properties of Progesterone (Animal Studies)

Dopaminergic neurons of the midbrain, pyramidal neurons of the hippocampus and cerebral cortex, Purkinje cells of the cerebellum, as well as neurons of the dorsal striatum and the caudate nucleus are known for their particular vulnerability to excitotoxic and ischemic damage (Gonzalez-Vidal et al., 1998; Xu et al., 2001). In cats, the administration of progesterone during experimentally induced global cerebral ischemia reduced the loss of these highly sensitive neurons, mainly within the dorsal hippocampus (subfields CA1 and CA2) (Cervantes et al., 2002). In rats, progesterone given before transient occlusion of the middle cerebral artery decreased the infarct size and consequent neurological deficits (Jiang et al., 1996; Kumon et al., 2000). In an analogous experiment on male mice the evaluation of motor ability through Grid and Rotarod assessment confirmed progress following progesterone treatment in both tests (Gibson and Murphy, 2004). Large increases of progesterone content in perifocal areas following injuries like spinal cord trauma suggest that the neurosteroid could be the physiological response to injury as an

attempt to cope with neurodegeneration (di Michele et al., 2000; Labombarda et al., 2006; Schumacher et al., 2007).

The potential role of progesterone as a neuroprotectant has also been evaluated in experimental models of traumatic brain injury (TBI). A frequently used model is the bilateral contusion of the medial prefrontal rat cortex which generates cognitive deficits in close analogy to human frontal lobe injuries (Hoffman et al., 1994; Stein, 2001). Traumatic brain injury leads to a significant inflammatory reaction that is generally accompanied by heavy gliosis and cell death in the vicinity of the lesion and a rise in cerebral water content in the form of oedema. Augmenting the intracranial pressure, the oedema is considered to be one of the most important negative factors for the outcome of TBI (Saatman et al., 2006). Again, observations of a gender-dependent difference in the outcome after brain injury, with a smaller water content in females (presenting higher endogenous levels of progesterone) when compared with males, suggested progesterone as a protective player (Roof et al., 1993). In various subsequent TBI studies progesterone was administered to male rats and in effect progesterone reduced both oedema and secondary neuronal losses and improved behavioural recovery (Roof et al., 1994; Roof et al., 1996; Thomas et al., 1999; Wright et al., 2001; Shear et al., 2002).

1.5.2 Neuroprotective Properties of Progesterone (Clinical Studies)

These encouraging results led to a translation from the laboratory to a pilot clinical trial in humans in order to test the safety and potential efficacy of progesterone as a neuroprotectant in acutely brain-injured patients. The randomized, double-blinded, placebo-controlled trial, named “ProTECT” was conducted in the United States and included 100 trauma patients of moderate and severe TBI who received rapid intravenous (i.v.) infusions of progesterone continuously for 3 days (Wright et al., 2005). In this trial no adverse events could be attributed to progesterone. The results showed that through i.v. administration steady state serum concentrations can be achieved which do not differ by sex or injury severity. While the trial lacked sufficient power to assess the definite efficacy of progesterone treatment, it showed possible signs of benefit. Progesterone-treated patients with severe TBI had a statistically significant decrease in one-month mortality and patients with moderate TBI had an improved functional outcome one month after injury

compared to patients given state-of-the-art treatment plus placebo (Wright et al., 2007). In 2008 these findings were replicated and extended in a Chinese trial including 159 patients with severe TBI, which reported a significant difference in mortality, lower intracranial pressure and improved functional outcomes one and six month after injury (Xiao et al., 2008). Despite the compelling outcomes demonstrated in animal and clinical studies, the specific molecular basis and mechanisms by which progesterone acts to enhance neuroprotection is still not understood in detail.

1.5.3 General Mechanisms of Action of Progesterone Within the CNS

1.5.3.1 Genomic Mechanism

Until recently, it was presumed that all steroid hormones regulate biological functions by genomic mechanisms. The genomic action of steroid hormones in the classical sense means steroid hormones enter the cell, either passively by diffusion, through the membrane, or assisted by any transporter, and subsequently bind to and activate intracellular receptors located in the cytosol or in the nucleus. Attachment of progesterone to the specific steroid receptor ligand-binding domain then induces a conformational change of the receptor and provokes the separation of the receptor from cytoplasmic chaperone proteins such as heat shock proteins. This leads to exposure of nuclear localization sequences or allows nuclear translocation and dimerization of the ligand-bound receptors and their binding to steroid response elements on the promoter regions of the target genes. Finally the binding on the promotor region regulates gene expression by interacting with the transcription machinery. Genes are transcribed into mRNA and the mRNA is in turn translated into protein molecules, which ultimately exert biological functions (Truss and Beato, 1993). These events require relatively long time to fully activate the specific biological response. Since the central mechanism takes place on the genome in the nucleus, these actions are termed ‘genomic effects’.

1.5.3.1.1 Isoforms of Progesterone Receptor

The effects of progesterone on gene expression are mediated by at least two intracellular receptor isoforms, PR-A and PR-B, which are generated from a single gene (Kastner et al., 1990; Conneely and Lydon, 2000). They only differ by an additional 164

amino acid segment in the N-terminal region of PR-B, called the B-receptor upstream segment (Takimoto et al., 2003).

The progesterone receptor (PR) is a member of the steroid receptor family, which is part of a larger nuclear receptor superfamily. These receptor proteins are related by sequence similarities within their "zinc finger" DNA-binding domains and by ligand-dependent regulation of specific gene transcription (Evans, 1988; Beato, 1989). Transcription activation is regulated by two transcription activation function (AF) domains, constitutive ligand-independent AF-1 in the N terminus and ligand-dependent AF-2 in the C terminal region. A DNA-binding domain and the hinge region are mapped to the central region of both receptors. Furthermore, a unique activation function domain, AF-3, is contained in the upstream segment of PR-B that is missing in PR-A. AF-3 allows binding of a subset of co-activators to PR-B that is not efficiently recruited by progestin bound PR-A (Giangrande et al., 2000). Thus, PR-A and PR-B display different transactivation properties that are specific to both cell type and target gene promoter context (Tora et al., 1988). Agonist-bound PR-B functions as a strong activator of transcription of several PR-dependent promoters and in a variety of cell types in which PR-A is inactive (Hovland et al., 1998). In these cell and promoter contexts in which agonist-bound PR-A is inactive, the PR-A possesses the capability to repress the activity of PR-B and even other steroid receptors including estrogens, androgens, glucocorticoids and mineralocorticoids, a phenomenon called “transrepression” (Li et al., 2004)

1.5.3.2 Non-genomic Mechanisms

In addition to their delayed action on gene expression in the cell nucleus, progesterone, like other steroids, elicits a variety of rapid effects on various signal transduction pathways and second messenger systems without the involvement of transcriptional modulation. Non-genomic signal perception occurs at the plasma membrane and cytoplasmic level but can also be observed within mitochondria (Razmara et al., 2008). The discovery of the non-genomic effects of progesterone originated with the aforementioned striking finding of Selye in 1941, who encountered the drastically conflicting magnitude of the time course between the rapid anaesthetic and the delayed anticipated hormone action of progesterone (Selye, 1941). With passing decades more experiments made rapid signal transduction of

hormone steroids apparent, such as acute cardiovascular effects of aldosterone (Klein and Henk, 1963). With a series of publications Pietras and Szego underlined the diversity of steroid action in cells and animals and in due course identified a high-affinity oestrogen binding protein (receptor) at the plasma membrane of cells that responded to oestrogen administration with cAMP generation and calcium changes (Szego and Davis, 1967; Pietras and Szego, 1975, 1977). This led to increasing interest and many experimental studies examined rapid effects of progesterone using mainly the two model systems of acrosome reaction in human spermatozoa and the induction of oocyte maturation in Xenopus laevis (Maller and Krebs, 1980; Tian et al., 2000; Qiu and Lange, 2003; Hammes and Levin, 2007). These experiments describe various potential 'non-genomic' mechanisms, showing that the actions of progesterone and other steroids are probably mediated by various pathways rather than only one. Rapid actions are suggested to be mediated through activation of intracellular signalling pathways, resulting in alterations in ion fluxes and intracellular free calcium concentrations occurring within seconds, and of other second messengers such as cyclic nucleotides and extracellular-regulated kinase 1 and 2 (erk 1/2), phosphoinositide-3 (PI3) kinase, mitogen-activated protein (MAP) kinase, Ras homolog A/ Rho-activated kinase-2 (RhoA/ROCK-2) cascade and G-protein activation which can take from a few minutes to several hours (Blackmore et al., 1990; Filardo et al., 2000; Bagowski et al., 2001; Peluso et al., 2001; Zhu et al., 2003; Luconi et al., 2004; Cai et al., 2008). However, progesterone membrane receptor-mediated pathways can also ultimately regulate transactivation of the nuclear progesterone receptor resulting in alteration in gene transcription, which is why the only characteristic common to all these non-classical steroid actions is rapid activation of intracellular signalling pathways. Depending on the system, they can vary from seconds, e.g. by opening of ion channels, to minutes or hours, in the case of apoptosis inhibition. Nevertheless they clearly contrast with genomic responses, which generally take from a few hours up to days to fully manifest themselves (Wehling et al., 2006)

1.5.3.3 Membrane Progesterone Receptors

1.5.3.3.1 Novel Receptors

Due to the technical complexity, investigators just recently started to characterize extranuclear progesterone binding and signal transduction. There is evidence for at least two types of novel membrane proteins, progesterone membrane receptors (mPRs) and progesterone receptor membrane component one (PGMRC1). The mPRs, (MW ~40 kDa) initially discovered in fish ovaries, comprise at least three subtypes, α, β and γ and belong to the seven-transmembrane progesterone adiponectin Q receptor (PAQR) family. The mPRs display high affinity, limited capacity, displaceable, specific progesterone binding and are directly coupled to G proteins activating inhibitory G proteins (Gi) and thereby downregulating adenylyl cyclase activity. The single-transmembrane protein PGMRC1 (MW ~26-28 kDa) was first purified from porcine livers and its cDNA was subsequently cloned from a variety of other tissues and species including rats (25-Dx) and humans (Hpr.6) by different investigators. PGMRC1 and the closely related PGMRC2 belong to the membrane-associated progesterone receptor (MAPR) family. The PGMRC1 protein displays moderately high binding affinity for progesterone but also binds other molecules such as testosterone and glucocorticoids. Despite recent advances, it has not been possible to date to describe the signal transduction pathways induced by binding of progesterone to these two families of novel membrane proteins. Amino acid sequence predictions suggest the involvement of tyrosine kinase, kinase binding, src homology domain 2 (SH2) and SH3 (Thomas, 2008).

1.5.3.3.2 Conventional Receptors

The conventional progesterone receptors have been shown to be targeted not only to the nucleus, but also to the cell membrane, where they interact with other proteins belonging to intracellular signalling pathways (Edwards, 2005). In form of a transient induction the N-terminal domain common to PR-A and PR-B selectively interacts with Src tyrosine kinase family members and activates c-Src and downstream MAPK (Erk-1/-2) (mitogen-activated protein kinase) signalling pathway in the absence of gene transcription (Leonhardt et al., 2003; Boonyaratanakornkit et al., 2008).

1.5.3.4 Corepressors and Coactivators

The multiplicity of regulatory effects that impact progesterone and other nuclear receptors is due in part to an intricate array of coactivators, corepressors, and cointegrators that are recruited to receptor-bound promoters. A subset of receptors binds corepressor factors and actively silences target gene expression in the absence of ligand (Gurevich et al., 2007). Corepressors are found within multicomponent complexes that contain histone deacetylase activity. Deacetylation leads to chromatin compaction and transcriptional repression. Upon ligand binding, the receptors undergo a conformational change that allows the recruitment of multiple coactivator complexes. Whereas some of these proteins are chromatin remodelling factors or possess histone acetylase activity, others potentiate transcription by direct interaction with the basic transcriptional machinery. The recruitment of coactivator complexes to the target promoter then causes chromatin decompaction and transcriptional activation. More than 300 cofactors have been described to date and some of them possess diverse additional functions, such as RNA chain elongation, splicing and termination (Aranda and Pascual, 2001; Lonard et al., 2007).

1.5.3.5 Modulation of Neurotransmission by Progesterone

One element of neuroprotection provided by progesterone and its metabolites is the inhibition of neuronal excitability. In this regard progesterone differs from estrogens, which in general have excitatory effects by potentiating the actions of excitatory neurotransmitters (Smith and Woolley, 2004). On the contrary, progesterone and its metabolites inhibit excitatory neurotransmitter receptors and stimulate inhibitory neurotransmitter receptors. Major effects of progesterone on a membrane neurotransmitter of neurons and glial cells are mediated by its metabolite allopregnanolone (3a,5a-tetrahydroprogesterone), which is a potent positive modulator of g-aminobutyric acid type A (GABA A) receptors (Majewska et al., 1986; Herd et al., 2008). This explains several of its psychopharmacological actions, in particular its anaesthetic, analgesic, and anxiolytic effects, as well as its role in stress, depression, memory, seizure susceptibility, and alcohol dependence (Morrow et al., 2001; Eser et al., 2006). The augmentation of inhibitory GABAergic transmission by 3α,5α-THP may preserve neurons from the effects of excessive excitatory neurotransmitter release in response to injury, given that after TBI, its administration reduces cell death, gliosis, and

functional deficits (Djebaili et al., 2005). In the range of high micromolar concentrations, progesterone inhibits the activity of the neuronal nicotinic acetylcholine receptor (nAChR) (Valera et al., 1992; Lena and Changeux, 1993).

1.5.3.6 Antioxidant Action and Lipid Peroxidation

Even though progesterone does not have the characteristic structure of an antioxidant, elevated serum levels have been shown to reduce free radical damage (Subramanian et al., 1993). Another important consequence of progesterone treatment is the reduction of lipid peroxidation. Thus, pregnancy was shown to reduce lipid peroxidation in brain homogenates and mitochondria (Garrel et al., 2010). The peroxidation of lipids involves complex enzymatic pathways as well as non-enzymatic mechanisms, such as free radical mediated peroxidation, which is reduced by the actions of different antioxidant enzymes (e.g. superoxide dismutases) (Moorthy et al., 2005; Niki et al., 2005).

1.5.3.7 Synopsis

The Mannheim classification scheme proposed by Falkenstein and co-workers seeks to describe and categorize potential mechanisms. The scheme is split into the two main categories ‘A - direct steroid action’ and ‘B - indirect steroid action’, which are in turn separated into a nonspecific (I) and a specific (II) category. The latter is again further divided into group ‘a - classic steroid receptor’ and ‘b – non-classic steroid receptor’ (fig. 6) (Falkenstein et al., 2000).

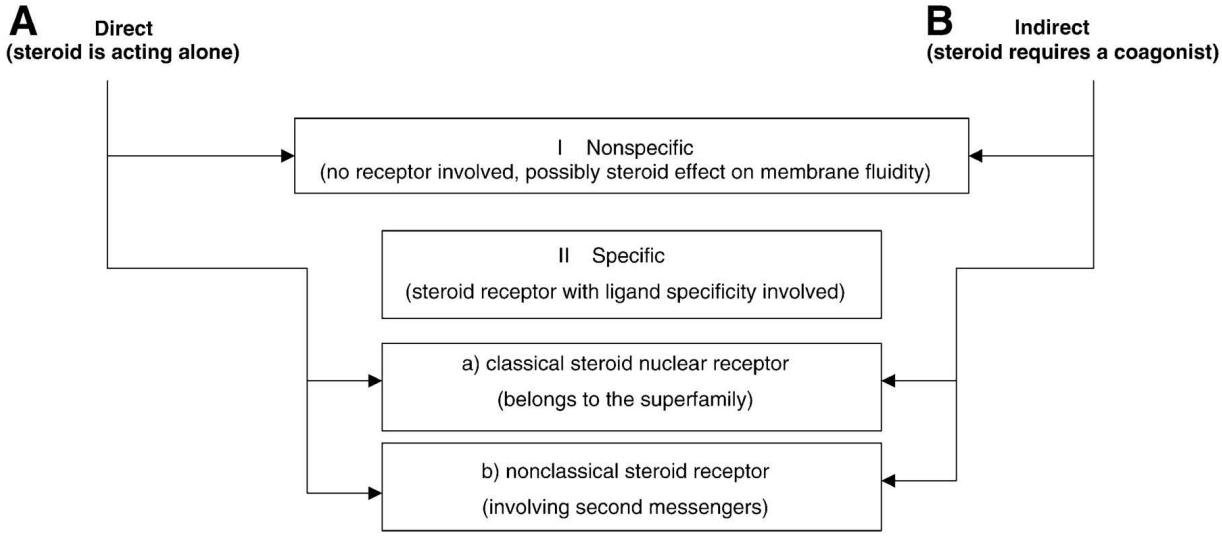


Figure 6
Mannheim Classification

Mannheim classification of nongenomically initiated (rapid) steroid actions.
(Falkenstein et al, 2000)

1.6 Animal Models

1.6.1 Models of Experimental CNS Demyelination

Since the human diseased CNS cannot easily be sampled, various models have been developed to gain ideas about the disease mechanisms and to study demyelination and remyelination in the CNS. There are four general categories of experimental CNS demyelination models.

I. Autoimmune-induced: experimental autoimmune encephalomyelitis (Rivers and Schwentker, 1935; Traugott et al., 1985)

II. Gliotoxin-induced: diphtheria toxin (McDonald and Sears, 1970), cuprizone (Blakemore, 1973), ethidium bromide (Yajima and Suzuki, 1979) and lysophosphatidylcholine (Hall, 1972)

III. Virus-induced: coronavirus (Herndon et al., 1975) and *Theiler's* murine encephalomyelitis virus (Lang et al., 1984).

IV. Myelin mutants: *Rumpshaker* and *Jimpy* mice (PLP mutants) *Shiverer* mouse and *Taiep* rat (MBP mutants), (Chernoff, 1981; Sorg et al., 1986; Griffiths et al., 1990; Duncan et al., 1992)

1.6.2 Experimental Autoimmune Encephalomyelitis (EAE)

The most frequently employed and well-studied model of autoimmune demyelination is the model of experimental autoimmune encephalomyelitis (EAE) (Gold et al., 2006). The origins of EAE can be traced back to the 1920s, when Koritschoner and Schweinburg produced spinal cord inflammation in rabbits by inoculation with human spinal cord and finally to the 1930s, when Rivers and Schwentker reproduced the encephalitic complications associated with Louis Pasteur's rabies vaccination by repetitive immunization of rhesus monkeys with CNS tissue (Koritschoner and Schweinburg, 1925; Rivers and Schwentker, 1935). It became apparent that the complications seen after vaccination have striking similarities with clinical, neuropathological and immunological aspects of multiple sclerosis in human. Ever since, EAE has been intensively employed in order to understand the pathogenesis and help identify potential therapeutic candidates for MS. It was obtained in several different species and induced by immunization with a large number of CNS-derived antigens. As a consequence, the pathogenesis, pathology and clinical signs of EAE vary (Steinman, 2001; Baxter, 2007). Furthermore the severity of the induced disease and the profile of its clinical deficiencies are determined by the dose and method of immunization. EAE is most commonly induced by active priming with CNS homogenate or components of myelin that are encephalitogenic, most notably myelin basic protein, proteolipid protein and myelin oligodendrocyte glycoprotein (Massacesi et al., 1995). The antigens are mixed with an adjuvant and inactivated *Mycobacterium tuberculosis* (complete Freund's adjuvant) to enhance the immune response. Immunization of these proteins in most strains causes paralytic disorder, generally beginning with the tail muscles and hind legs, and then progressing towards the upper limbs. The rapidly unfolding neurological defects are accompanied by dramatic loss of weight due to anorexia. The development of clinical EAE usually follows a predictable schedule. After a prodromal interval the actual disease develops 10-20 days post-immunization. EAE may be monophasic or take a fluctuating course with individual disease episodes separated by partial recovery phases, or chronic, where disease symptoms of the initial attack either stabilize at peak levels or gradually worsen over time. The disease may then either spontaneously resolve or end up in chronic paralysis.

The neuropathology of clinically afflicted rodents includes spinal cord demyelination, neuronal dysfunction, inflammatory cell infiltration, microglial activation, astrocytosis, axon loss and proliferation of oligodendrocyte progenitor cells (Penkowa and Hidalgo, 2003; Ayers et al., 2004; Papadopoulos et al., 2006a). Demyelination and paralytic episodes are associated with blood-brain barrier dysfunction and infiltration of myelin-specific inflammatory cells into the CNS (Ercolini and Miller, 2006). The protective myelin sheath is damaged as a result of several inflammatory mechanisms. Among them are cytokine- (i.a. IFN-γ and tumour necrosis factor alpha (TNF-α) and complement mediated damage, digestion of surface myelin antigens by macrophages, and direct damage by Th1 $CD4^+$ and CD8+ T cells, which lead to apoptosis of oligodendrocytes and microglia (Behi et al., 2005; Sonobe et al., 2007).

1.6.3 Focal Gliotoxin-induced Demyelination: Lysophosphatidylcholine (LPC)

Historically local actions of myelinolytic factors in general have been thought to be involved in the pathogenesis of demyelinating diseases. This encouraged experimental studies of the activity of lysophosphatides, i.a. lysophosphatidylcholine (LPC) (Thompson, 1961), in the course of which it could be demonstrated in different in vitro studies that LPC can cause demyelination in the central nervous system and that it is competent to produce complete solubilisation of brain myelin (Morrison and Zamecnik, 1949; Birkmayer and Neumayer, 1957; Gent et al., 1964). Several in vivo studies followed demonstrating the demyelinating capacity of lysophosphatidylcholine in mature, myelinated peripheral nerve fibres as well as in CNS fibres of dorsal white matter in adult animal spinal cord and cerebellar peduncle (Woodruff & Franklin, 1999) (Hall and Gregson, 1971; Hall, 1972; Blakemore et al., 1977). Injection of LPC appeared therefore useful as demyelination model. Hall and co-workers showed in their experiments that the intraspinal injection of LPC produces a pure, localized demyelinating lesion. The lesion appears already within 30 min secondary to the toxic effect of LPC on either the oligodendrocytes or the myelin itself without any involvement of other adjacent, non-myelin cellular components of the fibre (Hall, 1972). Only after 6-12 h of the toxin withdrawal, a transient T cell and neutrophil response in the CNS takes place which leads to recruitment of monocytes followed by

widespread of haematogenous macrophages that mediate rapid phagocytosis and facilitate the rapid clearance of myelin debris (Ousman and David, 2000). Already within the first week following LPC injection begins the process of spontaneous remyelination, comprising the proliferation and differentiation of oligodendrocyte progenitor cell (OPC), which is completed in most parts of the demyelinated area two weeks later (Jeffery and Blakemore, 1995; Larsen et al., 2003). The lysophosphatidylcholine model has since been employed by various investigators to create experimental demyelination by myelin breakdown and apoptosis of oligodendrocytes (Pavelko et al., 1998; Shields et al., 1999; Blakemore et al., 2002; Wallace et al., 2003). In a manner similar to that used in the EAE, the experimental model of demyelination based on the injection of LPC as a toxin has been employed as a model of multiple sclerosis (Blakemore and Franklin, 2008). Yet the use of the neurotoxic does not attempt to mimic MS with its complex aetiology and pathogenesis but is rather used to study the biology of remyelination. Comprising both demyelination as well as remyelination in a short time frame it is particularly suitable for studying the mechanisms and factors involved in promoting oligodendrocyte differentiation and proliferation.

1.6.3.1 Chemical Structure and Mechanism of LPC

Lysophosphatidylcholine (LPC), also known as lysolecithin, is a major lipid constituent in mammalian tissues. The LPC molecule is wedge shaped and consists of one long hydrophobic fatty acyl chain and one large hydrophilic polar choline headgroup, attached to a glycerol backbone. LPC possesses both hydrophilic and lipophilic properties. This amphipathic nature gives it surfactant and detergent-like properties.

The biochemical mechanism of the lysophosphatidylcholine generated demyelination is not yet fully understood. Part of the demyelination effect of LPC may be related to its detergent effect on membranes, which leads to secondary Ca^{2+} overload accelerating the demyelinating process. In addition to its action as direct solvent it is thought to cause myelin breakdown by increasing phospholipase A_2 activity contained in activated macrophages which degrades membrane phosphatidylcholine (lecithin) into lysophosphatidylcholine (lysolecithin) in form of a chain-reaction. Moreover, an indirect damaging effect on oligodendrocytes by latter disintegration of myelin seems plausible (Pavelko et al., 1998; Birgbauer et al., 2004).

1.7 Steroid Hormones, Pregnancy and Multiple Sclerosis

Multiple sclerosis is a devastating neurological disease that currently affects several million people throughout the world. Many treatments have been employed to alleviate the course of the disease and to avoid relapses. The fact that pregnant women suffering from multiple sclerosis are spared from relapses at the time that sex steroid hormones are at their physiological maximum prompted the use of neuroactive steroids as potential treatment for MS (Confavreux et al., 1998). Following this assumption, there is currently a clinical phase III trial ongoing, which involves steroid therapy, given immediately postpartum to prevent relapses of pregnant women suffering from MS (Vukusic et al., 2009). In this study, named *Popart-Mus*, an increasing number of pregnant patients are being recruited and treated with oestrogen/progesterone. First results are expected to become available by the end of this year. However, tissues to study the neurochemical basis for steroid treatment cannot come from human sources. Thus, animal models such as the ones employed in this thesis work may yield valuable data to support a rationale for steroid treatment of MS.

Relapse decline during pregnancy has also been observed in different animal studies. In an experimental autoimmune encephalomyelitis model using pregnant rabbits it was shown that pregnancy exerts a protective effect on the severity of EAE and the risk of EAE is even lower when immunization has been induced at the end of pregnancy (Evron et al., 1984). It has been proposed that the increased levels of circulating progesterone at the time of pregnancy may afford protection against MS (Vukusic and Confavreux, 2006). As mentioned in the introduction, progesterone receptors are found in spinal cord motoneurons and oligodendrocytes and it has been observed that progesterone influences myelin synthesis in the peripheral and central nervous system (Schumacher et al., 2004). In cultures of oligodendrocytes, the myelin-producing glia of the CNS, PROG increases the expression of the myelin basic protein (MBP) indicating strong stimulation of myelination (Ghoumari et al., 2003). Hence progesterone may contribute to oligodendrocyte progenitor proliferation and have a beneficial influence on the regeneration of myelin repair. In addition to neuronal and myelinating effects progesterone is thought to have modulating effects on the immune system.

1.8 Preliminary Data: Progesterone Application in EAE - Autoimmune-induced Demyelination (EAE Model)

A preceding study investigated the role of progesterone on spinal cord neuropathology and clinical outcome employing the before mentioned EAE model of autoimmune-induced demyelination (Garay et al., 2007).

The study included C57BL/6 female mice (9–11 weeks old) which remained untreated, received a single 20 mg or 100 mg progesterone pellet one week before EAE induction by immunization with the myelin protein "myelin oligodendrocyte glycoprotein" (MOG_{40-54}) together with Freund's adjuvant. This combination led to an encephalitogenic T-cell and demyelinating autoantibody response, caused extensive demyelination, and reproduced the complex range of pathological and clinical phenotypes which are associated with multiple sclerosis (Schluesener et al., 1987; Storch et al., 1998). More specifically, rodents with EAE presented the expected spinal cord demyelination, inflammatory cell infiltration, microglial activation, astrocytosis and neuronal dysfunction among other features that are typically seen in EAE (Papadopoulos et al., 2006a).

Progesterone was employed in order to determine whether the disease onset, clinical stages and neurochemical changes of the spinal cord could be delayed or ameliorated by the treatment with the neuroactive steroid. To this end the EAE clinical disease signs were evaluated along with the spinal cord tissues by means of Luxol fast blue histochemical staining, expression of myelin basic protein (MBP) and proteolipid protein (PLP) in mice with and without hormonal treatment.

1.8.1 Progesterone Effects in EAE Mice

The EAE experiment showed that progesterone led to a reduction in clinical severity. The implanted progesterone pellets modified neurological deficits of EAE mice in that clinical severity was significantly attenuated and the disease onset significantly delayed. Histopathological examination of the EAE experiment provided evidence that progesterone improves the spinal cord neuropathology. While a haematoxylin and eosin staining, commonly used to assess immune cell infiltration, revealed intense spinal cord infiltration of mononuclear cells in EAE mice, PROG treatment significantly ($p < 0.01$ at doses of 20 as well as 100 mg) dampened this inflammatory cell infiltration, thus exhibiting a down-

regulatory role upon the immune reaction triggered by the myelin protein immunization (figure 7).

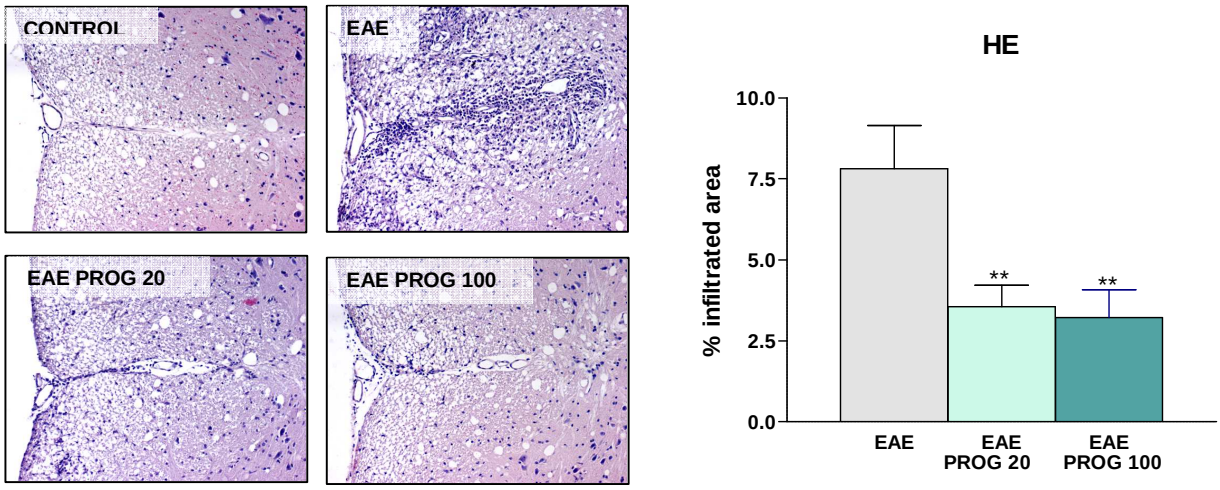


Figure 7. HE Staining.

Representative 100x magnified photomicrographs show a control mouse, intense infiltration of the dorsal funicular area in EAE and its recovery after treatment with 20 or 100 mg PROG pellets according to HE staining. Quantitative data for percent infiltration area is presented in graph on the right: EAE (gray column), EAE + PROG 20mg (light green column) and EAE + PROG 100 mg (dark green column). Cell infiltration in EAE was significantly decreased by PROG 20 and 100 mg pellets (**$p < 0.01$ for both doses; $n = 8–10$ mice per group).

Interestingly, percentage of demyelination in the EAE study was positively correlated with the area of inflammation according to the absence of Luxol fast blue staining for total myelin ($r = 0.99$, $p = 0.0019$) which was substantiated even further by immunohisto-chemical analysis of the expression of the two major CNS myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP) (figures 8a and b).

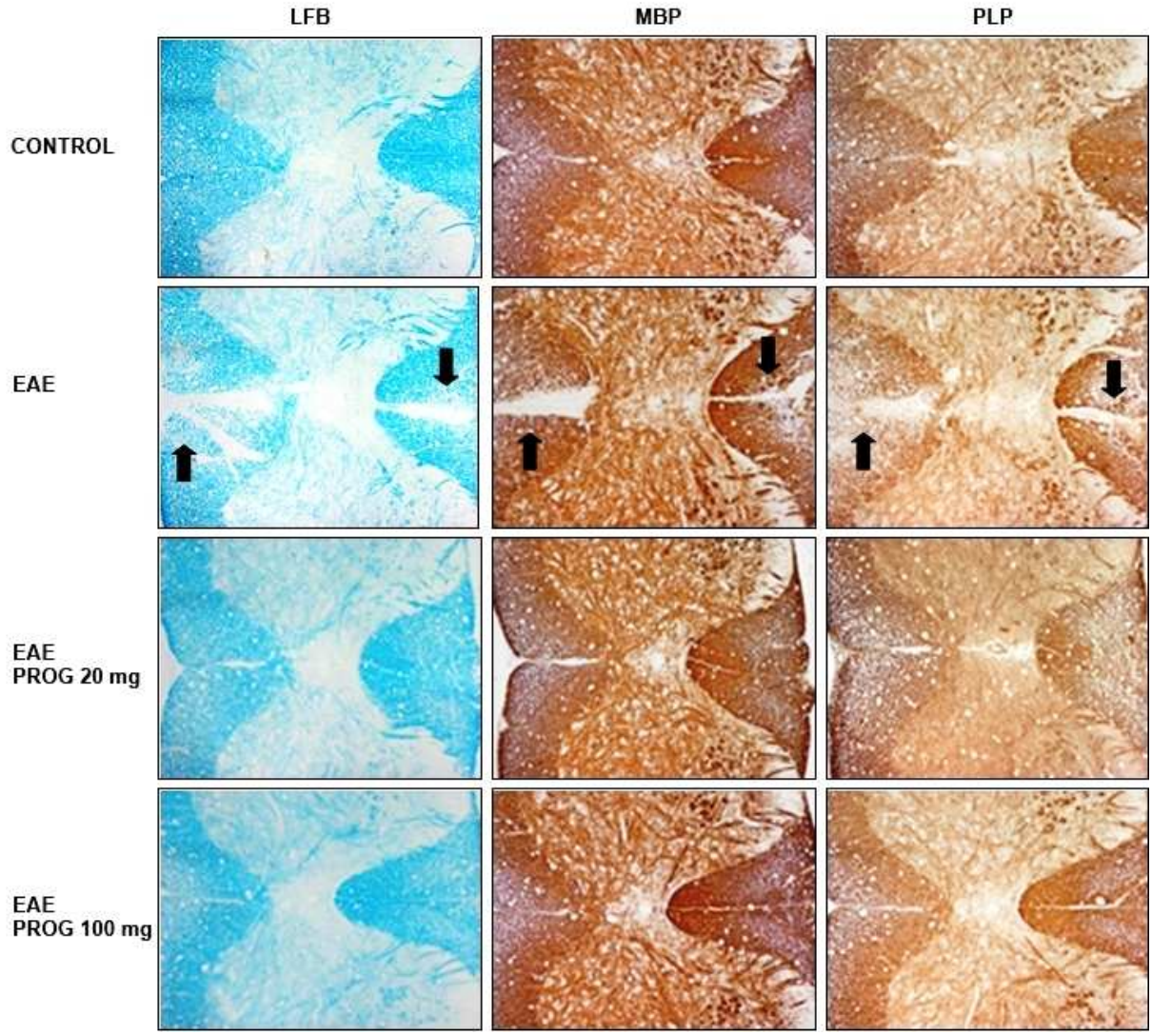


Figure 8a. Myelin protein stainings.

Representative photomicrographs (100x magn.) of LFB stain and immunoreaction for MBP and PLP. From top to bottom: CONTROL, EAE, EAE + PROG 20mg and EAE PROG 100 mg treated mice. Arrows in the EAE groups correspond to demyelinated areas free of LFB staining or MBP and PLP immunoreactivity. As can be seen in this figure, both doses of PROG were able to recover LFB staining as well as MBP and PLP immunoreaction intensity of EAE mice.

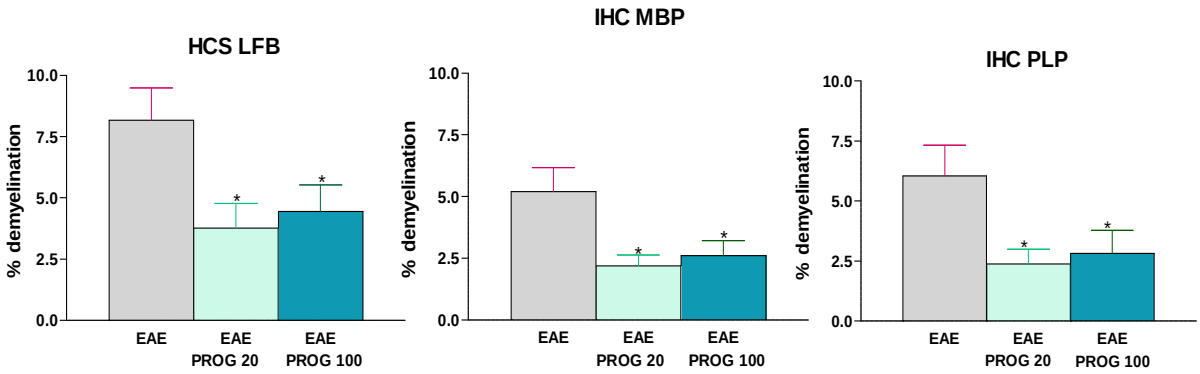


Figure 8b. **Myelin Protein Stainings.**

Quantitative data for percent of demyelination in respect to total white matter area according to Luxol Fast Blue staining (LFB), immunoreaction for myelin basic protein (MBP) and proteolipid protein (PLP). The three graphs represent EAE (gray columns), EAE + PROG 20mg (light green columns) and EAE + PROG 100 mg (dark green columns). PROG of 20 and 100 mg significantly reduced unstained area (*$p < 0.05$ for both doses vs. EAE, n = 8–10 mice per group).

The EAE model had been used as an approach to obtain information on whether progesterone has the potential to impact immune-mediated CNS demyelination. Indeed, PROG ameliorated disease severity and exhibited anti-inflammatory and myelin-protective effects, encouraging its potential therapeutic application in demyelinating diseases such as MS. Yet inflammatory models of demyelination and neurological impairment like EAE are based on systematic immunological induction and are thus associated with widespread involvement of not only large areas of the nervous system but also all kinds of immune cells. This makes it difficult to analyse and interpret specific components of de- and remyelination in a controlled manner. In contrast, the mechanism of gliotoxin-induced focal demyelination is independent of the activation of the peripheral immune system and chronic inflammation at the side of the lesion is minimal. Focal areas of demyelination can be induced in animals by injection of myelinolytic chemicals into white matter areas which specifically destroy glial cells.

2 HYPOTHESIS AND RESEARCH OBJECTIVES

2.1 Hypothesis and General Objectives

Neurosteroids influence the biochemical composition of myelin proteins and promote myelin renewal in a slow but continuous process of myelin maintenance in the adult human brain. Even though it is most likely that MS has manifold causes, one possibility contributing to the pathology could be a diminishment of neuroactive steroids leading to an absence of their protective, promyelinating influence. This absence would result in an increased vulnerability of the myelin constituents leaving the myelin structurally altered and less stable, ultimately resulting in demyelination.

The emerging findings regarding sources, mechanisms, and effects of progesterone challenge our traditional understanding of steroids and reveal novel so far undefined actions. Physiological existence of local synthesis and autocrine/paracrine activities are likely to play an important role in the viability of oligodendrocytes and in the formation of myelin sheath.

The aim of this thesis was to obtain a more detailed knowledge of the biological significance and the possible therapeutic value of progesterone in the CNS. It was of interest to examine whether progesterone ameliorates myelin impairment independently of the general activation of the immune system. To this end, the role of progesterone and its contribution on the myelin status were investigated at cellular and molecular levels using an approach in which myelin destruction is achieved by the local application of a gliotoxin.

2.2 Specific Objectives

To put these aims into practice a non-immune mediated demyelination model of toxin-induced origin following intraspinal lysophosphatidylcholine (LPC) injection was employed (referred to as “LPC model”). The specific aims were to assess whether progesterone treatment modifies:

a) the survival of oligodendrocytes and their production of myelin proteins indicating the product of myelin synthesis by mature oligodendrocytes and/or indirectly by measuring the number of myelinating cells (measured by Luxol fast blue histochemical staining)

b) the proliferation of the oligodendrocyte progenitor cells and their maturation needed for remyelination (using immunohistochemistry for NG2 and CC1)

c) the infiltration of inflammatory cells as indicative of the degree of microglial cell activity (evaluated by OX42 immunohistochemistry)

d) the response of the astrocyte population (by means of GFAP immunohistochemistry)

3 ANIMALS, MATERIAL AND METHODS

3.1 Experimental Animals

The experiment of lysophosphatidylcholine induced demyelination was carried out in C57BL/6 male mice aged 15 weeks. These mice were born and raised in the animal facilities of the IBYME Institute of Biology and Experimental Medicine or purchased from the Faculty of Veterinary Medicine, National University of La Plata. The mice were kept at a constant temperature (23℃) and humidity (40%), on a 12 h light–dark cycle, standard food and water was given ad libitum.

Animal care and all surgical procedures were performed in accordance with the “National Institutes of Health's Guide for the Care and Use of Laboratory Animals” (NIH Guide, Institute’s Assurance Certificate # A5072-01) and experimental protocols were approved by the Institute’s Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

3.2 Methods

3.2.1 Surgery of Toxin-induced Demyelination – Intraspinal LPC Injection

Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (25 mg/kg body weight) and xylazine hydrochloride (5 mg/kg body weight). Dorsal laminectomies were made in the upper thoracic region of the spinal cord and the dura mater incised close to the midline. A 34 gauge needle attached to a Hamilton syringe was used to inject 1 µl of a 1% solution of lysophosphatidylcholine (Sigma-Aldrich, MO USA) in sterile PBS, pH 7.4, with Evan's blue added as a marker. The needle was inserted into the anterolateral part of the spinal cord, 1 µl of the LPC solution injected, and the needle then slowly withdrawn. The surgical wound was sutured in two layers, and mice were allowed to recover. The day of lysophosphatidylcholine injection was designated day 0.

3.2.2 Progesterone (PROG) Treatment

Seven days prior to lysophosphatidylcholine injection 14 mice received hormone treatment. To this end, mice were implanted with a single 100 mg progesterone (Sigma-Aldrich, MO USA) pellet under the skin of the neck, whereas another group of 14 mice remained without steroid treatment and received an equivalent cholesterol pellet. The group assignment was carried out randomly.

3.2.3 Perfusion, Fixation, Dissection and Tissue Preparation

For histochemical examination experimental animals were sacrificed 7 days after lysophosphatidylcholine injection. To this end the animals were deeply anesthetized with ketamine and xylazine hydrochloride (50 mg/kg and 10 mg/kg body weight i.p. respectively) and intracardially perfused with 30 ml of diethylpyrocarbonate-treated (DEPC) 0.9 % sodium chloride (NaCl) followed by 30 ml of 4 % DEPC paraformaldehyde fixative (pH 7.2) at 4℃. Blood was collected from the heart at the time of sacrifice. The spinal cords were carefully removed. More precisely, the thoracic segments of the spinal cords were extracted cranial and caudal to the injection site, which were indicated macroscopically by the addition of Evan's blue dye at level T1–T3 approximately. The spinal cord tissues were then post-fixed in the aforementioned fixative for 2.5 h at 4℃. Eight entire thoracic segments of the spinal cord per group were processed for further inclusion in paraffin and six entire thoracic segments per group prepared to be frozen. Tissues to be stored frozen until further process were cryoprotected by immersion in 20% sucrose in phosphate buffer (pH 7.2) for 24 h at 4°C, then embedded in Tissue Tek OCT compound (Sakura Finetek, CA USA) and kept at -80℃ .

3.2.3.1 Paraffin Section Process

Spinal cord segments embedded in paraffin were cut with a microtome into consecutive transversal 5 µm sections and mounted on glass slides previously coated with 3.5 % gelatine. Before that the distinctive stainings sections were deparaffinised with xylene and rehydrated in descending alcohols.

3.2.3.2 Cryostat Section Process

Tissues stored frozen at -80℃ were cut transversally at 30 µm thickness in a cryostat (HM505N, Microm, Walldorf Germany) and mounted onto positively charged microscope slides to be stored again frozen at -80℃ until further processing.

3.2.4 Progesterone Serum Level Determination

At the day of sacrifice, blood was drawn from the heart and centrifuged to obtain the serum. The content of serum progestins were determined using a Coat-A-Count progesterone radioimmunoassay (RIA) kit (Diagnostic Product Corporation, CA USA) and results expressed as ng/ml serum.

Due to the relative specificity of RIA methods, the possibility of cross-reaction with progesterone metabolites existed in hormone-treated mice. Thus, steroid levels were reported as serum progestins referring to progesterone and its derivates (5α-DHP and 3α,5α-THP).

3.2.5 Determination of Cell Infiltration and Demyelination

3.2.5.1 Luxol Fast Blue (LFB) Histochemical Staining

Deparaffinised sections were stained with Luxol fast blue to mark the area of demyelination. LFB dye is commonly used for the demonstration of normal myelin. It belongs to the sulfonated copper phthalocyanine type and is the alcohol-soluble counterpart of the water-soluble Alcian Blue. Staining is due to lipoproteins, and the mechanism is an acid-base reaction with salt formation, where the base of the lipoprotein replaces the base of the dye causing a colour change with myelin fibres seen blue under the light microscope (Kluver and Barrera, 1953).

For LFB staining, spinal cord sections were deparaffinised and hydrated to 95% ethyl alcohol and left in 1% Luxol fast blue solution (Luxol fast blue 1 g, 95% alcohol 100 ml, 10% acetic acid 5 ml) at 60º C for 18 hours. After rinsing of excess stain in 95% ethyl alcohol and distilled water, slides were immersed first in lithium carbonate solution, then in 70% ethyl alcohol (for each 1 min), rinsed in distilled water and mounted with Permount as described by Kim et al. (Kim et al., 2006).

3.2.5.2 IHC for Detection of Glial Fibrillary Acidic Protein (GFAP) and Adenomatous Polyposis Coli (CC1)

The adenomatous polyposis coli (CC1) antibody recognizes antigens of mature oligodendrocytes. A weak labelling of astrocytes was however reported. To verify the specificity of CC1 staining, a double immunofluorescence procedure using the monoclonal CC1 antibody and a polyclonal glial fibrillary acidic protein (GFAP) antibody (marker of mainly mature astrocytes) was adopted, where single-labelled cells (CC1-positive/GFAP-negative) were considered oligodendrocytes (Horky et al., 2006). For double-labelling with GFAP-CC1, the 30 µm frozen sections were thawed, rinsed in PBS and PBS containing 0.5% Triton X-100. Sections were first blocked in 3 % goat serum solved in 0.5% Triton X-100 for 10 at 37℃, then with mouse IgG blocking re agent (Vector M.O.M. immunedetection kit, Vector Labs) for 1 h at room temperature and rinsed. After preincubation in M.O.M. diluent (600 µl of protein concentrate in 7.5 ml PBS) for 5 min at room temperature, sections were incubated at 4℃ wi th the mouse anti-CC1 monoclonal antibody (1/100, Calbiochem, CA USA) and rabbit anti-GFAP polyclonal antibody (1/250, Sigma-Aldrich, MO USA) diluted in PBS containing 2% goat serum and 0.1% Triton X-100 at 4° C overnight. Negative controls were prepared omitting the primary antibodies (each singular and jointly). Following the incubation with the primary antibodies, sections were washed again before application of the secondary antibodies: goat anti-rabbit IgG conjugated to Alexa 488 (1/500, Molecular probes, OR USA) and goat anti-mouse IgG conjugated to Alexa 555 (1/1000, Molecular probes, OR USA) for 30 min at room temperature. Incubation with secondary antibodies was followed with final washes, coverslipped using Fluoromount G (Southern Biotech, AL USA) as mounting media and kept in the dark at 4° C until analysis by confocal microscopy.

3.2.5.3 IHC for Detection of Neuron-Glial Antigen 2 (NG2) and Complement Receptor 3 (OX42)

It has been reported that neuron-glial antigen 2 (NG2) labelling in lesioned spinal cord is not restricted to oligodendrocyte progenitor cell (OPC) population, but also includes a subset of macrophages (McTigue et al., 2001). Thus, the phenotype of NG2+ cells was

determined using a double labelling immunofluorescence protocol, in which sections were exposed to the polyclonal rabbit anti-mouse NG2 antibody (1/250, Dr. William Stallcup, the Burnham Institute, CA USA), and the monoclonal complement receptor 3 (OX42) antibody, a marker of macrophages / microglia, (1/100, Chemicon, CA USA). It was considered that single-labelled NG2+/OX42- cells were indicative of OPC. For double-labelling with NG2-OX42, the 30 µm frozen sections were thawed, rinsed in PBS and PBS containing 0.5% Triton X-100 and then blocked with a 3 % goat serum-PBS solved in 0.5% Triton X-100. After additional washing in PBS the tissue sections were treated with mouse IgG blocking reagent (Vector M.O.M. Immunodetection Kit, Vector Labs) for 1 h at room temperature and rinsed again. The application of the M.O.M. diluent (600 µl of protein concentrate in 7.5 ml PBS) for 5 min at room temperature was followed by the incubation with the respective primary antibodies in 2 % goat serum containing 0.1 % Triton X-100 solution at 4° C overnight. Following the incubation with the primary antibodies, the sections were washed again before application of the secondary antibodies: goat anti-rabbit IgG conjugated to Alexa 488 (1:500, Molecular probes, OR USA) and goat anti-mouse IgG conjugated to Alexa 555 (1:500), Molecular probes, OR USA) for 30 min at room temperature. Negative controls were prepared omitting the respective primary antibodies. Incubation with secondary antibodies was followed with final washes to be mounted with Fluoromount G and kept in the dark at 4°C until analysis by confocal microscopy.

Summary of Antibodies Used for Immunohistochemistry

Antibody	Specificity
α-NG2 (rabbit, polyclonal)	oligodendrocyte precursor cells (neuron-glial antigen 2)
α-CC1 (mouse, monoclonal)	mature oligodendrocytes (adenomatous polyposis coli)
α-OX42 (mouse, monoclonal)	microglia, macrophages (complement receptor 3)
α-GFAP (rabbit, polyclonal)	astrocytes (glial fibrillary acidic protein)

3.2.6 Quantitative and Statistical Analysis

Values were generally expressed as means ± standard error of the mean (SEM). In all cases p-values < 0.05 were considered significant and indicated with asterisk(s) (* $p < 0.05$, ** $p < 0.01$). Graph Pad Prism 5.0 was used for graphic visualization.

3.2.6.1 Analysis of Luxol Fast Blue

To determine spinal cord demyelination produced by the toxin and the treatment effect of progesterone on this parameter, images were captured at 40x magnification by the use of an Olympus BH-2 light microscope coupled to a digital Panasonic GP-KR222 camera. Focal areas showing negative histochemical staining for LFB were delimited at several regions of the spinal cord (dorsal, lateral or ventral funiculus) by computerized image analysis using Bioscan Optimas VI software (Bioscan Optimas, WA USA) (Ferrini et al., 1995). Surface areas of these regions were added up and demyelination for each spinal cord section expressed as a percentage of the total surface area of white matter sampled (Mathisen et al., 2001; Papadopoulos et al., 2006a). Group differences for LFB staining were determined by one-way ANOVA, followed by post-hoc comparisons with the Newman–Keuls test.

3.2.6.2 Analysis of NG2, OX42, CC1 and GFAP Immunohistochemistry

Double-labelled fluorescent cells were examined under a Nikon Eclipse E-800 confocal scanning laser microscope (Nikon, Tokyo Japan). Images were acquired sequentially in a line-scanning mode through an optical section of 1 μm in the z-axis, and merged using Nikon EZC1 version 2.1 software. Quantitative analysis of the amount of NG2+ and Ox42+ cells was carried out by computerized image analysis using Bioscan Optimas II software. For statistical analysis the Student's t-test was applied.

4 RESULTS

As it was pointed out in the introduction, the central aim of this thesis was to study progesterone's role as neuroprotectant considering its possible utility in demyelinating diseases. In view of the fact that MS presents clinical and pathological heterogeneity, it seems reasonable to undertake different approaches to better understand steroid action in the setting of demyelination. In a preceding study progesterone effects were studied using the EAE animal model of autoimmune induced demyelination, that reproduces specific features of the histopathology and neurobiology of MS including a broad systemic immune response. The findings of the EAE study obtained in our laboratory strongly support the promyelinating and neuroprotective actions of progesterone. Yet the EAE experimental model relies entirely on the compromise of the immune system, suggesting that progesterone's main effect is the suppression of immune attack on myelin and myelin-producing oligodendrocytes.

In view of existing reports in the literature presented in the introduction it seems possible that progesterone might modulate myelinogenesis at the local level. To test this hypothesis, LPC was injected directly into the spinal cord which resulted in a focal demyelinated lesion. In contrast to the EAE model of demyelinating disease, the mechanism of demyelination in the toxin-induced model is unrelated to general immune system activation, allowing the evaluation of the remyelinating repair potential of progesterone treatment from a different perspective.

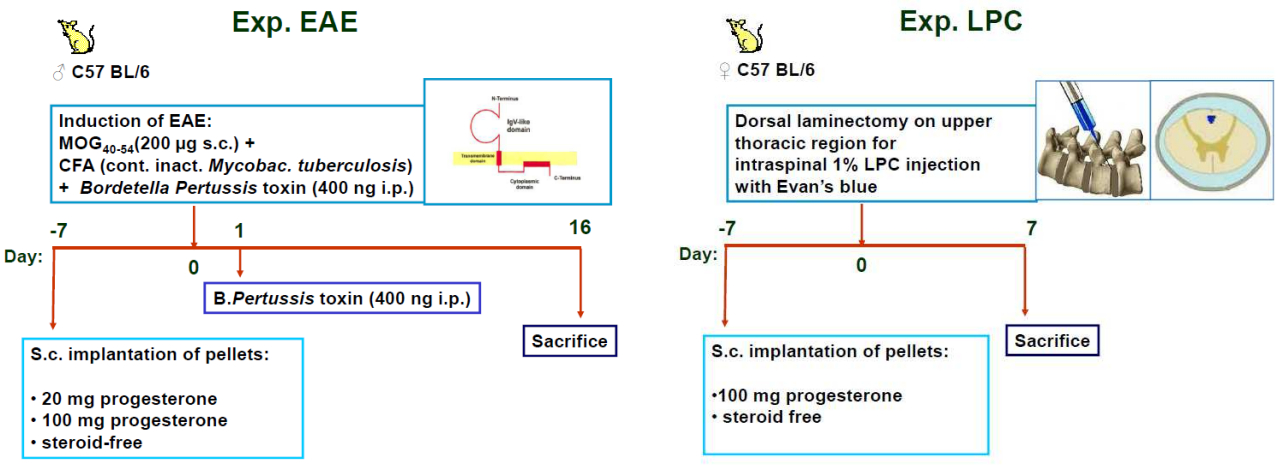


Figure 9. **Experimental Designs of the previous EAE and Actual LPC Experiment in Comparison.**

The experimental protocol involving lesioning with LPC was adapted from the preceding EAE experiment (figure 9). For the evaluation of demyelinating lesions using the model of chemical demyelination, one percent solution of lysophosphatidylcholine (LPC) was injected into the dorsal funiculus of the spinal cord. The demyelinated lesions, macroscopically visible due to aggregation of Evan's blue dye, appeared rostral and caudal to the injection site within an area ranging between 4 and 10 mm. The C57BL/6 male mice (15 weeks old) remained either untreated or received a single 100 mg progesterone pellet one week prior to intraspinal LPC injection and thus progesterone treatment lasted 14 days. Mice were sacrificed seven days after the surgical toxin application because it is known that at this time point the extent of the lesion is maximal and the expression of the myelin proteins MBP and PLP is reduced to a minimum as a result of lysophosphatidylcholine action (Woodruff and Franklin, 1999).

In order to evaluate whether progesterone is able to accelerate the remyelination process, a comparison between LPC-lesioned animals with or without treatment was carried out. For the histological assessment of spinal cord demyelination and remyelination, similar methods to the one described in the EAE experiment were applied; however, in this experiment a closer look was given at parameters reflecting the maturation grade of the oligodendrocytes.

4.1 Progesterone serum level determination

Steroid levels are reported as serum progestins (ng/ml) because of relative specificity of the RIA methods due to the possibility of cross-reactions with PROG metabolites in hormone-treated mice. The serum progestin levels were low in hormone-free mice (2.76 ± 0.95 ng/ml), and increased more than 25-fold in mice treated with 100 mg progesterone pellet (73.87 ± 8.88, $p < 0.0001$ versus non-treated mice).

4.2 Histochemical Results

4.2.1 Luxol Fast Blue Histochemistry

The demyelination degree of the lysophosphatidylcholine (LPC) lesioned spinal cord was determined by histochemical staining with Luxol fast blue (LFB). Intraspinal injection of

LPC resulted in a prominent focal demyelination 1 week after injury (Fig 10, upper photomicrographs). To test the putative neuroprotective function of progesterone, the lesion size of progesterone-treated mice was compared to that of untreated mice, evaluating sections all through the entire thoracic segment (site of injection) of the spinal cord. The quantitative evaluation of demyelination expressed as percentage of the total white matter was carried out as described in Materials and Methods. The study demonstrated that in progesterone-treated mice the extent of white matter lesions was significantly reduced in comparison to the lesions shown by the untreated mice (fig. 10, lower graph).

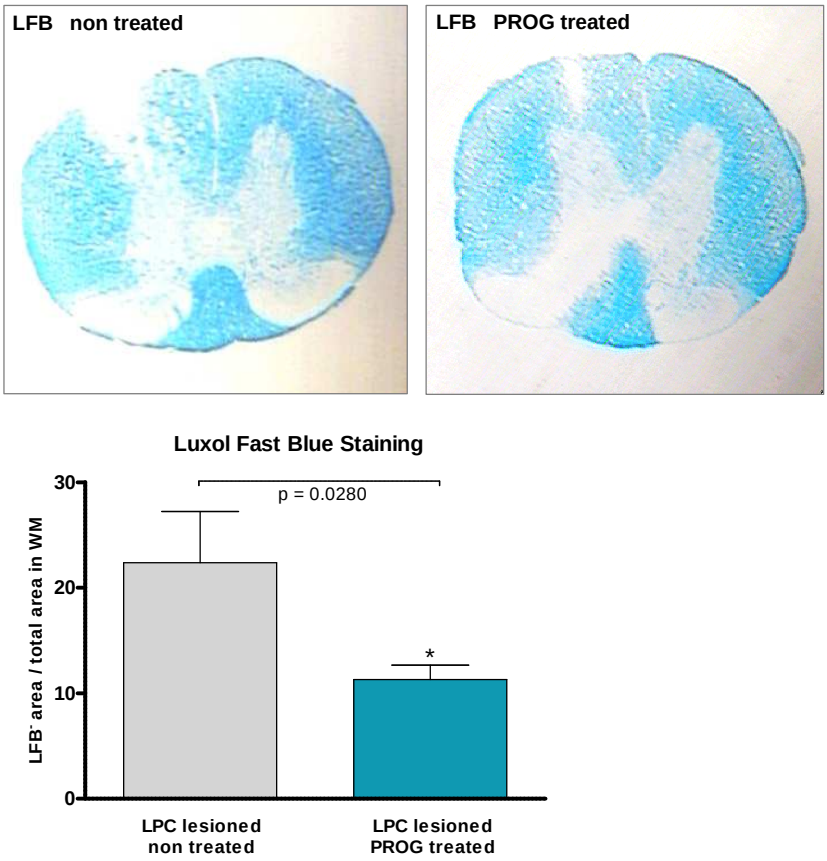


Figure 10. Demyelination Determined by LFB Staining.

The upper photomicrographs show the Luxol Fast Blue staining of representative transverse sections of the spinal cord. Progesterone administration had a protective effect against damage of white matter myelin. Quantitative evaluation (lower graph) of % demyelination with respect to total white matter according to Luxol Fast Blue staining confirmed that the extent of white matter lesions was less severe in progesterone-treated mice, as compared with untreated animals. Values are the mean ± SEM (n = 6-8 mice per group), *p< 0.05 (unpaired t test) with a 95% confidence interval of 1.41 to 20.70.

4.2.2 Lysophosphatidylcholine Induced Lesions

As a result of LPC injection, we observed a reactive cellular response histological visible as conglomeration of glial cells. It was equally possible that part of this response was due to the direct physical injury caused by the injection. Due to the spurious confluence of many cell types at the direct injection site, quantification of some sections mostly within the funiculus dorsalis was often difficult as exemplified in figure 11. Therefore, computerized image analysis was concentrated on the ventral and lateral parts of the spinal cord white matter.

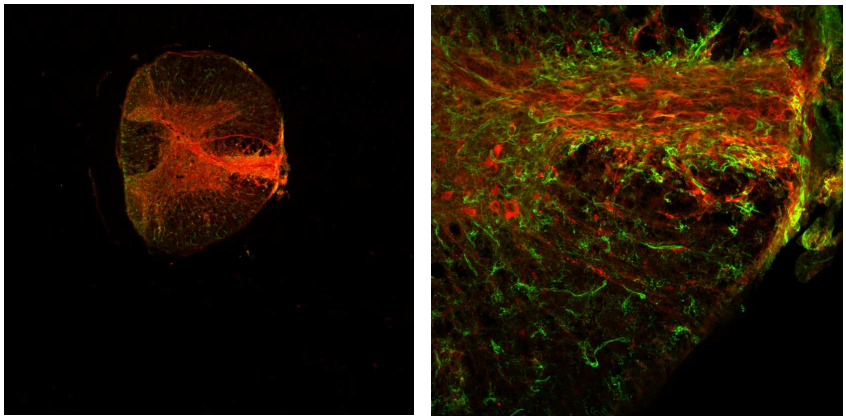


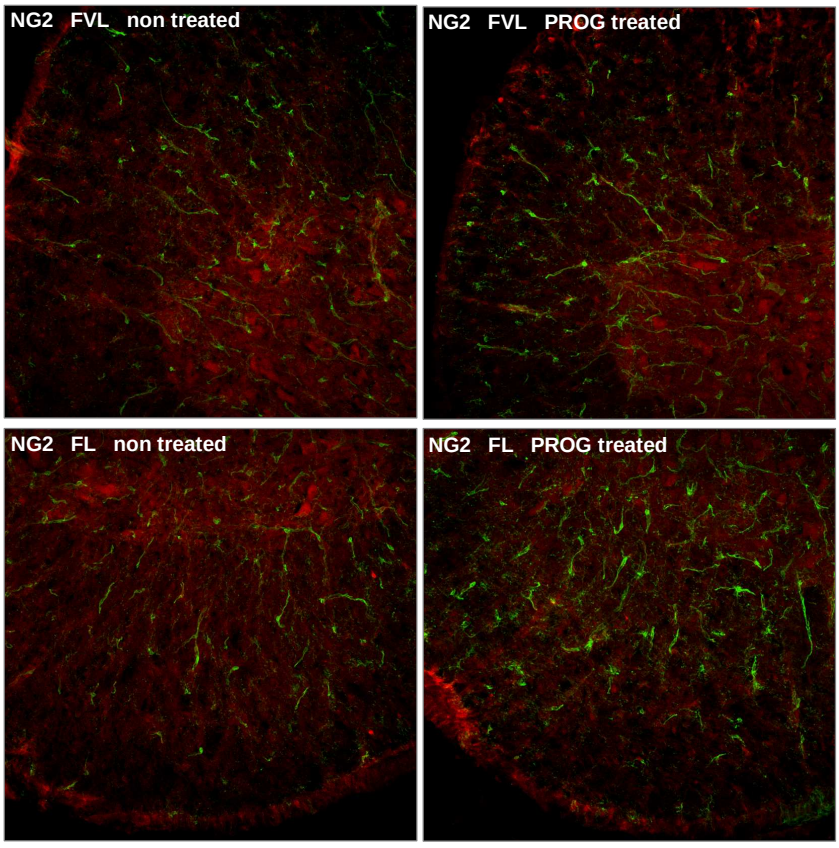
Figure 11. LPC-induced Glial Scar.

Exemplar of strong cell conglomeration caused by local LPC injection –here exc. at FVM (4x and 20x magnification).

4.2.3 NG2 Immunostaining

The effects of progesterone on oligodendrocyte-precursor cells (NG2-positive cells) were determined using a cell-specific marker and immunocytochemical techniques coupled to computerized image analysis. As shown in figure 12, progesterone treatment given seven days prior to the LPC induced demyelinating lesion moderately stimulated the proliferation of NG2-positive oligodendrocyte precursor cells. More precisely, progesterone significantly increased the number of NG2-positive cells in the lateral funiculus but not in the ventrolateral funiculus over levels of untreated mice. To discount the contribution of other cell types to NG2 quantification (activated microglia and macrophages), the number

of NG2-positve/OX42-negative cells was counted in a double immunofluorescence assay in which staining for NG2 was combined with anti-OX42 staining of the same sections. The single-labelled cells ($NG2^{+}/OX42^{-}$) were considered indicative of OPC, yet colocalization was very rarely observed. Thus, it is unlikely that microglia/macrophages produced an overestimation of the number of NG2+ cells.



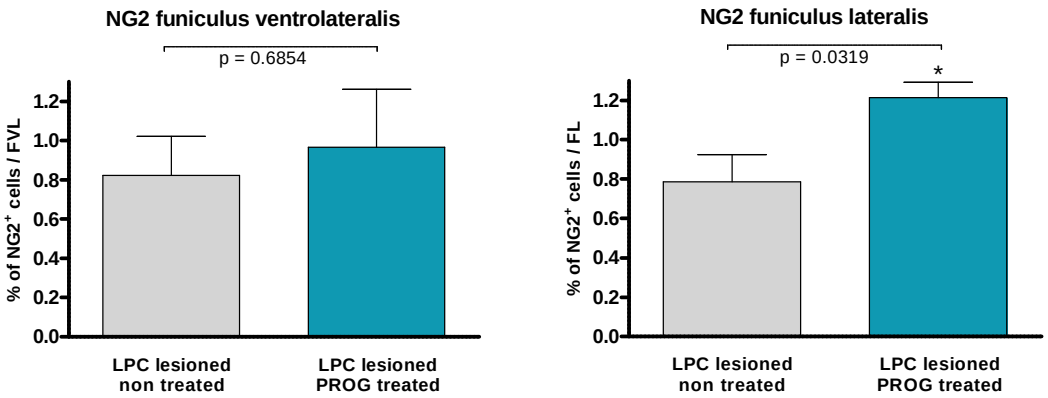
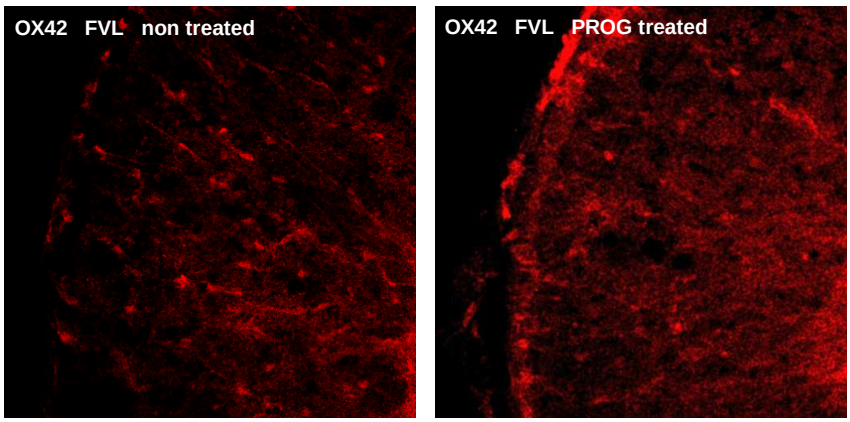


Figure 12. NG2 Immunostaining.

Quantitative evaluation of $NG2^+$ cells in FVL and FL in the demyelinated lesion area 7 days after LPC injection analyzed by immunohistochemistry in untreated and progesterone-treated mice. Results represent the mean percentage of $NG2^+$ cells per 300 μm^2 ± SEM (n = 5-6 mice per group). There was a significant difference (indicated with an asterisk) between treated and untreated groups *p< 0.05 in the FL but not in the FVL (unpaired t test). The 95% confidence interval was -0.64 to 0.93 in FVL and 0.05 to 0.80 in FL. The upper representative photomicrographs, obtained in the laser confocal microscope, show the counterstaining of NG2 (green) and $OX42^+$ cells (red) in FVL (upper row) and FL (lower row) of the spinal cord below the LPC lesion site in mice killed 7 days post-injection. Only very few $NG2^+$ cells colocalized with Ox42.

4.2.4 OX42 Immunostaining

Reactive microglia, identified by their intense OX-42 immunoreactivity and enlarged cell bodies, were additionally detected and measured in the white matter. Following progesterone treatment, microglial activation in response to LPC was noticeably depressed as shown by the significant decrease of the number of OX42 immunoreactive cells (fig. 13).



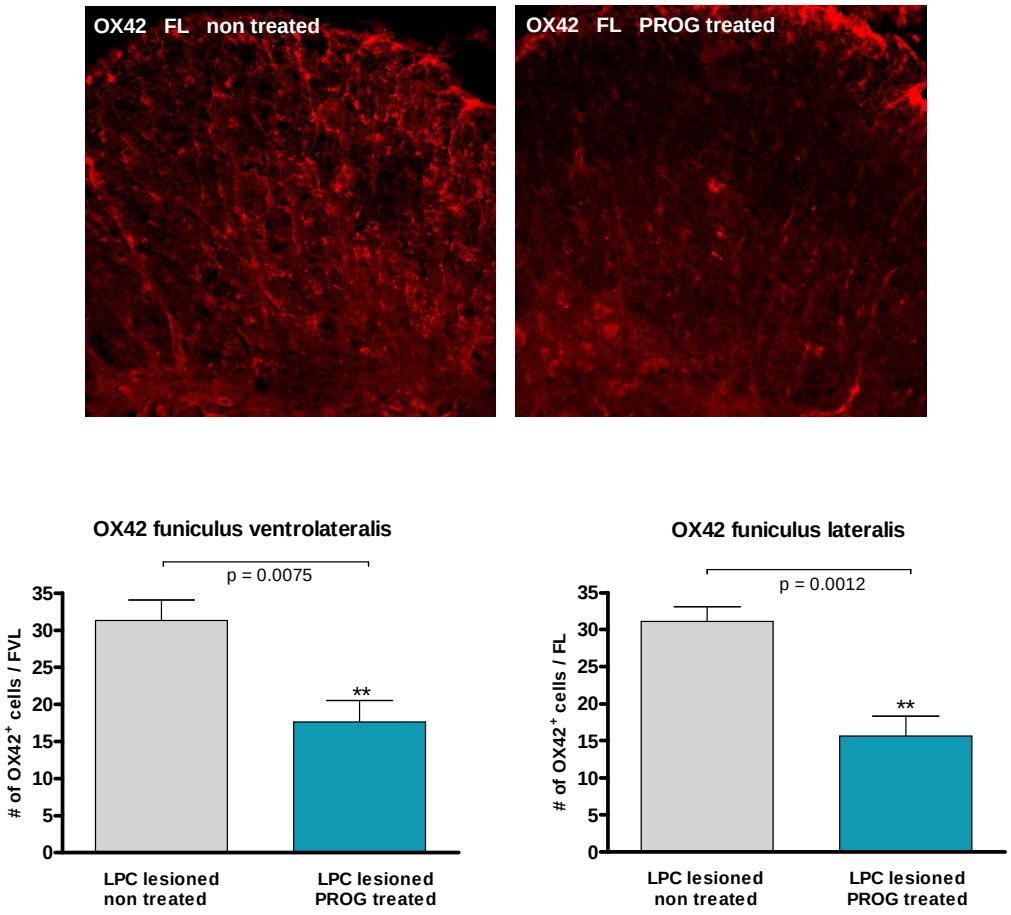


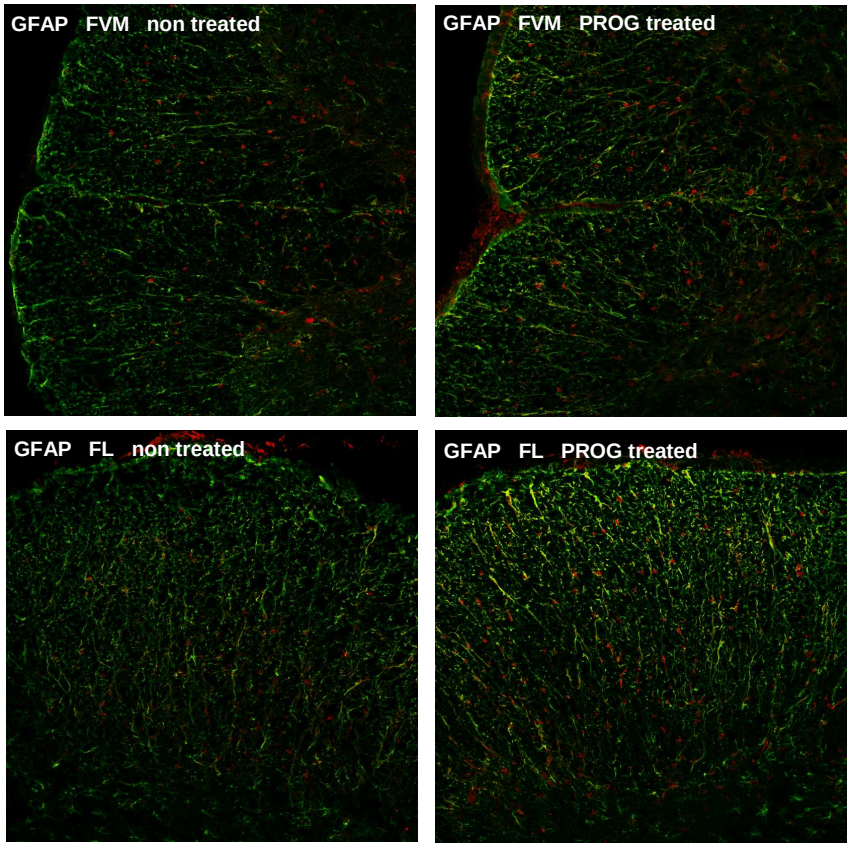
Figure 13. OX42 Immunostaining.

Quantitative evaluation of $OX42^+$ cells in FVL and FL in the demyelinated lesion area 7 days after LPC injection analyzed by immunohistochemistry in untreated and PROG-treated mice. Results represent the mean number of $OX42^+$ cells per 300 μm^2 ± SEM (n = 5-6 mice per group). The asterisk indicates significant difference between groups ** $p< 0.01$ (unpaired t-test). The 95% confidence interval is -22.73 to -4.67 in FVL and -22.99 to -7.97 in FL. PROG significantly decreased the density of $OX42^+$ cells in FL as well as FVL. The upper representative single channel confocal photomicrographs show $OX42^+$ cells (red) in FVL (upper image) and FL (lower image) of the spinal cord below the LPC lesion site in mice killed 7 days p.i.

4.2.5 GFAP Immunostaining

To further evaluate the effects of progesterone on neuroglial cells in the context of remyelination, the response of astrocytes to the gliotoxin was investigated. In previous studies it was shown that glial fibrillary acidic protein (GFAP)-labelled astrocytes are

strongly upregulated in response to CNS damage (Lee et al., 2006). In a study of penetrating spinal cord injury, progesterone, given for 3 days at the dose of 4 mg/kg, did not modify the already high GFAP immunoreactivity with the exception of an effect in gray matter astrocytes (De Nicola et al., 2003). In the present study there was also no statistically significant difference between untreated and steroid-treated mice between GFAP-immunoreactive astrocytes in the vicinity of the LPC induced lesion, neither in the white nor in the gray matter (fig. 14). Similarly, we did not observe a statistical difference in GFAP-expressing astrocytes, when comparing LPC injected mice with control mice receiving an injection of PBS as opposed to LPC (data not shown).



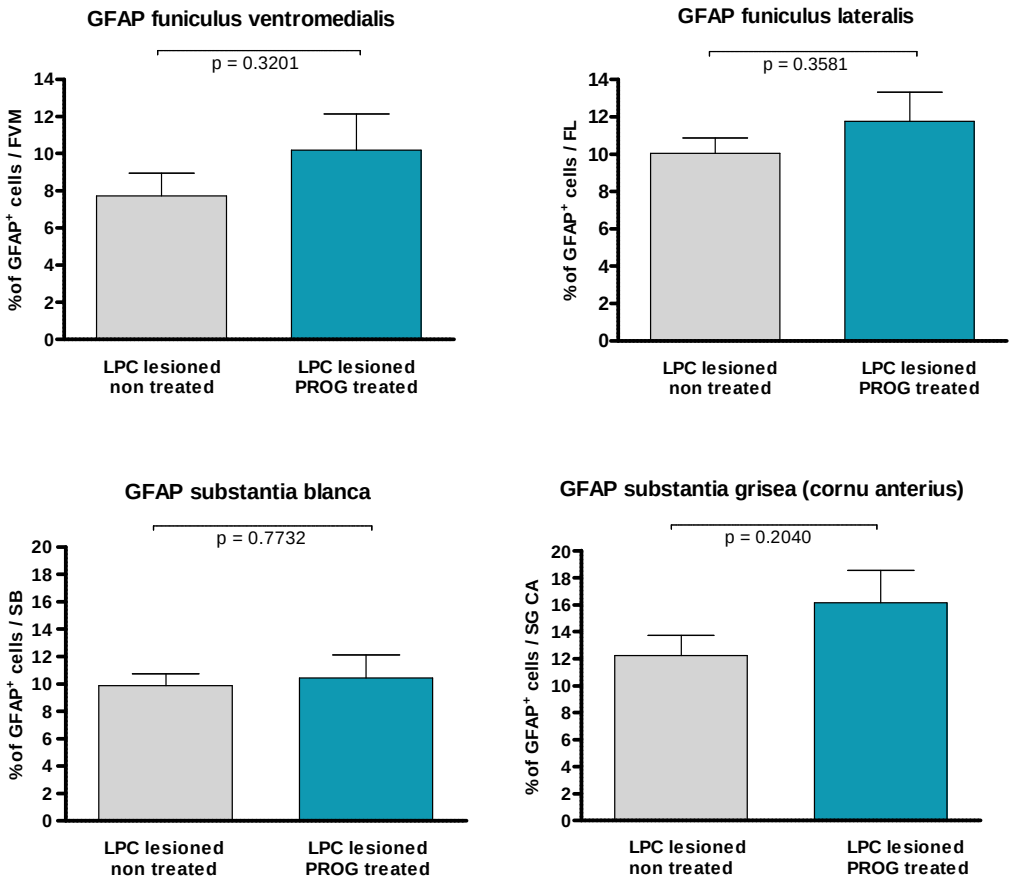


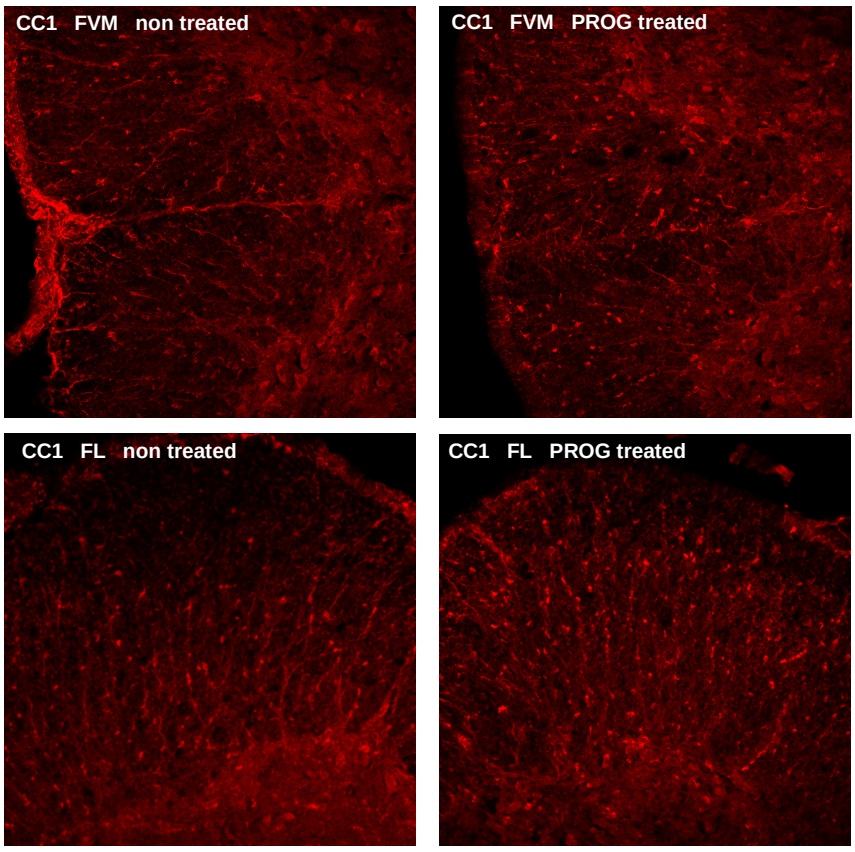
Figure 14. GFAP Immunostaining.

Quantitative evaluation of $GFAP^+$ cells in FVM and FL (upper histogram) and SB and SG (lower histogram) 7 days after LPC injection analyzed by immunohistochemistry in untreated and progesterone-treated mice. Results represent the mean percentage of $GFAP^+$ cells per 300 μm^2 ± SEM (n = 5 mice per group). Statistical comparison between groups was performed by unpaired t test. The 95% confidence interval is -2.87 to 7.77 in FVM, -2.34 to 5.76 in FL, -3.80 to 4.92 in SB and -2.61 to 10.45 in SG. The number of GFAP+ cells did not significantly vary after LPC injection in the demyelinated lesion area. Upper representative confocal photomicrographs show double staining of $GFAP^+$ (green) and CC1-oligodendrocyte labelled cells (red) in FVM (upper row) and FL (lower row) of the spinal cord below the LPC lesion site in mice killed 7 days p.i.

4.2.6 CC1 Immunostaining

The CC1 antibody has been shown to stain mostly mature oligodendrocytes, although astrocytes may cross-react (although feebly) with this antibody. We observed an increased immunoreactivity for CC1 in the lateral part of the white matter, which correlated with

increased immunoreactivity for NG2 (fig. 15). We also performed double-labelling of CC1 and the astrocyte marker glial fibrillary acidic protein (GFAP) in order to discount a contribution of the latter cell type to the CC1 data. Yet allocation was not always clear due to a peculiar staining pattern. Thus, it was difficult to differentiate CC1-positive cell bodies from GFAP-positive astrocytic processes even by confocal microscopy. However, the observation of the relative absence of influence on GFAP-positive cells, representing reactive astrocytes, following LPC injection (as described in 4.2.6) suggests that the significant CC1 increase in the FL portrays a response of mature oligodendrocytes. Interestingly, progesterone treatment led to a significant increase of CC1 in the funiculus lateralis but did not differ from untreated mice in the ventral funiculus. This is in agreement with the results obtained by NG2 immunostaining (see corresponding section 4.2.4).



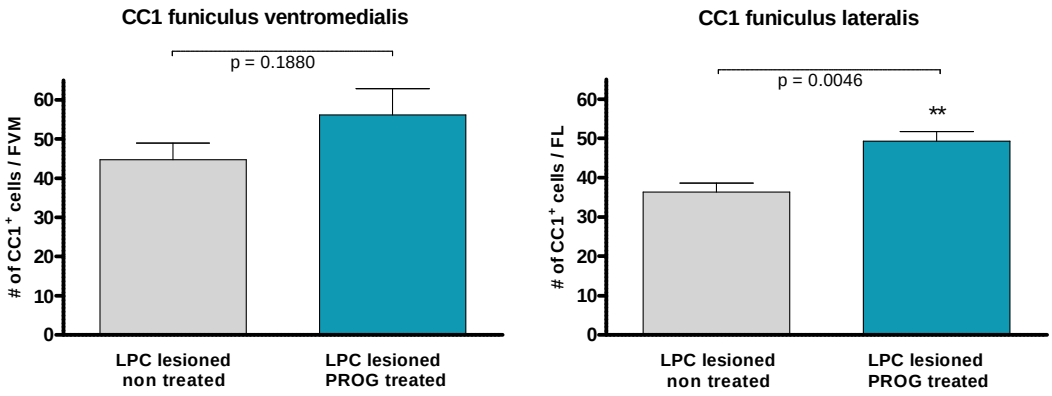


Figure 15. CC1 Immunostaining

Quantitative evaluation of $CC1^+$ cells in FVL and FL in the demyelinated lesion area 7 days after LPC injection analyzed by immunohistochemistry in untreated and progesterone-treated mice. Results represent the mean number of $CC1^+$ cells per 300 μm^2 ± SEM (n = 5 mice per group). The asterisk indicates significant difference between treated and untreated groups **p< 0.01 (unpaired t-test). The 95% confidence interval is -6.86 to 29.65 in FVM and 5.29 to 20.68 in FL. Progesterone significantly decreased the density of $CC1^+$ cells in FL but not in FVM. Upper representative single channel confocal photomicrographs show $CC1^+$ cells (red) in FVM (upper row) and FL (lower row) of the spinal cord below the LPC lesion site in mice killed 7 days post-injection.

In summary, the toxin-induced demyelination model showed that the significant increase in plasma progesterone via progesterone pellet implantation was able to attenuate the toxin neurochemical damage. Thus, progesterone ameliorated the degree of myelination according to measurements of Luxol fast blue staining, decreased the density of OX42-positive microglia/macrophages and increased the density of NG2-positive oligodendrocyte progenitors as well as the density of CC1-positve mature oligodendrocytes in the lateral part of the white matter as demonstrated by immunohistochemical analysis. These data support a promyelinating and anti-inflammatory effect of neuroactive progesterone, acting locally at the spinal cord level. Thus, previous results obtained with the EAE model are complemented with data obtained by the LPC model, indicating that in addition to a powerful suppression of the peripheral immune system, progesterone is able to locally modulate repair under conditions of demyelination and inflammation of the spinal cord. In other words, there are two sites of progesterone action, a peripheral and a local, that complement each other and counteract the neuropathological consequences of demyelination.

5 DISCUSSION AND PERSPECTIVES

Although inflammation is considered to be a primary feature of demyelinating plaques, recent reports indicate that demyelination may precede inflammation (Barnett and Prineas, 2004). Furthermore, differences in whether disease pathogenesis is initiated by demyelination or as preponderance of inflammation could be an explanation of the different disease courses observed in MS patients. A differentiation between primary inflammatory and primary demyelinating activities in lesions should therefore be considered. In the previously published EAE study, it could be demonstrated that progesterone improved the spinal cord myelin status subsequent to autoimmune triggered demyelination. This reflects progesterone's therapeutic suitability in immune triggered demyelination. We hypothesized furthermore that progesterone is likely to participate in the protection and regeneration of oligodendrocytes subsequent to demyelination in a local manner not only by influencing systemic inflammation response.

Hence the focal demyelination model was employed in order to understand if progesterone effects were entirely due to suppression of the activation of the peripheral immune system, or if a direct steroid effect in the spinal cord could also explain progesterone neuroprotective actions. It is known that a focal demyelination model can be achieved by injection or topic application of lysophosphatidylcholines (LPC). LPC is obtained from the partial hydrolysis of phosphatidylcholines which removes one of the fatty acid groups. Hydrolysis is generally the result of the enzymatic action of phospholipase A2.

Quantitative determination by Luxol fast blue staining gave evidence that successful focal loss of myelin in the spinal cord could be detected through areas lacking Luxol fast blue staining. Results obtained in mice with injections of lysophosphatidylcholine indicated that:

a) Progesterone treatment produced important changes in the myelin status observable in decrease of demyelination foci according to Luxol fast blue staining.

Thus also in the toxin-induced demyelination model, progesterone exerts myelin protecting effects as previously observed for the EAE demyelination model of autoimmune origin

(Garay et al., 2007) indicating that progesterone directly influences the survival of oligodendrocytes and their maintenance or production of myelin proteins.

b) In progesterone-treated mice, the number of NG2+ cells was increased in comparison to untreated mice injected with LPC.

This response suggests an effect on the oligodendrocytes, the myelin-synthesizing cells of the CNS. These cells are extremely labile to injury, toxins and neurodegenerative diseases, which provoke oligodendrocyte loss, axonal demyelination and impaired nerve conduction (Olby and Blakemore, 1996). However, remaining oligodendrocytes cannot repair myelin, and remyelination derives from recruitment of an endogenous population of oligodendrocyte precursor cells (OPC) that express the NG2 surface proteoglycan (McTigue and Tripathi, 2008). Normally, OPC remain in a quiescent state but rapidly proliferate in response to toxic demyelination. However, these NG2+ cells do not differentiate but rapidly evolve into myelin-forming oligodendrocytes if properly stimulated by several transcription factors, some of which are directly stimulated by progesterone (Labombarda et al., 2009). Therefore, it was quite important that under our experimental conditions, progesterone treatment decreased the extent of white matter lesions and increased the number of NG2+ cells in LPC-lesioned mice in comparison to mice injected with LPC but without progesterone. At the same time, progesterone treatment led to a significant increase of mature, differentiated oligodendrocytes labelled with the CC1 antibody at least in the funiculus lateralis, suggesting either that part of the NG2+ cells differentiated into mature, myelin-producing oligodendrocytes or that both oligodendrocyte stages were being protected from cell death during and after injury.

Other studies which examined the dynamic nature of oligodendrocyte death and replacement following spinal cord injury show that oligodendrocytes are lost early and then replenished within the first 2 weeks following injury (Zai et al., 2005; Horky et al., 2006; Tripathi and McTigue, 2007). To verify whether differentiation has taken place, a double-labelled analysis for mature oligodendrocytes (CC1) and bromodeoxyuridine (BrdU) using

confocal microscopy could be applied as demonstrated in a model of spinal cord injury (Labombarda et al., 2009).

Progesterone could influence oligodendrocyte survival and/or genesis by multiple external and internal signaling molecules. To the extracellular growth factors which are known to be upregulated in the vicinity of CNS lesions, belong brain-derived neurotrophic factor (BDNF), Neurotrophin-3, glial growth factor and fibroblast growth factor-2 (Lee et al., 2009; Linker et al., 2009). Data of our laboratory obtained in the *Wobbler* mouse model of motoneuron degeneration suggests for example that modulation of brain-derived neurotrophic factor is likely to be part of the progesterone activated-pathways affording a trophic protective environment (Gonzalez Deniselle et al., 2007). BDNF in turn could be the stimulator of intracellular signals such as the transcription factors Olig-1 and Olig-2 which are essential for the regulation of oligodendrocyte proliferation and differentiation (Talbott et al., 2005; De Nicola et al., 2009). It might also be of interest to study progesterone effects on the transcription factor peroxisome proliferator-activated receptor delta (PPAR-δ), which is known to play a global role in cellular differentiation and has been shown to be expressed in the CNS by oligodendrocytes and neurons (Benani et al., 2004; Hall et al., 2008; Muller et al., 2008). The relation of oligodendrocyte differentiation and this transcription factor has recently been studied in a model of spinal cord injury without any treatment applying a triple-label of the above mentioned CC1,BrdU and PPAR-δ (Almad and McTigue, 2010). The study showed two interesting details which help to understand the LPC experimental results. First of all the results obtained in the model of spinal cord injury proved once more that NG2-positive oligodendrocyte progenitor cells have the potential to proliferate after injury. More remarkably, the same study shows that within one week following injury, new mature CC1-positive BrdU positive oligodendrocytes are formed. Therefore, this paper is in line with our suggestion that it is indeed possible that new oligodendrocytes differentiated from proliferating progenitors during LPC-induced demyelination. Secondly, as mentioned above, the transcription factor PPAR-δ may be involved in the dynamic oligodendrogenesis detected during CNS lesions making it, along with progesterone treatment an interesting target worthy of further study (Almad and McTigue, 2010).

c) It is equally important that our results provided strong evidence that progesterone treatment prevented the strong microglial activation caused by LPC, as demonstrated by the diminished number of OX42 immunoreactive cells.

The OX42 antibody recognizes complement receptor type 3 (CR3) in mononuclear phagocytes and is commonly used to determine the activated microglia (Ling et al., 1990). Microglial activation is a common response to injury, neurodegeneration and application of neurotoxins to the central nervous system (O'Callaghan et al., 2008). In these situations, microglial cells change the phenotype from small, star-shaped cells to amoeboid subtypes showing strong labelling for OX42. Our demonstration that progesterone treatment decreased the density of OX42-positive cells indicated that progesterone shows potent anti-inflammatory effects *in situ*. There is a single paper in the literature showing that administration of a glucocorticoid in mice after intraspinal LPC injection arrests the inflammatory reaction (Pavelko et al., 1998). This glucocorticoid-like effect of progesterone may be explained by the fact that the glucocorticoid receptor and the progesterone receptor share the same hormone response element in the DNA (GRE/PRE) (Lee et al., 2009).

Activated microglia is believed to contribute to MS pathology, possibly, at least in part, due to the production of nitric oxide and TNF-α, molecules which can be toxic to CNS cells, including oligodendrocytes. The phenomenon of microglial immunomodulation has been reported by several authors using different animal models. It was demonstrated that, similarly to the effects observed during pregnancy, progesterone stimulates a change from a T helper cell 1 (Th1) pro-inflammatory response to a T helper cell 2 (Th2) anti-inflammatory response (Gibson et al., 2005; Pettus et al., 2005). It thereby alters the profile of cytokine secretion by activated lymphocytes, increasing the production of non-inflammatory, non-cytotoxic interleukins (IL) such as IL-3, IL-4 and IL-10 and reducing the production of inflammatory, cytotoxic cytokines including interferon delta (IFN-δ), TNF-α and IL-2 (Druckmann and Druckmann, 2005). Progesterone-dependent immune-modulation, in part mediated by a protein called progesterone-induced blocking factor (PIBF) (Szekeres-Bartho and Wegmann, 1996), seems plausible considering that during

pregnancy a protective immunological environment is necessary to prevent abortion. Thus progesterone plays a modulatory role in the interaction between the neuroendocrine and the immune systems, constituting a novel therapeutic intervention with respect to the treatment of autoimmune diseases such as MS. Given that our laboratory focuses on the biochemical neuroendocrinology, detailed immunomodulatory parameters were not examined in this study. However it might be interesting to further analyze this issue with the help of neuroimmunologists (a study concerning this issue will be carried out in the future in collaboration with the neuroimmunologist Dr. Gabriel Rabinovich at the IBYME Buenos Aires).

The findings presented here are consistent with a hypothesis regarding the pathophysiology of MS based on the instability of myelin. It suggests that myelin components breaking adrift would need to be eliminated by phagocytosis through microglial cells. The more myelin debris and phagocytic cells there are, the greater the likelihood that a fraction will be drained into the lymphatic system. Whereas the myelin proteins are inaccessible to immunosurveillance under normal conditions (being on the other side of the blood-brain barrier), they would now be present in elevated amounts and most likely over a longer period within an immunoregulatory environment and thus lead to the activation of the inadequate immune response. In that way it is favourable that progesterone seems to diminish broad systemic immune reaction as observed in the EAE model. Yet, as seen in the LPC model, also around the CNS lesions themselves the myelin debris triggers an undesirable local inflammatory response and contributes to the progressive neuropathology and functional deterioration. Until recently there were no studies on how exactly the myelin, as endogenous inflammatory stimulus, initiates this inflammatory reaction. Interestingly, new data show that the complement receptor 3 (CR3) may play a crucial role in the initiation of this inflammatory response. The myelin debris generated in the injured CNS or demyelinating diseases appear to stimulate macrophages by binding to its CR3. This leads to the activation of nuclear factor kappa of activated B-cells (NF-κB) through the focal adhesion kinase/ phosphotidylinositol 3-kinase/ protein kinase B (FAK/PI3K/PKB) pathway which initiates the inflammation cascade by

upregulating gene expression of pro-inflammatory mediators and downregulating anti-inflammatory cytokines (Sun et al., 2010).

Applying this new data to our results suggests that the progesterone treatment-attributed reduction of CR3 expression (evaluated by OX42 immunohistochemistry) may contribute to fewer CR3-myelin interactions, avoiding the undesirable microglial activation.

d) GFAP-expressing astrocytes did not significantly vary after LPC injection in the area surrounding the demyelinated lesion.

Reactive astrogliosis has become a pathological hallmark of CNS structural lesions; it starts when trigger molecules produced at the injury site drive astrocytes to leave their quiescent state and become activated. This process is morphologically and biochemically characterized by upregulation of the intermediate filament glial fibrillary acidic protein (GFAP), increased cell proliferation and cell hypertrophy.

It is commonly accepted that LPC causes astrocyte reactivity, changing their gene expression resembling the pattern of immune cells. The toxin-activated astrocytes can release pro-inflammatory mediators that attract macrophages and microglia and induce their local and distal proliferation (Sheikh et al., 2009). In the present study there was no statistically significant difference between GFAP-immunoreactive astrocytes in the vicinity of the LPC induced lesion in the white or gray matter between untreated and steroid-treated mice. Therefore, the astrocyte reaction under these conditions may be insensitive to progesterone levels reached under our experimental protocol, and its down-regulation may need an additional regulatory factor, i.e., the addition to estrogens, higher or more prolonged progesterone treatment or other factors.

While reactive astrogliosis was classically considered to be detrimental to CNS function, it has been suggested to be beneficial in an acute inflammatory environment soon after demyelination (Williams et al., 2007). Activated astrocytes have been shown to help maintain a functional environment and influence OPC survival, differentiation and migration (Dziembowska et al., 2005). GFAP-expressing astrocytes are thought to be necessary for remyelination. Once stimulated, they produce trophic factors, increase the uptake of toxic

levels of glutamate and potassium and release lactate as glucose precursor in neurons (Simard and Nedergaard, 2004).

There are many different theories about the relationship between inflammation and neurodegeneration in MS. EAE is a model in which inflammation causes demyelination, the LPC model, by contrast, is one where demyelination causes inflammation. Cumulatively the results obtained confirm previous data and indicate that progesterone possesses OPC survival promoting activity regardless of the demyelination origin. Progesterone seems to influence multiple neuroglial cell types including oligodendrocytes and microglial cells in the pathophysiological environment following injury induced by lysophosphatidylcholine.

Together with previous observations of progesterone neuroprotective effects observed following traumatic brain injury, kainic acid damage to the hippocampus and models of permanent focal brain ischemia as well as glutamate exposed motoneurons, the data presented in this thesis work provide additional proof that progesterone, a hormone deeply involved in pregnancy and sexual behaviour, displays unique properties remote and quite different from its reproductive effects in models of neuroinflammation. (Yu, 1989; Ogata et al., 1993; Ciriza et al., 2004; Ibanez et al., 2004; Gonzalez Deniselle et al., 2007; Sayeed et al., 2007; MacNevin et al., 2009). Future work is needed to unveil the clinical relevance of the present experimental study.

In perspective, as future therapies will have to tackle both damage repair and attenuation of the ongoing disease process, progesterone is likely to be included in a therapeutic trial suppressing disease-related inflammation, demyelination and enhancing myelination. New data evolving from different approaches will help to improve the understanding of the mechanisms affected by progesterone and other neurosteroids.

SUMMARY

This thesis comprehends the study of *in vivo* neuroendocrine effects of progesterone on myelination and its possible therapeutic value in multiple sclerosis. Progesterone has long been considered a female sex hormone that is primarily involved in regulating pregnancy. Yet interestingly also neurons and glial cells synthesize progesterone *de novo* and express progesterone receptors (Mariott et al. 2008 *Glia* 56: 686-98; Schumacher et al. 2008 *Curr Opin Pharmacol* 8**:** 740-6). Recent research shows that progesterone is a potent pleiotropic neuroactive steroid that can protect and promote myelin renewal in the slow but continuous process of myelin maintenance in the adult central nervous system. Regeneration of myelin is mediated by oligodendrocyte precursor cells, which are widely distributed throughout the central nervous system (CNS).

In multiple sclerosis (MS), the most common demyelinating disease and cause of neurological disability in young adults, there is a progressive and irreversible loss of myelin and the remyelination process fails. MS disease activity diminishes during the third trimester of pregnancy and increases during the first three months post-partum which coincides with increasing high levels of female sex steroids during pregnancy followed by dramatic decline immediately after delivery (Confavreux et al. 1998 *N Engl J Med* 339: 285-291).

An ongoing clinical trial aims to prevent MS relapses related to the post-partum condition by administering progesterone in combination with estradiol (Vukusic et al. 2009 *J Neurol Sci* 286: 114-8). Data generated from another clinical trial found evidence that progesterone is safe for use in patients suffering from traumatic brain injuries and may reduce the risk of death and long-term disability (Wright et al. 2007 *Ann Emerg Med* 49: 391-402). While these trials are already enrolling thousands of patients, it seems important to gain more knowledge of the biological significance of progesterone in the CNS.

Since preclinical data of various experimental CNS lesion models including a preceding experiment of autoimmune induced encephalitis (EAE) with progesterone treatment indicate promotion of myelin viability along with anti-inflammatory protection, it was our objective to investigate whether progesterone ameliorates myelin impairment also independently of general immune system activation (Garay et al. 2007 J Steroid Biochem

Mol Biol. 107: 228-237, Gibson et al. 2008 Brain 131: 318-28). Thus, the effects of exogenously applied progesterone on myelin protection and renewal were examined in spinal cord lesions of demyelination in a murine model using C57BL/6 mice. Demyelination was focally induced by direct intraspinal injection of the gliotoxic detergent lysophosphatidylcholine (LPC).

The histopathological examinations revealed that progesterone reduced the size of LPC demyelinated lesions as seen by histochemical and immunohistochemical staining of myelin proteins in animals receiving progesterone treatment compared to untreated animals. Specifically, the experiments indicated that progesterone treatment (a) produced important changes in the myelin status of the spinal cord according to the decrease of demyelination foci measured by Luxol fast blue staining. Progesterone treatment (b) increased the number of NG2-positive oligodendrocyte-precursor cells and (c) diminished the number of the local LPC-triggered reactive microglia identified by OX42 immunoreactivity in comparison to untreated mice injected with LPC alone, while (d) progesterone administration did not significantly change glial fibrillary acidic protein (GFAP)-labelled astrocytes after LPC injection in the area surrounding the demyelinated lesion.

Thus the results of this thesis using the non-immune mediated toxin-induced model run in parallel with previous results obtained in the EAE model. While progesterone prevented excessive microglial activation in the context of local reactive inflammation, the model suggests that progesterone shows potent myelin protecting activities which are independent from the systemic immunomodulation and involve recruitment and proliferation of oligodendrocytes.

ZUSAMMENFASSUNG

Die vorliegende Arbeit untersucht den neuroendokrinen Einfluss von Progesteron auf die De- und Remyelinisierung im zentralen Nervensystem (ZNS) und seinen möglichen therapeutischen Einsatz in Multiple Sklerose (MS).

Progesteron ist uns bisher als weibliches Sexualhormon bekannt, unter anderem in seiner Funktion zur Aufrechterhaltung der Schwangerschaft. Bemerkenswert ist jedoch, dass nicht nur Rezeptoren für das Steroidhormon im gesamten ZNS verteilt gefunden werden können, sondern auch dass es dort von Nerven- und Gliazellen lokal synthetisiert wird (Mariott et al. 2008 *Glia* 56: 686-98; Schumacher et al. 2008 *Curr Opin Pharmacol* 8**:** 740-6). Neue Forschungsergebnisse deuten darauf hin, dass es sich bei Progesteron um ein leistungsfähiges pleiotrophisches Neurosteroid handelt, welches den langsamen, aber kontinuierlichen Prozess der Aufrechterhaltung und Erneuerung der von Oligondendrozyten gebildeten Myelins im adulten ZNS schützt und fördert.

MS, die häufigste chronisch-entzündliche, demyelinisierende Erkrankung des ZNS, ist charakterisiert durch eine progrediente, irreversible Zerstörung der Nervenzellen-umhüllenden Myelinscheide. Studien zeigen, dass die Aktivität der Krankheit im Verlauf einer Schwangerschaft deutlich abnimmt und nach Entbindung wieder zunimmt, welches mit der drastischen Erhöhung während fortschreitender Schwangerschaft sowie dem postpartalen Abfall der Konzentration dieses Sexualhormons korreliert (Confavreux et al. 1998 *N Engl J Med* 339: 285-291).

In einer laufenden klinischen Studie scheint die Einnahme von Progesteron kombiniert mit Östradiol den postpartalen MS-Rezidiven vorzubeugen (Vukusic et al. 2009 J Neurol Sci 286: 114-8). Daten einer weiteren klinischen Studie demonstrieren, dass Progesteron im Rahmen von traumatischen Gehirnverletzungen Mortalität und Langzeit-Behinderung reduziert (Wright et al. 2007 *Ann Emerg Med* 49: 391-402). Während die klinischen Studien bereits tausende Probanden rekrutieren, erscheint es wichtig die biologische Bedeutsamkeit von Progesteron im ZNS genauer zu erörtern.

Präklinische Daten aus experimentellen Tiermodellen auf der Basis verschiedener ZNS-Läsionen, darunter einem für MS häufig genutztem Tiermodell der experimentell

autoimmun-induzierten Encephalitis (EAE), unterstützen die These, dass Progesteron antiinflammatorisch und protektiv auf Neurone und dessen Myelinschicht wirkt.

Ziel dieser Arbeit ist es, zu untersuchen, ob Progesteron Myelinschäden auch unabhängig von seinem Einfluss auf das allgmeine Immunsystem direkt schützt (Garay et al. 2007 J Steroid Biochem Mol Biol. 107: 228-237, Gibson et al. 2008 Brain 131: 318-28). Anhand eines geeigneten Tiermodelles zur Untersuchung der De- und Remyelinisierung wurde die Wirkung von exogen verabreichtem Progesteron auf den Schutz und die Erneuerung von Myelin untersucht. Hierzu wurde das gliotoxische Detergens Lysophosphatidylcholin (LPC) lokal in das Rückenmark von C57BL/6 Mäusen injiziert. Die histopathologischen Analysen mittels immun-/ histochemischer Nachweise zeigten, dass Progesteron die Grösse der durch LPC verursachten demyelinisierten Läsionen im Vergleich zu unbehandelten Tieren reduziert.

Im Detail zeigte das Experiment, dass die Therapie mit Progesteron (a) signifikant den Myelin-Status verändert, da es die demyelinisierten Foki, sichtbar durch Luxol-Fast-Blau Färbung, verringert. Durch die Progesterontherapie wurde sowohl (b) die Anzahl der NG2-positiven Oligodendrozten-Vorläuferzellen erhöht, als auch (c) die Anzahl der durch die LPC-verursachten reaktiven Ox42-positiven Microglia-Zellen jeweils im Vergleich zu unbehandelten Mäusen reduziert, während die Progesteronbehandlung (d) GFAP-markierte Zellen im Bereich der durch LPC bewirkten demyelinisierten Läsion nicht beeinflusste.

Die Arbeitsergebnisse, die unter Zuhilfenahme des immununabhängigen Demyelinisierungmodelles durch lokale Toxinverabreichung gewonnen wurden, stimmen mit vorhergenden Ergebnissen des EAE Modells überein. Während Progesteron eine übermäßige Mikrogliaaktivierung auch im Rahmen einer lokalen Entzündungsreaktion eindämmt, zeigt dieses angewandte Modell weiterführend auf, dass Progesteron starke Myelin-schützende Eigenschaften besitzt, welche nicht unter dem Einfluss systemischer Immunreaktionen stehen und die Rekrutierung und Proliferation von Oligodendrozyten beinhaltet.

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ERKLÄRUNG

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