EXPERIMENTAL NEUROINFLAMMATION:

A FOCUS ON MITOCHONDRIA, OXYGEN AND FUNCTION

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i. ABSTRACT

There is increasing evidence for "hypoxia-like" conditions in some inflammatory multiple sclerosis (MS) lesions raising the possibility that the tissue is deficient in energy. Accordingly, mitochondrial abnormalities have been described in some MS tissue, including reduced mitochondrial gene transcripts in MS motor cortex and decreased mitochondrial complex IV expression.

To explore the role of mitochondrial defects in neuroinflammatory lesions, experimental inflammatory lesions were induced by the intraspinal injection of lipopolysaccharide in rats under general anaesthesia. The tissue was snap-frozen for histochemical assessment of mitochondrial complex II and/or IV activity at various intervals (1-28 days) post injection: the activity of the complexes was compared with porin expression or complex IV subunit I expression (presence of mitochondria or protein respectively). The oxygen concentration within the dorsal column was determined at 24 hours after lesion induction *in vivo* using an oxygen-sensitive optical probe. EMG potentials were also recorded at the foot dorsum in response to stimulation of the sciatic nerve. Presence of reactive oxygen and nitrogen species were examined by immunohistochemistry and by the fluorescent marker dihydroethidium (DHE) *in vivo*.

Complex IV activity within the motor neurons was decreased 1 day after injection, further decreased by day 2, and returned to pre-injection baseline by day 5. Decreased complex IV activity exactly coincided with a temporary reduction in motor neuron excitability as measured by H reflex and F wave amplitude, which was maximal at day 2. The oxygen concentration within the dorsal columns at the site of the LPS lesion was significantly elevated when compared with the saline-injected and naïve control animals. DHE fluorescence revealed that superoxide production was increased at the lesion site and immmunohistochemistry revealed oxidative stress to DNA, lipids and protein.

LPS-induced neuroinflammation results in a reversible and coincident decrease in both complex IV activity and motor neuron excitability, which was strengthened by

the result that intraspinal LPS-injections causes hyperoxia, presumably from mitochondrial dysfunction. Nitric oxide has been implicated in neuronal dysfunction and although a causal relationship has not been established, observations support an interpretation that inflammation-mediated NO causes mitochondrial damage, increased oxygen concentration and formation of reactive oxygen species (ROS). These actions illustrate a vicious circle where ROS induce further damage, which results in energy deficiency displayed by reduced neuronal excitability and neurological deficits. The observations are consistent with energy deficiency and reduced function in neuroinflammatory lesions similar to those found in MS

ii. STATEMENT

I state that the data presented in this thesis is based on experiments conducted by me, Frida Laulund, with a few exceptions:

Some of the immunohistochemistry was done in collaboration with Peter Anderson, Ph.D.
All of the electrophysiology (except for the ischemia experiments) was performed in collaboration with Andrew Davies, Ph.D., analysis of recordings were not.
The experiment were the motor neurons were located by electrophysiology was solely the product of work by professor Kenneth Smith, Ph.D. and Andrew Davies
All the mitochondrial activity assays (with few exceptions) were conducted by Dr Don Mahad, Ph.D. and his collaborators at University of Newcastle (Graham Campell, Iryna Ziabreva and Mark). Tissue was prepared but rarely processed by me.
Plastic sections were prepared by Daniel Morrisson, Ph.D.

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Inflammation within the CNS may impair neuronal function in experimental models of MS by causing a dysfunction of mitochondria.

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To characterise the immunohistochemistry of Pattern III model lesions to find clues as to if there might be an energy deficiency of the cells and through which mechanism it would occur.

To determine if the oxygen concentration of the Pattern III model lesions is abnormal.

To develop and characterise a novel model involving the ventral horn motor neurons, and to use this model to gain insight as to how the excitability of the neurons is affected by neuoinflammation.

To study what effect neuroinflammation has on the activity of the mitochondria in the Pattern III model lesion and the ventral horn model lesion.

iiiii. ACKNOWLEDGEMENTS

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iiiii. LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AIF	apoptosis inducing factor
APCs	antigen presenting cells
AREs	antioxidant response elements
ATP	adenosine triphosphate
ATPase	complex V of the mitochondrial electron chain
CAP	compound action potential
CIS	clinically isolated syndrome
CNP	2,3-cyclic nucleotide 3-phosphodiesterase
CNS	central nervous system
COX	cytochrome oxidase
CSF	cerebrospinal fluid
DC	dorsal column/-s
DNA	deoxyribonucleic acid
EAE	experimental autoimmune/allergic encepahalomyelitis
EBV	Epstein-Barr virus
eNOS	endothelial NOS
ETC	electron transport chain
EPR	electron paramagnetic resonance
eu	endotoxin units
GSH	glutathione
H_2O_2	hydrogen peroxide
HIF	hypoxia inducible factor
HO-1	heme oxygenase 1
HREs	hypoxic response elements
Ig	Immonoglobulin
iNOS	inducible NOS
LPS	lipopolysaccharide
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging

MRS	magnetic resonance spectroscopy
MS	multiple sclerosis
NAA	N-acetylaspartate
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAWM	normal appearing white matter
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
NOX	NADPH oxidase
Nrf2	nuclear related factor E2
$O_2^{-}\cdot$	superoxide
OH^-	hydroxyl ion
PBS	phosphate buffered saline
PARP	poly ADP ribose polymerase
PLP	proteolipid protein
PP	primary progressive
PPP	Pattern III + prephagocytotic
PT	permeability transition
RA	room air
RNS	reactive nitrogen species
ROS	reactive oxygen species
RR	relapse remitting
sGC	soluble guanylate cyclase
SD	sprague dawley
SDH	succinate dehydrogenase
SOD	superoxide dismutase
SP	secondary progressive
TBS	tris-buffered saline
TLRs	toll-like receptors
TNF	tumor necrosis factor
UCP	uncoupling protein
VSMCs	vascular smooth muscle cells
VEGF	vascular endothelial growth factor

Q_{O2}	oxygen consumption
$\Delta\Psi_m$	mitochondrial membrane potential

1. INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) that affects approximately 85,000 patients in the UK alone, and 2.5 million worldwide (reviewed by Compston and Coles, 2002). The neurological deficits can typically be traced to a conduction deficit along axons. The pathophysiology of MS is complex but is traditionally thought to involve a myelin-targeted activation of the autoimmune system. However, recent studies suggest that inflammation have a prominent role in the display of the transient symptoms taking place during clinical relapses and that a deficit in the energy-producing metabolism of the mitochondria may play a part in the lesion progression. To test these two claims, two inflammatory models were examined for neuronal function and mitochondrial activity.

1.1 CLASSIFICATION OF THE DISEASE

Patients with MS are described as having different phases of the disease depending on the frequency and duration of the symptoms (Steinman, 2001). The majority of MS patients have a disease course initiated with relapsing/remitting symptoms (RR) MS, followed after several years by a secondary progressive (SP) phase in which relapses are uncommon (Figure 1). Some patients have a progressive disease course from onset and are characterized as primary progressive (PP). Occasionally patients may have a benign disease course where symptoms are mild or even non-existent. Examples of symptoms are visual dysfunction, hearing impairment or balance problems, but also more severe disabilities such as paralysis. MS patients are characteristically more easily tired upon physical or cognitive tasks, and they will take longer to recover from normal tasks (Fisk et al., 1994).

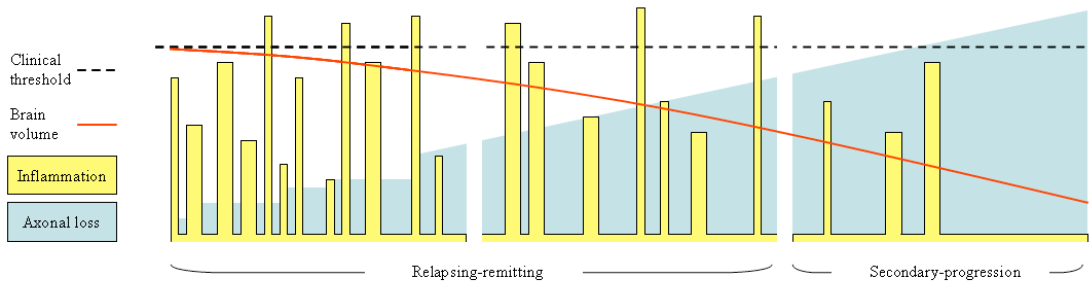

Figure 1. MS is hallmarked with an initial relapsing-remitting phase where inflammation is prominent and plasticity and remyelination is common. Most patients progress to a secondary phase where inflammation becomes more sporadic and where demyelination, axonal loss and gliosis becomes more prominent. Figure adopted from Compston and Coles, 2002.

1.1.1 Relapses

The occurrence of new symptoms is termed a relapse and it can often be attributed to a period of axonal conduction block. This deficit in conduction can be caused by demyelination of the axon, or perhaps inflammation. Normally the nodal axolemma generates a surplus of the current needed to depolarize the next node, but in demyelinated axons this current is lowered to a critical level, close to, or below, the threshold required for signal propagation (Smith, 1994). Conduction in demyelinated axons is therefore insecure and easily affected by external influences, resulting in some of the more unexpected symptoms expressed by patients (e.g. exacerbation of symptoms by warming of the body). The demyelination of the axolemma can cause the axons to become hyperexcitable, a property that can result in "extra" sensations, or so-called positive symptoms, e.g. tingling sensations in parts of the body, or visual effects such as sparks of light when the eyes are closed.

1.1.2 Demyelination

Loss of function was once almost entirely attributed to demyelination, but a role for inflammation has emerged as not only potentially important but maybe even a dominant factor in the occurrence of the neurological symptoms displayed in patients (Moreau et al., 1996, Youl et al., 1991). Indeed, one biopsy study concluded "..inflammation alone may be sufficient to cause significant clinical deficits without demyelination" (Bitsch et al., 1999b). Nitric oxide (NO) has been suggested as a mediator of the inflammation-induced effect on conduction properties (Redford et al., 1997, Shrager et al., 1998). The production of NO in inflammatory states is associated with the inducible form of nitric oxide synthase (iNOS) and both NO and iNOS are detected at high concentrations in MS lesions (Oleszak et al., 1998, reviewed by Smith and Lassmann, 2002, De Groot et al., 1997). Nitric oxide is known to act as a reversible inhibitor of axonal conduction, especially in demyelinated axons (Redford et al., 1997, Kapoor et al., 1999) and if axons are electrically active they are especially prone to suffer irreversible axonal injury and destruction (Smith et al., 2001).

The pathology associated with relapses is difficult to determine, but in one case study from a 14 year old patient who died shortly after a relapse, Barnett and Prineas (2004)

found that there was only very limited myelin loss but major oligodendrocyte apoptosis. Inflammatory cells, such as ramified microglia as well as complement were present but did not seem to be the cause of the lesion.

1.1.3 Remission

In relapsing remitting disease, patients typically recover the function lost during relapses, which can occur by a number of mechanisms. The most obvious one is resolution from the primary inflammatory insult. Demyelinated axons may also be structurally repaired by remyelination, by either endogenous Schwann cells or by precursor oligodendrocytes. In either case, conduction can be effectively fully restored (reviewed by Waxman, 2001).
When axons still are demyelinated they can recover function by an increase in the expression of sodium channels along the demyelinated axolemma which can support conduction across the lesion (reviewed by Compston et al., 2005). This type of repaired conduction is unstable and subject to a low safety factor.
There may also be a reorganisation of the neuronal network where parallel motor pathways maybe be recruited to aid in the signal propagation as well as new dendritic branching and increased synapse-density and distribution with respect to the soma.

Pathologically, the RR MS phase is characterised by inflammation and the formation of new white matter lesions with inflammatory infiltrates into the surrounding tissue with demyelination and some associated axonal loss (reviewed by Compston et al., 2005).

Lesions, where conduction is impaired, may often occur in “silent” areas of the brain and may hence not give cause to symptoms. There may therefore be a lot more activity to the disease than apparent to the clinician or patient. This means that although the patient seems to have a remission and feel fully restored, hidden pathology may still be present and ongoing.

1.1.4 Progression

Patients may start to experience a progression of symptoms and continued development of negative symptoms from the initiation of the disease (PP), or after repeated episodes of negative symptoms and remissions (RR). Pathological studies in these patients often discover significant areas of atrophy in both grey and white matter as well as changes in the NAWM (reviewed by Compston et al., 2005). The permanent neurological deficits have been reported to be more related to axonal loss than to demyelination (Kornek and Lassmann, 1999).

1.2 PHYSIOLOGY AND ANATOMY OF AXONS AND MYELIN

The human brain contain neurons in the order of 10^{11} of which each neuron may be receiving signals from up to 10,000 other neurons (Kandel et al., 2000). The neurons are greatly outnumbered by glial cells (90% of the total amount of cells in the human brain) which surround and support the neurons.

1.2.1 Myelin-producing glial cells

Two forms of glial cells produce myelin to insulate axons; oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system (Kandel et al., 2000). The myelin layer around the axons acts to increase the speed of impulse propagation by decreasing membrane capacitance, and it also increases the effective membrane resistance, thus protecting the electrical potential from dissipating out of the axon. An oligodendrocyte outgrowth winds itself in layers around a segment of the axon and thereby forms the compact spiral of myelin sheath: a single oligodendrocyte may be connected to up to 50 axons (Baumann and Pham-Dinh, 2001). Between internodes of myelin there is a node of Ranvier where sodium channels are clustered, bordered in the juxtaparanodes by potassium channels.

1.2.2 Neuronal signal conduction

In order to transfer information, neurons pass signals over distances through action potentials. The neuron is at rest negatively charged with respect to the extracellular fluid. An action potential is the rapid reversal of this state where the inside of the cell becomes transiently positively charged in relation to the outside of the cell. The frequency and pattern of action potentials constitutes the code which relays information.

The voltage across the membrane can be monitored with microelectrodes and recorded with an oscilloscope. A resting neuron is polarized at about -70 mV. Upon stimulation, sodium channels open, allowing positive sodium ions to enter the cell and rapidly depolarise the neuron, which is denoted the rising phase. The voltage over the

neuronal membrane will go from negative to positive, hence becoming more positive in respect to the extracellular fluid. This is termed an 'overshoot'. The falling phase of the action potential is the repolarisation of the membrane which is due to opening of potassium channels and the release of positive potassium ions from the neuron. The neuron potential where here become more negatively charged compared with the resting membrane potential causing an 'undershoot', or 'after-hyperpolarisation'. Finally there is a gradual restoration of the resting membrane potential. The whole event takes about two ms. The sodium and potassium ions are pumped back in an energy requiring manner by the Na^+/K^--ATPase pump, which repolarises the neuron in preparation for the next action potential (Skou, 1962). Two potassium ions are pumped out and three sodium ions in for each adenosine triphosphate (ATP) molecule consumed (reviewed by Scheiner-Bobis, 2002). Neurophysiologists can alternate the stimulation by adjusting duration of the pulse, amplitude of the input current and frequency of the stimulation.

1.2.3 Demyelinated axons

In demyelinated axons, a loss of signal propagation is caused by an insufficient density of sodium channels expressed along the exposed axonal segment. The resulting inward sodium current may not be sufficient to support the conduction (reviewed by Smith, 2007). Another phenomena found in MS patients is nodal widening where the paranodes are demyelinated, or retracted, which may be sufficient to block conduction (reviewed by Smith et al., 2005).

1.3 PATHOLOGY OF MS

The pathology of MS is hallmarked by the demyelinated plaque, which may occur at any place of the central nervous system where myelin sheaths are present (reviewed by Compston et al., 2005). Demyelination is accompanied by astrocytic scarring, which together with the multiple, focal lesions gives the disease its name; multiple sclerosis.

Plaques are generally sharply demarkated lesions, round or oval in shape, usually centred on medium sized blood vessels. Sometimes the lesions may extend along the path of blood vessels, giving an appearance referred to as Dawson's fingers. The demyelinating process is associated with inflammation throughout the CNS and inflammatory cells are often located around small or medium-sized veins, in the perivascular cuffs.

Both relapsing and progressing MS patients display both actively demyelinating and chronic lesions. Early, acute or active lesions are typically identified by the infiltration of numerous inflammatory cells, especially macrophages containing myelin and tissue debris within them, indicative of myelin degradation. Early lesions may also have extensive "shadow plagues", composed of remyelinated axons. Chronic active lesions display, in contrast to acute active lesions, ongoing demyelination only at the border of the plaque.

Chronic inactive lesions are hallmarked by demyelinated plaques in the absence of ongoing myelin destruction. They also display the presence of astrocytic scars and some preservation of axons (reviewed by Compston et al., 2005). The plaques occur preferentially in the optic nerves, periventricular white matter, brain stem, cerebellum, and spinal cord white matter.

1.3.1 Heterogenity of MS lesions

There are complex variations in the pathology of demyelinated lesions, suggesting that MS may be confounded of a group of diseases instead of one disease caused by one common factor. One group of researchers has divided the active lesion into four

different patterns, termed Patterns I-IV (Lucchinetti et al., 2000). Although the authors suggest that the patterns may represent distinct cohorts of patients, other groups claim that the different pattern scan be detected in the same patient (Barnett et al., 2009). It may be that the patterns represent different stages of lesion progression. Different groups of scientists have suggested that one type of lesion, referred to as 'primary', 'Pattern III' or 'prephagocytotic' (depending on which team of researchers is quoted) is an early type of lesion, preceding the formation of the variety of active lesions found. The primary Pattern III, or pre-phagocytotic lesion, shall be referred to as the PPP lesion hence forth.

The pathology of the PPP lesion involves activation of primarily the residing microglia and apoptosis of oligodendrocytes (Barnett et al., 2009, Lucchinetti et al., 2000). Importantly, the PPP lesion has been described as hypoxia-like based on the type of demyelination taking place (Felts et al., 2005, Lassmann, 2003, Marik et al., 2007). In addition, the myelin component that is primarily affected in the PPP lesions is the myelin-associated glycoprotein (MAG), which is located within the myelin layer closest to the axon. The resulting inside-out demyelination is similar that found in ischemic patients suffering from white matter demyelination (Aboul-Enein et al., 2003) and in cuprizone-induced mitochondrial poisoning (Johnson and Ludwin, 1981).

1.3.1.1 Patterns I-IV

Patterns I and II show close similarities with T cell-mediated or T cell plus antibody-mediated autoimmune encephalomyelitis, respectively (Lucchinetti et al., 2000). The other patterns, III and IV, are highly suggestive of a primary oligodendrocyte dystrophy, reminiscent of virus- or toxin-induced demyelination rather than autoimmunity. In the study by Lucchinetti and colleagues, it is suggested that the target of injury (myelin or oligodendrocytes) and the mechanisms of demyelination vary distinctly in the four different sub-groups of the disease. Active lesions are characterised by the presence of macrophages and activated microglia containing phagocytosed myelin, positive for myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and proteolipid protein (PLP), together with a reduced density of myelinated fibres. In this thesis, the Pattern II and III lesions will be

discussed in more detail as experiments have been conducted on models of these two Patterns.

1.3.1.2 Pattern II

Pattern II morphology in acute lesions is similar to that of Pattern I with T-lymphocyte and macrophage infiltration (Lucchinetti et al., 2000). However, Pattern II differs from Pattern I in that there is immunoglobin (Ig), mainly IgG, and c9neo complement deposits at sites of active demyelination. Both Pattern I and II were centred on small veins and venules and had sharp demarcated edges with perivenous extensions. The edge of the active lesions showed loss of oligodendrocytes and demyelination but oligodendrocytes seems to reappear in the inactive lesion centre and remyelination is common with clear shadow plaques. Loss of all myelin proteins seems to occur simultaneously.

1.3.1.3 Pattern III

The pathology describing MS Pattern III lesions is consistent with the presence of T-lymphocytes and activated macrophages and microglia (Lucchinetti et al., 2000). There was no apparent deposition of immunoglobulin (Ig) or complement proteins and the borders of the plaques were diffuse with no detection of remyelinating shadow plaques. The inactive centres of the lesions were almost completely devoid of oligodendrocytes. Unlike Pattern I and II, the demyelinated lesions were not centred on veins, instead, a ring of preserved myelin was observed around inflamed vessels within the demyelinated plaques.

The most striking feature of pattern III lesions was the specific loss of myelin-associated glycoprotein (MAG), despite the presence of PLP, MBP and 2,3-cyclic nucleotide 3-phosphodiesterase (CNP) within the damaged myelin sheaths, implying that these lesions represent “new” pathological sites. At sites of MAG loss there were nuclear condensation and DNA fragmentation, implying a role for apoptosis. The pattern III lesions were observed to resemble ischemic brain lesions, partly because of the loss in MAG-expression (Aboul-Enein et al., 2003) but also the labelling of

hypoxia inducible factor-1α (HIF-1α) which is regarded as a marker for decreased oxygen tension within tissues (Semenza, 2001, Sharp and Bernaudin, 2004). Pattern III morphology was found in acute lesions, seldom in chronic cases and most often in patients with onset of the disease less than two months before the autopsy/biopsy (Lucchinetti et al., 2000). The pathology of Pattern III lesions have been described to resemble that of Balo's concentric sclerosis (Stadelmann et al., 2005), where alternating layers of preserved and destroyed myelin as well as the expression of HIF-1α, indicating tissue precondition (Bergeron et al., 2000, Sharp and Bernaudin, 2004).

1.4 PATHOGENESIS

The initiating sequence to the formation of MS plaques is unknown but imaging and pathological studies establish that demyelination, inflammation and degeneration contribute in varying degrees to the heterogeneity of MS (Lucchinetti et al., 2000, Bielekova et al., 2005, Lublin and Reingold, 1996). There is a general consensus that an inflammatory reaction against an unknown CNS antigen may be the triggering event which results in the pathological tissue loss found within lesions. Although no common antigen has been found to date, the hypothesis is strengthened by the susceptibility of certain animal strains to induction of demyelinating lesions by immunisation with CNS antigens in the commonly used model of MS; experimental autoimmune encephalomyelitis (EAE).

1.4.1 Inflammation

The inflammatory component of MS is thought to be mediated by self-reactive T cells targeting major components of the myelin sheath; myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), MAG or proteolipid protein (PLP) (reviewed by Noseworthy et al., 2000). Activated macrophages may be recruited to the CNS by cytokines secreted by these T cells. Histological examination of MS lesions shows that activated macrophages and microglia contain myelin debris, consistent with the classical view that it is the immune cells themselves that cause the demyelination. Alternatively, the macrophages and microglia may act as scavengers, cleaning up debris from necrotic oligodendrocytes.

There is evidence that inflammation directly may cause axonal loss and dysfunctional impulse conduction (Bitsch et al., 2000, Moreau et al., 1996, Youl et al., 1991). A paper by Bitsch and colleagues reported that in a single case with Marlburg's disease, inflammation may be enough to cause clinical deficits by arrested impulse conduction, even without demyelination (Bitsch et al., 1999b).

1.4.1.1 Introduction to the innate and adaptive immune system

The immune system consists of several parts that work together to bacteria, viruses and parasites. The first line of defence is the physical barrier of our skin and the mucus membranes that cover lungs, reproductive and tracts (reviewed by Sompayrac, 1999). namely theinnate and adaptive immune systems. The innate system is consistently in place but lacks memory . The antigen-presenting cells (APCs) work like sentinel and patrol the tissue for invading pathogens, recognised in part via toll-like receptors (TLRs). The APCs can engulf pathogens into phagosomes, which the contents enzymes . APCs will process and present the antigen to the third line of defence; the adaptive immune system. Furthermore, activated APCs produce cytokines which can enter the blood stream and recruit more immune cells to the tissue. Other players of the innate immune system includes complement proteins, which can create holes in the bacterial wall, and natural killer (NK) cells which target bacteria, parasites, virus-infected cells, and cancer cells.

The adaptive immune system only exists in vertebrates and it is directed specifically against the pathogen and consists of T cells and antibody-producing B cells. Memory is also achieved, enabling a rapid response to the specific pathogen if ever re-infected. The specificity is possible thanks to antibodies which can recognize foreign molecules. The most common antibody type is Immunoglobin G (IgG) that makes up 75% of the antibodies in the blood, but there are also IgA, IgD, IgE and IgM. When a specific B cell recognise a foreign epitope, perhaps through display of the antigen by an APC, the B cell will be activated and divide repeatedly and can within a week make 20,000 identical B cells. These can recognize and make antibodies to the specific pathogen which upon binding doesn't kill the pathogen, but labels it for destruction. T cells have the same diversity as B cells but are matured in the Thymus instead of the bone marrow and recognise antigens presented on the surface of APCs and infected cells and secrete cytokines and modulate immune-function.

During the maturation of T cells of the innate immmune system, the cells are 'taught' to distinguish between self antigens and foreign epitopes in a processes denoted self-tolerance, including both a positive and negative selection. Positive selection selects for adequate binding to the antigen-presenting major histocompatibility complex

(MHC); only cells with high enough affinity to MHC are given the survival signals necessary for further maturation. In the negative selection, cells which bind with too high affinity to self-structures will receive an apoptosis signal. However, sometimes T and B cells which are self-reactive are released and can upon stimulation start to attack endogenous structures causing an autoimmune response.

1.4.1.2 Nitric oxide

Nitric oxide (NO) is an important signalling molecule and plays a role in both endocrine and paracrine signalling. Importantly, NO production is increased by inflammation and elevated concentrations of NO are present within MS lesions (De Groot et al., 1997, Oleszak et al., 1998). NO is synthesised from the amino acid L-arginine by nitric oxide synthase (NOS), which exists in three isoforms; endothelial, neuronal and inducible NOS (eNOS, nNOS and iNOS respectively). Depending on where NOS is expressed it has different functions. eNOS is expressed in endothelial cells (Gruetter et al., 1979, Napoli and Ignarro, 2009) and has a role in regulating vascular tone, and, therefore, in controlling blood pressure (reviewed by Fleming, 2009). nNOS is expressed in neuronal cells and it is important for cognitive function, synaptic plasticity and regulation of temperature, sleep patterns, etc (Bredt et al., 1990, Hars, 1999). eNOS and nNOS are constitutive forms of the enzyme and require Ca^{2+} coupling for activation/function. iNOS on the other hand is not normally expressed in the CNS but it will, once translated, swiftly produce a large amount of NO molecules since iNOS already contains the required co-factors calmodulin and Ca^{2+}. iNOS can be expressed by most cells, but it is especially expressed by cells of the macrophage/monocyte lineage, which produce it in response to various tissue insults, and to inflammation (De Groot et al., 1997, Liu et al., 2001, Oleszak et al., 1998). In this thesis, all three isoforms are important since nNOS is expressed in neurons, astrocytes and epithelial cells, eNOS in the CNS vasculature and iNOS on macrophages penetrating the CNS tissue in response to inflammation. Increased levels of NO and its derivates have been found in the CSF of MS patients during relapse (Brundin et al., 1999, Yamashita et al., 1997). Furthermore, the inducible form of NOS is expressed in MS lesions (Bagasra et al., 1995, Bo et al., 1994, De Groot et al., 1997, Hooper et al., 1995).

NO is a fascinating molecule and has a range of effects. It has a half life in tissues of one to ten seconds and it easily passes through cell membranes (Chiueh and Rauhala, 1999). NO in itself is not very reactive or detrimental in low concentration but it readily reacts with superoxide to form peroxynitrite ($ONOO^-$) (Rubanyi and Vanhoutte, 1986) which is a potent oxidising agent and reactive nitrogen species (RNS) which causes cellular damage. In particular, $ONOO^-$ can permanently modify proteins (e.g. inactivating enzymes) by nitrating tyrosine residues. Interestingly, the absence of T cells within the PPP lesion and the LPS-DC lesion may be caused by exceptionally high NO concentrations through two mechanisms; NO may interfere with antigen presentation (Sicher et al., 1994) and inhibit T cell proliferation (Albina and Henry Jr, 1991).

1.4.2 Demyelination

Damage to myelin may either be from direct insult to myelin lipids or proteins, or arise indirectly via damage to the oligodendrocyte cell-body. Major effort has been invested into finding ways to promote the remyelination of existing lesions, for example by using stem cells or the implantation of oligodendrocyte precursor cells (Blakemore et al., 2002, Keirstead and Blakemore, 1999, Pluchino et al., 2005). Almost all demyelination seen in MS lesions occurs in conjunction with inflammation, and indeed active lesions are characterised by myelin debris-containing macrophages (reviewed by Compston et al., 2005). The mechanisms by which inflammation could promote demyelination are not fully understood but several mediators can cause direct damage to myelin and oligodendrocytes, including tumour necrosis factor (TNF)-1α, myelin specific immunoglobulins, NO and reactive oxygen species (ROS ($O_2^{-}\cdot$, H_2O_2, OH^-)) (Bo et al., 1994, Selmaj and Raine, 1988, Bagasra et al., 1995).

1.4.2.1 Inflammation-mediated cytotoxicity

Oligodendrocyte and myelin cytotoxcity may be induced by macrophages or T cells. Macrophages may induce tissue damage by secreting toxic products in an antigen—independent and non-selective manner. However, myelin and oligodendrocytes have a higher susceptibility, followed by neurons and axons. In contrast, microglia and astrocytes are relatively resistant. Damage to myelin and oligodendrocytes may also

be mediated by reactive oxygen species released by microglia and macrophages. Indeed lipids are more easily accessible and do not display anti-oxidant defences. Lipid peroxidation studies in serum and CSF samples from MS patients report varying results (reviewed in (Smith et al., 1999)) but in autopsy tissue lipid peroxidation products have been found in active and chronic plaques (Newcombe et al., 1994), and nitrotyrosine has been found in demyelinating and remyelinating lesions (Jack et al., 2007).

Antigen-dependant cytotoxicity is mediated by T cells which may cross the BBB or blood-SCF barrier upon activation in the periphery. After re-activation by an antigen-presenting cell the T cells can induce cell death through direct binding to death receptors on the cell surface, such as CD95L, or by release of cytotoxic granules, e.g. perforin and granzyme B. Increased expression of CD95L in oligodendrocytes, astrocytes and macrophages has indeed been found in MS plaques (Bruno and Cedric, 1997, Dowling et al., 1996). Another study reported that CD95L was found predominantly in oligodendrocytes in increased concentrations in chronic lesions (D'Souza et al., 1996).

The hypothesis that components of the myelin-sheath are the target of an antigen-directed inflammatory attack is still debated (Polman and Killestein, 2006). There are reports describing that serum samples from patients displaying clinically isolated syndrome (CIS) were predictive of their clinical progression based of the presence of anti-MOG and anti-MBP antibodies (Berger et al., 2003, Tomassini et al., 2007), but other authors failed to reproduce these data (Lim et al., 2005, Kuhle et al., 2007, Pelayo et al., 2007b, Rauer et al., 2006, Pelayo et al., 2007a). Where a positive correlation was observed it was most often consistent with more frequent or earlier relapses (Berger et al., 2003, Tomassini et al., 2007). Alternatively, an antigen-targeted attack may be directed against ROS or RNS-induced damaged lipids or proteins. Such an effect has been observed in animals models of carbon monoxide (CO) poisoning, where altered MBP proteins have initiated an adaptive immune response (Thom et al., 2006).

1.4.2.2 Non-immune mediated oligodendrocyte death

A common observation in the PPP lesions is oligodendrocyte apoptosis (Barnett and Prineas, 2004, Lucchinetti et al., 2000, Marik et al., 2007). Apoptosis may of course be induced by macrophages and T cells by binding to the death receptors on the cell surface, however, the apoptosis seen in the PPP lesion appears to by caused by an energy deficiency and indeed, oligodendrocytes are, in addition to neurons, particularly sensitive to oxygen deficiency (Petito et al., 1998). Oligodendrocytes are in addition, sensitive to excitotoxic damage (glutamate and kainate), and in an EAE trial, a kainate antagonist ameliorated the disease (Pitt et al., 2000).

1.4.3 Atrophy

The predominant neuropathology of MS is myelin loss with axonal preservation (Noseworthy et al., 2000) but the preservation is relative and there are several accounts of early and irreversible axonal degeneration (Simon et al., 1999, Chard et al., 2002, Pirko et al., 2007, Chard et al., 2004). Even though MS is generally thought of as a white matter (WM) disease, pathology can be found in the cortex and in deep cerebral nuclei (Cifelli et al., 2002, Brownell and Hughes, 1962, Kutzelnigg et al., 2005, Kidd et al., 1999). The GM lesions are thought to be less inflammatory than the WM lesions, but reactive microglia are still part of the pathology (Peterson et al., 2001). Demyelination of the cortical lesions has been reported, but it does not seem to be T-cell mediated (Bo et al., 2003).

Classically, GM loss is explained as secondary to WM lesions, axonal damage and Wallerian degeneration (Cifelli et al., 2002, Bermel RA et al., 2003, Simon et al., 2000). However, atrophy of the GM has been seen early in the disease progression, even before the onset of fixed deficits (Dalton et al., 2004, De Stefano et al., 2003, Sastre-Garriga et al., 2005, Tiberio et al., 2005, Chard et al., 2002), and in some MS cases the white matter injury is too small to explain the axonal damage present (Bozzali et al., 2002). This early loss of axons is particularly noticeable in PP MS patients where loss of brain volume occurs so early that inflammation-mediated demyelination does not appear to be an important part of the mechanism (Filippi et al., 2003).

The atrophy in MS patients is widespread and may affect all areas of the brain (Geurts et al., 2005), as well as the brain stem (Cifelli et al., 2002) and spinal cord grey matter (Gilmore et al., 2009). Several papers report that the majority of brain volume loss occurs within the GM (Dalton et al., 2004, Quarantelli et al., 2003, Sanfilipo et al., 2005, De Stefano et al., 2003, Sastre-Garriga et al., 2005, Tiberio et al., 2005). Other papers have shown that atrophy affects both GM and WM equally (De Stefano et al., 2003) or, indeed WM dominantly (Kutzelnigg et al., 2005). A dissociation has been suggested between neurodegeneration and inflammatory demyelination (reviewed by Lassmann, 2003), but a correlation has also been described between inflammation and axonal pathology in both white and grey matter (Frischer et al., 2009).

1.5 MODELS OF MS

There is no perfect model of MS lesions although clinical and pathological hallmarks can be imitated by the injection of parts of, or whole, myelin proteins in a model of MS termed experimental autoimmune/allergic encephalomyelitis (EAE). This is the most commonly used model for MS and resembles, if anything, Pattern I or II lesions (Storch et al., 1998). In turn, the PPP lesion is reproduced in a model of MS induced by the intraspinal injection of the pro-inflammatory agent lipopolysaccharide (LPS) into Sprague Dawley (SD) rats (Felts et al., 2005, Marik et al., 2007). This type of experimental lesion has been suggested to display the same neuropathological features not only as seen in the PPP lesion, but also as those seen in stroke (ischemic/hypoxic) patients (Lassmann et al., 2007, Marik et al., 2007).

1.5.1 EAE as Pattern II model lesions

Experimental autoimmune encephalomyelitis (EAE) was accidentally discovered when Pasteur and his disciples were vaccinating people against the rabies virus (Baxter, 2007). Rabies virus was propagated by intracranial inoculation in rabbit brains and the CNS tissue was air-dried (inactivation of the virus) before administration as a vaccine to patients. As the success rate started failing, the rabbit CNS tissue was air-dried more briefly to make the vaccine more potent, and subsequently the patients started showing the clinical signs of MS and their autopsies showed demyelination and lymphoid infiltrates. This failed treatment illustrates the autoimmune character of EAE, and possibly of MS. Both the clinical and histopathological characteristics of EAE are very similar to MS (Wolf et al., 1947). However, EAE is induced by a known autoantigen whereas there is debate about whether MS is an autoimmune disease (reviewed by Gijbels et al., 2000).

EAE can be induced in various species and, depending on the host, a wide array of protein or peptides are encephalitogenic. The various forms of EAE can be used to model different aspects of MS. The animals usually exhibit inflammatory demyelinating lesions in the CNS, and neurological deficits such as weakness and paralysis (reviewed by Compston et al., 2005). Both whole CNS homogenate (spinal cord) and myelin preparations of proteins or peptides like MBP, PLP, MOG and glial

fibrillary acidic protein (GFAP) can be used to induce active EAE. EAE can also be induced by passive transfer of T cells, previously activated to a specific myelin protein.

The histopathology of EAE is similar to that of MS, starting with perivascular infiltrates of lymphocytes, monocytes/macrophgaes and eosinophils (hence the old name allergic encephalomyelitis), and prominent meningeal inflammation (reviewed by Gijbels et al., 2000). Demyelination, with a relative sparing of axons occurs in most models, and after a slow disappearance of infiltrates the demyelination is typically repaired by remyelination, in conjunction with astrogliosis.

1.5.2 LPS induced Pattern III model lesions

LPS is a major component of the outer membrane of Gram-negative bacteria. LPS is an amphiphilic molecule with a lipid part that is the immunostimulatory or 'endotoxic' component and that interacts with macrophages, polymorphonuclear leukocytes, platelets, and vascular endothelial cells (Acosta, 2001, Galanos, 1975). Via these cells, LPS is able to activate a multitude of endogenous immunomodulatory or inflammatory compounds such as cytokines, interleukins, tumour necrosis factor-α and eicosanoids (Cybulsky et al., 1988). The local injection of LPS may therefore induce inflammation in most tissues within minutes by causing infiltration of neutrophils and monocytes from the surrounding bloodstream (Blouin et al., 2004). LPS can also act as a non-hypoxic stimulus to increase the expression of hypoxia-related genes (Alexander and Rietschel, 2001). In small amounts, LPS can be beneficial by up-regulation of the immune system, but in higher doses it may lead to pathological reactions, for example, as seen in septic shock (Issekutz, 1981, Cybulsky et al., 1988).

The results presented in this thesis are based on models induced by LPS-injections into the spinal cord of experimental rats. The LPS-induced dorsal column model (LPS-DC lesion) has proven to be a valid representation of Pattern III lesions as seen by morphological and histological markers (Felts et al., 2005, Marik et al., 2007). The model was characterised by a major increase in inflammatory cells from 8 h post injection consisting of macrophages, and less prominently T-cells, in the absence of B

cells. Demyelination occurred between days 7 to 14 post-injection, and remyelination was commonly observed after 14 days (Felts et al., 2005, Marik et al., 2007). Amyloid precursor protein (APP) could be detected in axons, as well as some Wallerian-type degeneration (Felts et al., 2005, Marik et al., 2007). MAG expression was found to be missing prior to loss of other myelin proteins, indicating an 'inside-out' demyelination. Tissue preconditioning was indicated by the presence of the heat shock protein (HSP)-70.

1.6 POSSIBLE ENERGY FAILURE

There have been several suggestions of a possible energy failure in MS lesions, especially in the lesions where the mechanism of myelin degradation and the presence of oligodendroglial apoptosis is reminiscent of the pathology found in ischaemic or stroke patients (Aboul-Enein et al., 2003, Barnett and Prineas, 2004). An inadequate energy supply to the cells may arise from several causes, including: 1) a metabolic strain from the increased activity from both constitutive and infiltrating cells; 2) insufficient nutrient delivery to the inflamed site; 3) hypoxia; 4) genetic or environmental effects injurious to mitochondria, such as causing damage to complex I-V. Although macrophages may function well in anaerobic conditions, a property which is essential for their activity in walled-off infected areas, neurons and oligodendrocytes are particularly sensitive to oxygen deprivation (Petito et al., 1998).

If there is a shortage of energy in the cells within lesions in MS patients it would help to explain the occurrence of symptoms. Not only do MS patients often experience a feeling of fatigue, but neuronal conduction is also impaired. The action of the ion pumps responsible for creating the resting membrane potential in neurons and axons is an extremely energy-demanding mechanism. Thus, in neurons, as well as in other cells, sodium and potassium ions are pumped across the membrane by the ATP-dependant Na^+/K^+-ATPase transporter. In the case of local energy deprivation, arising perhaps from an insufficient oxygen delivery or from mitochondrial inhibition with subsequent lowered ATP levels, there may be an inability of the axon to maintain its resting membrane potential and hence conduction will be impaired.

Strengthening an argument that there is an energy deficit in MS patients is the fact that axonal injury predominantly affects smaller axons (Evangelou et al., 2001) thereby leaving thicker axons preserved in chronic lesions (Shintaku et al., 1988). This effect may be a sign of the higher metabolic load on smaller axons compared to their mitochondrial mass (Stys, 2005).

1.6.1 Mitochondria

Mitochondria have a multitude of influences on cell function, but are most obviously an important energy supplying organelle of the cell. Energy is stored in chemical form in the molecule ATP, which is produced by a series of reactions during which oxygen is reduced to water. Mitochondria are important in calcium storage when the intracellular free calcium concentration rises above a critical threshold, and in the signal cascade initiating apoptosis (Smaili et al., 2003). Apart from these largely beneficial functions, mitochondria are also a major producer of superoxide radicals ($O_2 \cdot^-$) which can be detrimental to the cell, although they may also function as signalling molecules. All of these functions can influence the mitochondrial membrane potential ($\Delta\Psi_m$) and, in turn, the adenosine di/triphosphate (ADP/ATP) ratio, which influences glycolysis, the activity of the Na^+-K^+-ATPases, and thereby Na^+-dependent signal propagation along axons.

1.6.1.1 The structure and function of mitochondria

The mitochondria are surrounded by a double membrane of which the inner one protrudes into the matrix forming cristae. These projections increase the area of the inner membrane, and as this is the location of the five proteins responsible for ATP production, the proliferation of membrane allows for increased presence of these proteins. There are five complexes in the respiratory chain (Figure 2). Complex I, also known as NADH dehydrogenase, removes an electron from NADH meanwhile transporting the proton out into the intermembrane space. Complex II, or succinate dehydrogenase, also removes an electron from its substrate, but this time from succinate. Electrons freed at complex I and II are transported via co-enzyme Q to complex III, cytochrome bc_1, which passes electrons further on to complex IV, cytochrome c oxidase, via cytochrome c. Complexes III and IV also transfer protons from the matrix in the process. At complex IV, the electrons are finally passed on to oxygen which is then reduced to water. The protons that are transported into the inter membrane space create the $\Delta\Psi_m$ which provides the electrochemical gradient that drives protons through complex V, the ATP synthase, thereby generating ATP.

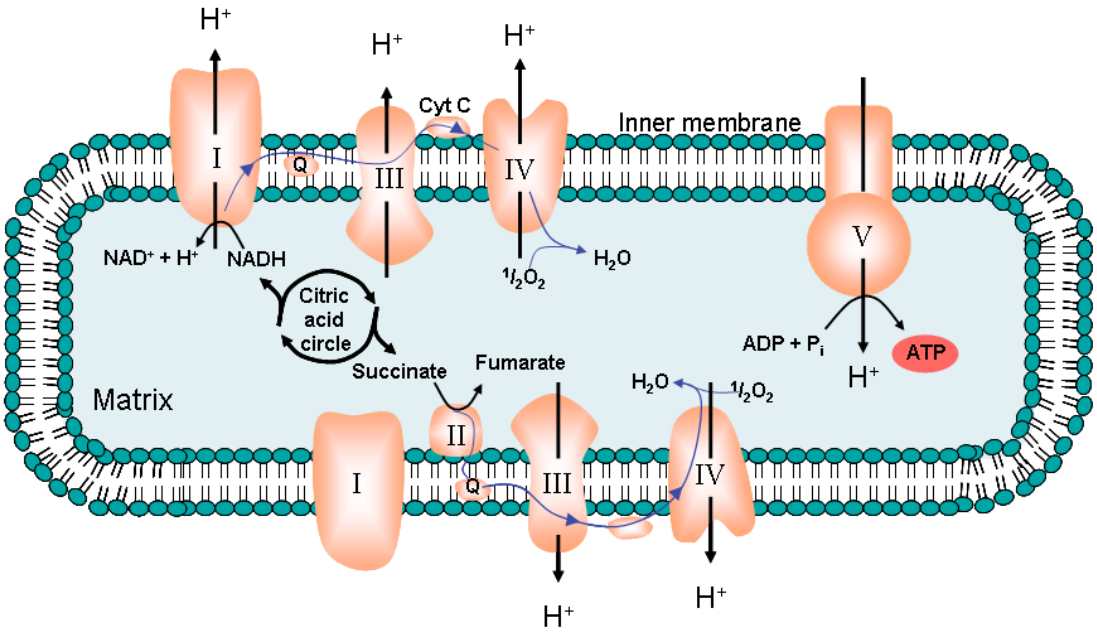

Figure 2. The electron transport chain is located in the inner mitochondrial membrane and consists of complex I-V

It is believed that mitochondria were not originally an endogenous organelle, and so it may be helpful to consider the different locations of the mitochondrial DNA. Mitochondria are thought to have originated from an internalised respiring α-proteobacterium which started a symbiosis with the host cell, in which nutrition and protection was provided for the organelle in exchange for hydrogen (ATP substrate for the early host cell), and thereby the cell became able to respire and tolerate higher concentrations of oxygen (Cavalier-Smith, 1987, Lane, 2005). During evolution the incorporated bacteria became true organelles with most of their DNA stored in the nucleus of the cell, rather than in the mitochondria themselves, and indeed eukaryote mitochondria today retain DNA in the mitochondrial matrix which only transcribes for parts of complexes I, III, IV and V (Nass and Nass, 1963, Young et al., 2008, Schatz et al., 1964). The retention of some of its genes allows the mitochondria to regulate the aerobic metabolism taking place, and this is of importance since an unregulated build up or inhibition of aerobic metabolism, e.g. because of insufficient metabolites, will increase the detrimental escape of ROS from the respiratory chain (Allen and Raven, 1996). The ROS can damage DNA, and the mutational effects of ROS on the mitochondrial DNA can be illustrated by the fact that mitochondrial genes have a 20 times faster evolution rate compared to nuclear genes (Lane, 2005).

1.6.1.2 Cell death

Cells may respond to detrimental cellular stress by two main mechanisms (with a spectrum of variations between): either they will undergo ATP-dependant apoptosis or they may die by an inflammatory-inducing necrosis. Both pathways involve a loss of the membrane potential but whereas apoptosis is a tightly-regulated process, necrosis is caused by external factors and may through uncontrolled release of digestive enzymes cause damage to surrounding cells.

Apoptosis may be activated by intrinsic factors, e.g. energy loss or DNA damage, or by external stimuli, e.g. TNF signalling, but is initiated in the mitochondria and is dependant on a depolarisation of the mitochondrial membrane potential (Marchetti et al., 1996, Zamzami et al., 1995b). Important players in the apoptosis machinery are the permeability transition (PT) pore and the large family of proteins, referred to as the Bcl-2 family. The PT pore will upon opening dissipate the $\Delta\Psi_m$ through release of hydrogen ions, which is consistent with an increase in intracellular calcium

(Macho et al., 1997). The loss of $\Delta\Psi_m$ precedes a burst of reactive oxygen species (ROS)(Macho et al., 1997, Zamzami et al., 1995a), as well as the release of cytochrome c (Kantrow and Piantadosi, 1997, Liu et al., 1996) and apoptosis inducing factor (AIF) (Susin et al., 1996) from within the mitochondria. The actual degeneration of the cell is mediated by a cascade of caspases which literally slice up the contents of the cell. Cellular fragments are encapsulated by the membrane and 'bleed' away from the cell, to be absorbed by surrounding cells.

1.6.1.3 MS and mitochondria

There is increasing interest in the ways in which MS might affect mitochondria function, and vice versa. For instance Dutta and colleagues could detect both a decrease in mitochondrial gene transcripts, and in the activity of complexes I and III in the normal appearing motor cortex of MS patients (Dutta et al., 2006). However, even though this study was performed with matched controls, their gene expression patterns were not characterised at the same time, and the MS patients were severely disabled (Expanded Disability Status Scale [EDSS] score, >7.0) which might have affected the results. Damage of mitochondrial DNA has also been detected in chronic active lesions of MS (Lu et al., 2000), and a vital subunit of complex IV in the mitochondrial electron transport chain has been reported to be decreased in oligodendrocytes in active lesions from Pattern III lesions (Mahad et al., 2008).

Mitochondrial stress will initiate a stress response which may act to protect the cell against further metabolic challenges. This preconditioning is mediated by stress genes, of which one, heme oxygenase (HO)-1, is found within actively demyelinating MS lesions and in the EAE model of MS (Schipper, 2004, Emerson and LeVine, 2000). HO-1 is activated by heat shock, ischemia, glutathione-depletion, hyperoxia and disease states (Blouin et al., 2004, Fukuda et al., 2002, Hellwig-Burgel et al., 1999, Kietzmann et al., 2003, Stiehl et al., 2002, Racay et al., 2009, Ren et al., 2008).

A recent study of chronically demyelinated axons of MS patients noticed that the expression of Na^+-K^+-ATPases was decreased (Young et al., 2008). Even earlier in the disease, the decreased expression of mitochondrial complex V (ATPase) indicates

a deficiency of mitochondria when comparing normal white matter with an actively demyelinating plaque of acute MS (reviewed by Smith and Lassmann, 2002).

Interestingly, N-acetylaspartate (NAA) which is a molecule used to assess axonal loss in the CNS by MRS, has been linked to energy metabolism in neuronal cells (Arnold et al., 1994, Bitsch et al., 1999a, Davie et al., 1999, Ebisu et al., 1994, Harms et al., 1997, Sager et al., 1995), and it is therefore possible that MRS studies may indicate areas of mitochondrial deficiency as well as axonal depletion (Bates et al., 1996).

1.6.1.4 Anaerobic metabolism

Alternative pathways to the aerobic metabolism of the mitochondria seem to be activated in MS patients. Increased lactate levels seen by MR spectroscopy have been reported(Simone et al., 1996, Miller et al., 1991, Arnold et al., 1990), and the increase in lactate concentration has been correlated with the number of inflammatory lesions found (Lutz et al., 2007). Additional indicators of activated anaerobic metabolism have been found in MS patients, e.g. a significant increase of fructose in the CSF in MS patients with clinically isolated syndrome (CIS) both with, and without, inflammatory active plaques (Lutz et al., 2007). CSF samples are also reported to display an increased lactate concentration as well as sorbitol and fructose in primarily and secondary progressive MS, but also, in a lesser degree, in relapse remitting MS patients (Regenold et al., 2008). Lactate in itself may not be proof of an energy deficiency because astrocytes can produce lactate for the neurons to utilise. The increase in lactate levels may alternatively be explained by the biased nature of macrophages; they have the ability to switch from aerobic to anaerobic metabolism as needed, and the increase in lactate may thus be due to an increased presence of macrophages in the tissue. The reports of increased lactate are contradicted, for example in a recent study where significantly decreased lactate concentrations in the CSF in early MS patients were reported (Fonalledas Perello et al., 2008).

1.6.1.5 NO-mediated mitochondrial inhibition

The most likely effect on the mitochondria in MS is probably mediated via the increased iNOS concentrations in inflammatory lesions (Oleszak et al., 1998,

reviewed by Smith and Lassmann, 2002, De Groot et al., 1997), which is consistent with those found in EAE (Lin et al., 1993). As described more thoroughly above, the inducible form of nitric oxide synthase, iNOS, produces nitric oxide continuously at high concentrations (reviewed by Alderton et al., 2001), but more importantly, NO may act as an inhibitor of mitochondrial complex IV (Brown and Cooper, 1994, Cleeter et al., 1994, Giulivi, 1998, Schweizer and Richter, 1994, Takehara et al., 1996), by competing with oxygen at the catalytic site. NO is known to act as a reversible inhibitor of axonal conduction, and especially demyelinated axons are vulnerable (Redford et al., 1997, Kapoor et al., 1999), and this effect is possibly a direct result of inhibition of the mitochondrial respiratory chain. Alternatively, NO or peroxynitrite may S-nitrosate the mitochondrial complexes, which has been reported for complex I (Clementi et al., 1998, Chiueh and Rauhala, 1999, Moncada and Erusalimsky, 2002). Inhibition in any step in the respiratory chain will not only lead to decreased ATP levels (Beltran et al., 2000, Brown et al., 1995, Brorson et al., 1999), but also increase the production of ROS (Lane, 2005). NO-mediated mitochondrial inhibition may predictably play a role in MS, and this is consistent with the finding of toxic hypoxia in some lesions: such 'hypoxia' results from an energy deficit due to mitochondrial inhibition, but the inability of the mitochondria to use the available oxygen means that an actual state of hyperoxia may prevail. Alternatively, NO may mediate its cytotoxic effect on neurons by increasing intracellular Ca^{2+} levels (Brorson and Zhang, 1997).

NO may also be produced by the neuronal form of NOS (nNOS) inside the mitochondria (Elfering et al., 2004). The two constitutive forms of the NOS enzyme are most likely important for cellular signalling, and administration of NO may even be beneficial in low concentrations by promoting cell growth, but is certainly detrimental in higher doses (Bolaños et al., 2008). iNOS is expressed in macrophages in injured tissue and a positive correlation has been seen between the degree of injury and the number of iNOS positive macrophages present (Aboul-Enein et al., 2006).

Experiments on isolated mitochondria and cells have revealed a rapid collapse of the $\Delta\Psi_m$, ATP synthesis and ATP depletion upon administration of NO (Angeles and Juan, 2001, Schweizer and Richter, 1994). When NO is added to neurons and astrocytes, both cell types decrease their ATP production but astrocytes will

additionally activate their glycolysis (Almeida et al., 2001). The results from two studies where ATP generated by glycolysis enter the mitochondria is consistent with the view that such ATP may help to maintain the $\Delta\Psi_m$ via the ATPase through reversible transportation of protons (Almeida et al., 2001, Beltran et al., 2000a). Importantly, an inhibition of the mitochondrial complexes from S-nitrosation may be reversed by glutathione. Hence, NO and/or peroxynitite may cause some of their cytotoxic effects on cells by inhibiting their intracellular antioxidant defences via depletion of the reduced glutathione pool, making the cells more susceptible to ROS-mediated damage (Clementi et al., 1998, Radi et al., 1991), and exacerbating mitochondrial inhibition (Dahm et al., 2006).

1.6.2 Oxygen utilisation of the CNS

The brain is metabolically very sensitive which is amply demonstrated in the case of stroke patients where large areas of brain may be terminally affected by a decrease in blood flow. Neurons of the brain are dependent upon a nearly continuous supply of sufficient oxygen and, indeed, if the blood flow is stopped for even 8-10 seconds, unconsciousness will occur (Sife, 1998).

1.6.2.1 Blood perfusion in the CNS

Although the brain represents only 2 % of the total body weight, it receives 20 % of the resting cardiac blood output. There are several safety mechanisms to secure the blood supply, including the circle of Willis, which supplies both halves of the brain if one of the internal carotid arteries is blocked. Another safety system is the collateral blood supply which activates small blood channels that connect major vessels and grow in size to divert the blood around an occlusion (Fields et al., 1965).

The metabolic rate of the gray mater is three to four times higher than that of the white matter, which correspondingly contains many fewer capillaries (Buxton, 2002, Hasegawa and Ravens, 1968). This lack may make the whiter matter more susceptible to hypoxia and less capable of achieving a diversion of blood from collateral supply. However, since the oxygen supply of the CNS tissue is well regulated (in the absence of an occlusion), it is likely that an altered oxygen

concentration within the brain in patients is either caused by deficits of the regulation of perfusion, or fluctuations/inhibition of the cellular oxygen utilisation.

1.6.2.2 Blood perfusion in MS

MRI studies imply that MS patients display an alteration in blood flow during early lesion development (perhaps representative of the PPP lesion), including an increase in both blood volume and cerebral blood flow (Wuerfel et al., 2004, Wuerfel et al., 2007). In contrast, in *established* lesions of both relapsing-remitting, secondary progressive, and primary progressive patients the perfusion is reported to be decreased compared with controls (Adhya et al., 2006, Ge et al., 2005, Inglese et al., 2007a, Inglese et al., 2007b). The contrast between these reports may be due to a compensatory action of the vasculature at later time-points, or to the fact that there is less tissue surviving that requires perfusion.

1.6.2.3 Oxygen consumption and inflammation

The oxygen concentration in the tissue will be affected by the amount of oxygen used by the cells, which in turn is dependent upon the level of neuronal activity. The blood flow to the site of action is regulated so that the oxygen concentration within the tissue is maintained. However, in inflamed or degenerative tissue there have been conflicting reports of oxygen consumption. In 1963, Manhold and Volpe reviewed and re-examined the existing observations and found that the reported utilisation of oxygen (Q_{O2}) for the first five days after tissue injury are inconsistent (Manhold and Volpe, 1963). However, they concluded that the variation is dependent on the strength of inflammation and cell proliferation, i.e. Q_{O2} is low at the peak of inflammation and at low cell proliferation, but high at the peak of proliferation, usually with the repair stage post injury (Manhold and Volpe, 1963). Interestingly, the LPS-DC model of MS lesions used in this thesis is characterised to have the peak of inflammation at 24 hours from induction. Taken at face value, these data (Manhold and Volpe, 1963) indicate that the oxygen consumption may be low at this time point. However, measurement of the actual oxygen consumption would be required to determine whether such a decrease in oxygen utilisation was due to decreased perfusion of the site, or due to an inhibition of the mitochondria (the latter would result in a local elevation of oxygen). Importantly, mitochondrial inhibition would not only be detrimental due to the loss of ATP, but also because the subsequent increase in oxygen may be toxic to the tissue through increased ROS production (Haugaard, 1968).

1.6.2.4 Oxygen and MS

There are several lines of evidence suggestive of an altered oxygen concentration in MS lesions, both from clinical studies as well as in EAE. The disease plaques have been linked with abnormal vasculature (Zamboni et al., 2009), higher concentrations of vascular endothelial growth factor (VEGF) (Proescholdt et al., 2002, Su et al., 2006), and increased ROS, all of which are consistent with altered oxygen concentrations. Moreover, MS lesions are normally centred around a vessel, consistent with a role for infiltrating immune cells, but, in contrast, demyelination in Pattern III lesions is often absent around inflamed vessels indicating that another

mechanism may be involved (Lucchinetti et al., 2000). In particular, this pathology is consistent with the hypothesis that Pattern III lesions are hypoxic. Arguing against hypoxia however, is the consideration that if the inflammation present in MS lesions can reduce the pH of the tissue, this should in turn facilitate the release of oxygen molecules from haemoglobin according to the Bohr effect (Riggs, 1960): in this regard the oxygen concentration at the inflamed site should increase.

1.6.2.5 Inflammation and hypoxia in MS

Inflammation is a prominent component of MS and macrophage activation is an early event in inflammation. One of the roles of macrophages is to protect the tissue against possible pathogens by walling off the inflamed area and this isolation is achieved by total or partial occlusion of the local blood supply, which may cause local hypoxia (Saadi et al., 2002). The activity of the macrophages themselves is not affected in hypoxic regions because these cells can function via the anaerobic metabolism of glucose (Butterick et al., 1981). Macrophages accumulate in hypoxic sites and will try to protect/precondition the tissue against further ischemic insult by up-regulation of the transcription of hypoxia-inducible factors (HIFs) 1 and 2 (Talks et al., 2000, Bernard Burke, 2002). Interestingly, labelling of HIF-1α is seen in increased amounts in Balo's concentric sclerosis lesions (Stadelmann et al., 2005) and in the Pattern III lesions of MS (Marik et al., 2007). The consequences of hypoxia can include blood brain barrier dysfunction, inflammation, demyelination and eventually, axonal loss (James, 2007), all of which are observed.

1.6.2.6 Hypoxia inducible factor

HIF-1 is composed of two subunits: HIF-1α and HIF-1β (Wang and Semenza, 1995). When oxygen is present, HIF-1α is marked for degradation by ubiquitin (Jaakkola et al., 2001) or alternatively, HIF-1α is acetylated under normoxic conditions which targets the transcription factor for protesomal degradation (Jeong et al., 2002). During hypoxia, these two pathways are blocked or down-regulated, respectively, which permits HIF-1 protein stabilisation. Once stabilised, the heterodimer can bind to the hypoxic response elements (HREs) of genes such as glucose transporters, glycolytic enzymes and vascular endothelial growth factor (VEGF), thereby controlling

angiogenesis, glycolysis, mitochondrial function, cell growth and survival (Webb et al., 2009). (Non-hypoxic stimuli, such as LPS, can also increase the number of hypoxic genes activated (Blouin et al., 2004)).

1.7 REACTIVE OXYGEN SPECIES

The term reactive oxygen species (ROS) is used to describe more or less unstable molecules, to describe reactive oxidising agents that react with neighbouring molecules thereby potentially causing conformational changes that can result in loss of function. Some ROS molecules (e.g. the hydroxyl radical) are so reactive that they will react with the closest molecule available, whereas others have a longer half-life and can travel further until they encounter a molecule with which they can react more easily. ROS can be a side product of aerobic metabolism but they may also be used by the immune system to kill pathogens. These two sides illustrate the beneficial as well as the detrimental side of ROS. On one hand it will aid in the fight against pathogens and on the other it will react with proteins, DNA or lipids (Figure 3), thereby disturbing mitochondrial function which may result in cell death (Bolanos et al., 1997). The slow build up of ROS-mediated damage has also been implicated in the ageing process (Beckman and Ames, 1998, Harman, 1956).

The ROS molecules are usually oxygen derived, for example superoxide ($O_2\cdot^-$), hydroxyl ($OH\cdot$), peroxyl ($RO_2\cdot$), and alkoxyl ($RO\cdot$), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). The oxygen species often easily react with NO, forming reactive nitrogen species (RNS); peroxynitrite ($ONOO^-$), nitrogen dioxide ($\cdot NO_2$) and dinitrogen trioxide (N_2O_3). Catalyzed by iron, hydrogen peroxide can produce the hydroxyl radical in a reaction known as the Fenton reaction (von Sonntag, 2006). Both ROS and RNS have been indicated as a player or mediator in MS. However, the extent and possible contribution to the pathology is unknown.

1.7.1 ROS production

In vivo, ROS can be produced enzymatically and non-enzymatically. The main formation of ROS takes place in the respiratory chain of the mitochondria where oxygen usually is the end-receiver of electrons and is reduced to water. However, in 1-2 % of these reactions the oxygen is non-enzymatically reduced to superoxide instead, a free radical and precursor to other ROS.

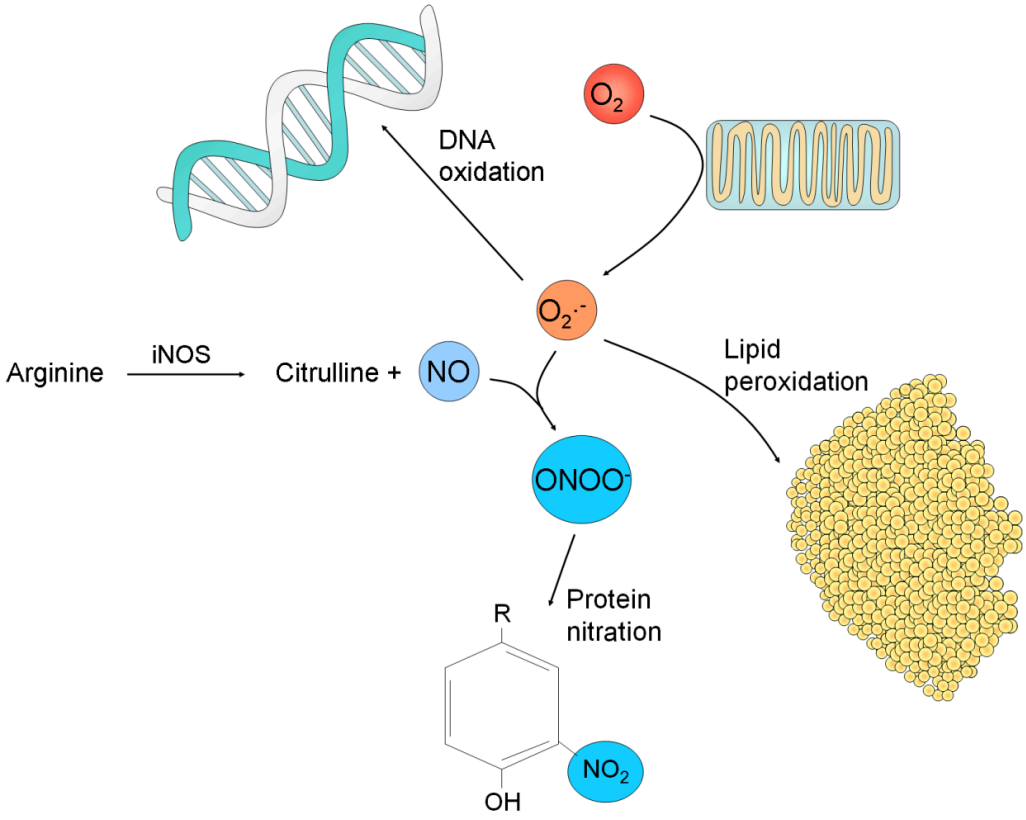

Figure 3. Activity of ROS and RNS through oxidation or DNA or peroxidation of lipids or tyrosine residues of proteins.

Complexes I and III are considered to be the main producers of ROS in the mitochondria (Turrens et al., 1982a, Turrens et al., 1982b, Barja, 1999). ROS are more likely to be produced if one of the mitochondrial complexes are compromised and will then be free to attack surrounding complexes, thereby promoting even more ROS formation (Zeevalk et al., 2005).

ROS can also be enzymatically produced, for example by xanthine oxidase, cyclooxygenase, lipoxygenase, uncoupled eNOS, cytochrome p450 and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase or NOX). NOX stands out because it is the only known enzyme family solely dedicated to ROS production. All the other mentioned enzymes produce ROS as a product (or probably by 'mistake'). NOX2 produces superoxide by taking an electron from NADPH and coupling it to an oxygen molecule. The resulting superoxide is used by the immune system to degrade foreign particles and bacteria and is activated in inflammation (Owen, 1994, Roos et al., 2003).

1.7.2 Inflammation and ROS

There is an excessive generation of ROS in inflammatory states, primarily generated from activated macrophages and microglia (Hartung et al., 1992). The superoxide produced by NOX 2 can cause damage to the endothelial cells, increasing the permeability of the microvasculature and BBB, and also hence promoting inflammatory cells to enter the site of inflammation (Afonso et al., 2007, Haddad, 2002, Lagrange et al., 1999, Olesen, 1987, Schreibelt et al., 2006, van der Goes et al., 2001). Binding of leukocytes to the adhesion molecules of the endothelium, vascular cell adhesion molecule-1 (VCAM-1) increases the local ROS production through activation of NOX (Cook-Mills, 2002, Tudor et al., 2001). There are many indications that overproduction of ROS may be detrimental but antioxidant therapies have so far not proven to be beneficial in patients when a multitude of studies were summarized (Bjelakovic et al., 2007, Bjelakovic et al., 2008). In fact, it has been reported that a mutation in the gene for one of the NOX2 subunits, neutrophil cytosolic factor 1 (Ncf1), results is an augmentation in the severity of EAE, due to downregulation of T cells (Hultqvist et al., 2007).

1.7.3 ROS in MS

ROS are implicated in the pathophysiology of acute and chronic neurodegenerative diseases (Gilgun-Sherki et al., 2001) since free radicals can cause damage to lipids, proteins and DNA (Lewen et al., 2000). A mitochondrial dysfunction and the subsequent reactive oxygen species have been implicated in the pathogenesis of multiple sclerosis (Andrews et al., 2005, Kalman et al., 2007, Lu et al., 2000). Interestingly, the degeneration of myelin by macrophages is ROS dependent (van der Goes et al., 1998).

Of the damage ROS can cause, DNA damage might have the biggest impact on cell survival, both because it impairs protein expression but also because the DNA repair mechanism uses up a lot of ATP, exacerbating any respiratory deficit. DNA is normally repaired by the action of poly ADP ribose polymerase (PARP) through removal of oxidised bases. The enzyme is activated by DNA strand breaks or kinks and ROS. However, during severe oxidative stress it would be beneficial for PARP not to be activated since over-action of PARP can lead to energy deficiency and cellular death through depletion of nicotinamide adenine dinucleotide (NAD) and ATP (Na, 1985, Ha and Snyder, 1999, Yu et al., 2002).

There are increased levels of oxidised DNA in the NAWM of MS patients (Vladimirova et al., 1998). The effect on mitochondrial function by ROS may be important because mitochondrial DNA mutations are seen ten times more frequently than nuclear DNA mutations (Mecocci et al., 1993). Furthermore, activation of PARP, the DNA repairing enzyme, is also seen in rodent- and marmoset models of EAE (Besson et al., 2003, Scott et al., 2001, Scott et al., 2004, Tiina M. Kauppinen, 2005). In the marmoset model of EAE, the PARP positive cells are primarily astrocytes, but PARP is also present in neurons and oligodendrocytes, at the borders of the lesions (Tiina M. Kauppinen, 2005).

In addition to DNA oxidation, increased peroxidation of lipids and antioxidant activity in peripheral and serum leukocytes has been reported in MS patients (Koch et

al., 2006). Furthermore, an altered oxygen concentration (which is indicated in MS) may be harmful to the cells since both hypoxia and hyperoxia increase the number of breaks in the DNA in proportion with the partial oxygen tension and the resultant ROS (Cacciuttolo et al., 1993).

1.7.4 Iron and ROS

Iron is an essential nutrient and is used by cells as an oxygen carriers or redox catalysts. Iron is usually bound up in a heme complex since iron in its free form can mediate the production of damaging reactive oxygen species (Bozho et al., 2009). In the CNS, iron is important for both myelination and inflammation (Pujol et al., 1992). Staining for iron in normal healthy people shows that, in the CNS, oligodendrocytes contain the highest amounts of iron (Connor et al., 1992, Honda et al., 2004, House et al., 2007), although a correlation has also been observed with increased age in the general cell population (Berg and Hochstrasser, 2006, Dexter et al., 1987, Jenner and Jenner, 1991, Peter et al., 1989, Youdim et al., 1993). In disease states there are reports of iron deposits in Alzhemier's disease (Drayer et al., 1987, Bakshi et al., 2002, Mohit et al., 2009, LeVine, 1997) and Parkinson's disease (Forge et al., 1998, Levine and Chakrabarty, 2004). In the case of MS patients, the amount of iron labelling as seen by T2 hypointensity MRI scans is increased (Grant et al., 2003). Iron seems to have an important role in the pathogenesis of multiple sclerosis, or at least in models of the disease, since mice fed a low iron diet did not develop EAE compared to controls (Ghio et al., 2003) and administration of deferiprone, an iron chelator, ameliorated EAE in mice (Mitchell et al., 2007).

1.8 ANTIOXIDANTS

Antioxidants are the body's natural defence against ROS and can protect the tissue by acting as free radical scavengers, preventing the oxidation of other molecules. The activation of antioxidant enzymes are regulated by the transcription factors nuclear-related factor E2 (Nrf2) and antioxidant response elements (AREs). There is a multitude of antioxidants, both endogenous molecules such as glutathione, uric acid and NADPH and exogenous antioxidants for example vitamins C and E and flavonoids, as well as enzymes such as catalase, superoxide dismutase (SOD), and other various peroxidases (Blokhina et al., 2003).

1.8.1 Antioxidants in MS

There are several indications of decreased antioxidant defences in MS patients, but it is not known if the decrease reflects depletion by increased oxidative stress or whether there are constitutively lower antioxidant levels in these patients. Oligodendrocytes are likely to be more sensitive to oxidative damage than other CNS cells due to lower antioxidant defences and the increased presence of iron (Smith et al., 1999).

Serum and CSF levels of antioxidants are used as a measurement of antioxidant concentration and have been repeatedly shown to be decreased in MS patients. The serum concentration of uric acid, which is a very potent scavenger of hydrogen peroxide, is reduced compared with controls (Rentzos et al., 2006, Toncev et al., 2002). Other natural antioxidants with significantly lower levels in MS patients are glutathione (GSH, red blood cells) and SOD (cerebrospinal fluid) (Calabrese et al., 1994, Langemann et al., 1992, Zagorski et al., 1991, Ronquist and Frithz, 1979). SOD and glutathione peroxidase gene transcripts were seen to be increased by 14-20 times that in NAWM (Tajouri et al., 2003). Although ROS concentrations are difficult to measure and hard to validate it is likely that they have an impact on the disease progression of MS.

The clinical score of animals with EAE improved when administered several different antioxidants, e.g. lutein (Lutskii and Esaulenko, 2007, Hendriks et al., 2005), uric acid (Hooper et al., 2000), flavonoids (Hendriks et al., 2004), bilirubin (Liu et al., 2003),

N-acetyl-L-cysteine (Lehmann et al., 1994) and alpha lipoic acid (Marracci et al., 2004, Morini et al., 2004, Schreibelt et al., 2006). Additionally, the treatment of catalase, but not SOD decreased the severity of EAE (Ruuls et al., 1995). Together these data indicate that insufficient antioxidant defences, especially in oligodendrocytes, might lead to induced ROS damage and hence contribute to the pathology of MS lesions.

2. EFFECT OF LPS-INDUCED INFLAMMATION ON THE OXYGEN CONCENTRATION *IN VIVO*

2.1 INTRODUCTION

Multiple sclerosis (MS) is a complex disease with genetic and environmental influences. The variations in the age of onset, disease course and severity have made some investigators suggest that it is a group of diseases instead of one disease brought on by one common factor. Indeed, the pathology of MS patients has been divided into four categories, termed Patterns I–IV (Lucchinetti et al., 2000). One morphological pattern, referred to as Pattern III, was found in acute lesions, seldom in chronic cases and most often in patients with onset of the disease less than two months before the autopsy/biopsy. Pattern III has hence been suggested to represent an early lesion which may evolve to display the various pathological features found in active lesions (Lucchinetti et al., 2000). Since the initiation of the disease itself is still elusive, it is highly interesting to study a lesion which may represent the early events taking place during the disease course. A similar 'prephagocytotic' lesion is reported to precede inflammatory demyelination as seen in active lesions (Barnett et al., 2009).

A model lesion of Pattern III pathology has been invented in our laboratory (Felts et al., 2005) which is induced by an intraspinal injection of the pro-inflammatory agent lipopolysaccharide (LPS) into the dorsal column (DC) of the spinal cord and which displays inflammation, demyelination and partial remyelination. The mechanism underlying the demyelination in the LPS-DC model has been described as hypoxia-like (Felts et al., 2005, Marik et al., 2007, Lassmann, 2003), and in this respect the model is similar to that in the Pattern III lesion in MS patients (Aboul-Enein et al., 2003), and in a cuprizone-induced CNS model (oligodendrocyte-specific mitochondrial deficiency) (Johnson and Ludwin, 1981), which, in turn, is similar to the mechanism of demyelination that can occur in ischaemic, stroke lesions (Aboul-Enein et al., 2003). This pathology is believed to arise from an energy-deficiency within the oligodendrocytes, but it is not clear why the deficiency arises. There are two likely hypotheses. On the one hand, it could arise from poisoning of

mitochondrial enzymes by diffusible products of inflammation, such as nitric oxide, while on the other it could arise from an inadequate supply of oxygen to allow the mitochondria to function effectively. Both mechanisms would have the same consequences, namely a shortage of ATP production. However, to devise an effective therapy it is necessary to be able to discern between the two mechanisms. If the energy deficit is due to poisoning of the mitochondrial electron transport chain, we have reasoned that the oxygen concentration within the lesion should increase, because oxygen is no longer being utilized in the mitochondria. However, if, on the other hand, the deficit is due to true hypoxia, the oxygen tension should be low. Therefore, in order to gain insight into the cause of the hypoxia-like demyelination, we have aimed to measure the oxygen concentration within the 'early' LPS-DC lesion, *in vivo,* at the peak of inflammation occurring at 24 hrs post-injection.

2.2 MATERIALS AND METHODS

2.2.1 Experimental design

The model Pattern III lesion, induced by the intraspinal injection of LPS into the dorsal columns were studied *in vivo* at the peak of inflammation for abnormal oxygen concentrations. To explore the dependence of the results on the oxygen concentration of the inspired 'air', we tested the effects of varying the inspired oxygen concentration. Blood samples were taken for blood gas analysis.

All animal procedures were carried out according to the UK Home Office regulations and the Animals (Scientific Procedures) Act (1986). Animals were obtained from Harlan (Bicester, UK) or Charles River (Tranent, UK) and were allowed free access to food and water. Animals were left for a minimum of seven days in the biological service unit for acclimatisation before any procedures were conducted. All animals with surgical recovery received 10 µl Vetergesic (0.3 mg/ml buprenorphine hydrochloride, Rechitt Benckiser Healthcare, York, UK).

2.2.2 Original experimental plan

The initial experimental plan was to use oxygen-sensitive microelectrodes to map the 3-dimensional distribution of oxygen within inflammatory lesions, correlating the findings with the distribution of blood vessels. These plans were pursued with only variable success for 18 months due to instability of the recordings. An expert in the field, Dr. Julian Millar, kindly assisted in later experiments but even with his advice and participation it was not possible to obtain reliable data. Although initially optimistic, Dr. Millar and we decided that the plan of creating a map of oxygen distribution within the inflammatory lesion was not achievable with the micropipette technology currently available. Attention was transferred to an alternative technology, oxygen-sensitive optical probes, "OxyMicro" (WPI, Stevenage, UK). However, our reluctance more promptly to use such probes was because they have a much larger tip diameter than micro-pipettes (50 µm cf. 8.5 µm) and this prevents high resolution recordings and also causes more significant tissue damage. Our determinations of

oxygen concentration were therefore made at only two depths within the lesion, along a single track.

Realising that determinations of oxygen concentration within tissues are prone to artefact, we planned to confirm our findings by also measuring the oxygen concentration by an entirely different technique, in the belief that similar findings obtained by different techniques would have especial validity. We therefore explored the use of electron paramagnetic resonance (EPR) imaging and contacted the expert on this methodology in the UK, Dr. Philip James, University of Cardiff. Dr. James was very encouraging and helpful, and experiments were planned, but a series of delays ensued, due to an unfolding litany of political, regulatory and legal problems in Cardiff. These always appeared to be on the verge of being solved, but in the event they persisted over at least 18 months and in fact no experiments could be achieved. This series of delays not only prevented progress in EPR, but also delayed other experiments in the belief that the superior EPR method would soon be available.

2.2.3 Induction of the model Pattern III lesion

Adult male Sprague Dawley (SD) rats (200-250 g) were anaesthetised (~2% isoflurane in oxygen, Merail, Harlow, UK) and the site of operation shaved and sterilised with iodine. A quarter-laminectomy was performed at the caudal end of the twelfth thoracic vertebra and a small incision made in the dura and pia to permit the unobstructed entry of a glass microinjection pipette. The pipette was held in a micromanipulator and inserted at two sites, 0.5 mm apart in the rostro-caudal axis, as close to the ventral vein as possible, with a trajectory of 19 degrees to the vertical. Two injections (0.5 µl LPS (100 ng/µl, from *Salmonella enterica* serotype typhimurium or abortus equi, Sigma) were made along each track at depths of 400 and 700 µm. The injection sites were marked with a small amount of sterile charcoal. Control animals received injections of saline alone, with the lesion site marked in the same way. To determine the precise location of such injections within the dorsal columns, a marker injection of dilute India ink was occasionally used. The ink solution was prepared in sterile saline which was centrifuged to remove larger ink fragments that may otherwise have blocked the injection pipette.

2.2.4 Design of microelectrodes

Oxygen-sensitive microelectrodes were produced in-house. Single carbon filaments were placed inside glass capillaries (Harvard Apparatus, Edenbridge, UK) which were pulled using a micropipette puller (Sutter Instruments, Novato, USA) into two separate micropipettes of which one would contain the carbon filament. Excess carbon was removed using a heated wire, leaving an approximately 1-2 μm carbon tip protruding from the micropipette. The carbon filament was connected to an insulted wire, bare at its tip, using electrically-conducting epoxy (Chemtronics, Kennesaw, USA) within the pipette. The wire was secured to the end of the micropipette with glue. Before starting an experiment, the microelectrodes were left to stabilize in Ringer's solution at pH 7.4. A silver-AgCl coated electrode (Diamond General, Michigan, USA) was used as a reference electrode to allow polarisation of the oxygen-sensitive microelectrodes at -0.75 V to try to ensure a stable reading of the oxygen concentration (Figure 4).

2.2.5 Calibration of oxygen sensors

Before use, the oxygen-sensitive OxyMicro probe and/or the microelectrode were calibrated in a two point determination in Ringer's solution bubbled with either nitrogen (0% oxygen) or room air (21% or 12 mmHg oxygen).

2.2.6 Experimental protocol

Animals were anaesthetised with isoflurane and body temperature maintained at 37.0 $\pm 0.5^{o}$C with a heating pad. Breathing rate and end-tidal CO_2 levels were monitored via tracheotomy and end-tidal capnometer. Blood pressure was monitored via cannulation of the left carotid artery and maintained by intravenous administration of saline. A partial laminectomy was performed between the T8 and L3 vertebrae and the animal stabilised

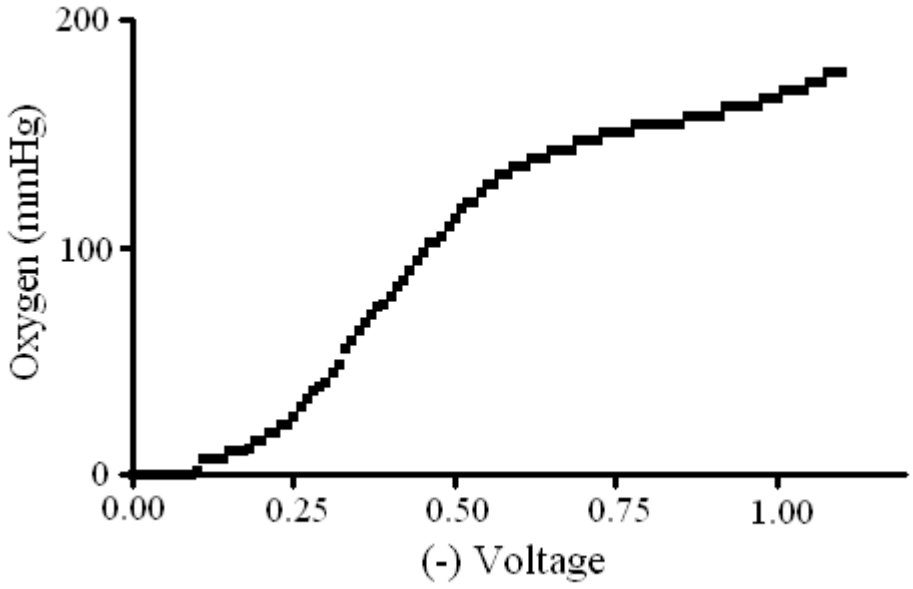

Figure 4. Oxygen-voltage curve. Between voltages -0.6 to -1.0, a plateau region is reached where the output is dependent on the diffusion of oxygen to the electrode. Below -0.6 V, oxygen is reduced at the electrode surface in a voltage dependent matter and above 1.0 V, non-specific reduction starts to occur.

in a stereotaxic frame to reduce breathing movements being relayed to the spinal cord. Warm Ringer's solution (pH 7.4) was added to the bowl-like space created by raising skin flaps, and an infrared light was used to keep the spinal cord and pool maintained at a constant temperature of 35^{o}C (to avoid overheating of the cord tissue and animal).

The dura and pia were removed at sites of interest and an oxygen electrode and/or OxyMicro probe, supported in a micro-manipulator, were inserted close to the central vein at an angle of 19 degrees to the vertical, and measurements taken at 500 and 1000 μm depths. Measurements were taken at the site of the lesion and from other sites (approximately 1.5 cm rostral and caudal to the lesion) to compare the oxygen concentration between the lesioned and control tissue, as well as in white versus grey matter. Naïve or saline-injected animals were used as controls. Oxygen concentrations were sampled every 10 seconds, with the OxyMicro probe was calibrated before and after the measurements: measurements drifting more than 5 % during the experiment were disregarded. Data was saved via a Picologger (ADC-11, Pico Technology, St Neots, UK) with accompanying software and transferred to an Excel (Microsoft Co., USA) spreadsheet for analysis.

To ensure that the oxygen saturation in the blood was consistent between the different groups, a cohort of rats had 150 μl blood sampled from the tail artery at the start and sometimes the end of the experiment and analyzed with the Gem Premier 3000 (Instrumentation Laboratory, Lexington, USA). In another subset of experiments, blood oxygen saturation, heart rate and breathing rate were monitored using a MouseOx Pulse Oximeter (Red box direct, Dublin, Ireland) and a sensor clipped to the foot pad. The data obtained from the MouseOx was saved in Notepad (Microsoft, USA).

2.2.7 Characterising the properties of the OxyMicro probe

The oxygen-sensing reliability of the OxyMicro probe was assessed using an experimental ischaemic lesion within the rat spinal cord induced by the intraspinal injection of the potent vasoconstrictive agent endothelin-1 (Bei, 2009). The model created within our group by Fengfeng Bei Ph.D. allows the simultaneous monitoring of changes in blood flow with a laser Doppler flowmeter (PF3 Periflux, Perimed, Stockholm, Sweden), blood pressure by carotid cannulation, and the functional properties of the axons by electrophysiology.

Animals were anaesthetised with isoflurane (2% in oxygen), intubated and the left carotid artery cannulated for monitoring of blood pressure and supply of sterile saline and neuromuscular blocking agent. The spinal cord was exposed by a laminectomy at the vertebral level T10 – L2. The animal was ventilated and a neuromuscular blocking agent (1 ml gallamine triethiodide, 40 mg/ml/hour in sterile saline, Concord Pharmaceuticals Ltd, Dunmow, UK) was slowly infused via the carotid artery to prevent any electromyographic (EMG) signals from the musculature from interfering with the electrophysiological findings. Conduction along the axons within the dorsal columns was monitored by recording the compound action potential (CAP) from the base of the tail in response to electrical stimulation of the axons at 50 Hz via a computer-programmed pulse generator (Master-8, A.M.P.I., Jerusalem, Israel) with needle electrodes positioned above the thoracic vertebrae rostral to the intended lesion. Stimuli were at supramaximal intensity (1.5 times the voltage giving a CAP of maximal amplitude) and recordings were averaged (n=20), displayed on a digital oscilloscope (Gould 4164, Gould Instruments, Essex, UK) and saved onto a PC using Transition2 software (Gould Instruments, Essex, UK) every two minutes. Local ischemia was induced at the L1 vertebral level by the direct intraspinal injection of 5.7 µg endothelin-1 (8x 0.5 µl, Alexis, Exeter, UK) in sterile saline, with the injections placed as illustrated in Figure 5A. The experimental arrangement and position of the OxyMicro probe and the Doppler blood flow probe is shown schematically in Figure 5B. The oxygen concentration and blood pressure recording were saved to computer as described above.

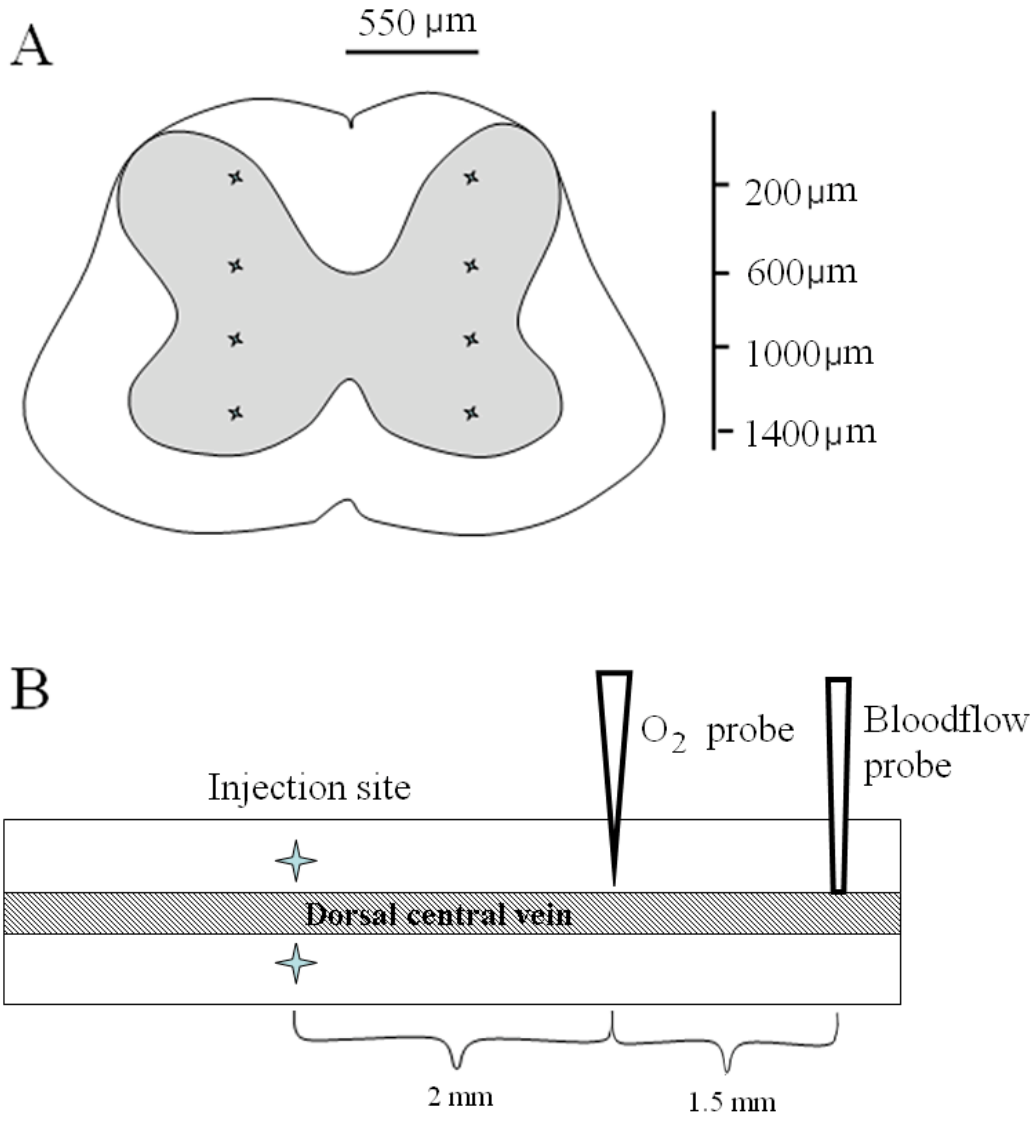

Figure 5. Experimental arrangement for the endothelin-induced ischemic lesion. (A) Endothelin-1 was injected at the eight different sites illustrated. (B) Placement of the oxygen-sensitive OxyMicro probe and the Doppler blood flow probe on the exposed spinal cord.

2.2.8 Statistical analysis

Where we examined the correlation or statistical significance between two or more groups we utilized the GraphPad Prism 4 software (GraphPad Software Inc., USA). Each group that was compared for significance differences consisted of at least three animals. Unpaired, two-tailed student's t-tests were used to compare two groups with each other, significant values were considered to be * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

2.3 RESULTS

2.3.1 Verification of injection site

India ink was used as a marker to determine the precise location of solutions injected into the rat dorsal columns (Figure 6A). The India ink was located within the dorsal column white matter, but was distributed quite widely, although largely unilaterally. There was a tendency for the marker to travel along the plane formed between the white and grey matter, and along the sulcus that can sometimes be discerned within the dorsal columns, especially when larger volumes are used (1 µl compared with 0.5 µl). In another experiment, India ink was injected diluted ($^{1}/_{8}^{th}$ concentration) with the standard concentration of LPS so that ED1 labelling could be correlated with the distribution of the injected liquid (Figure 6B). This one animal was culled 24 hrs after the injection and the photograph shows how the liquid gathers at the bottom of the funiculus and at the border with the grey matter. The presence of activated macrophages and microglia (ED1) is seen in brown, and India ink in black.

2.3.2 Characterising the properties of the Oxy-Micro probe

The sensitivity of the microelectrode and the OxyMicro probe for oxygen was determined by immersing the probes in Ringer's solution, pH 7.4, and continuously measuring the output while the solution was alternately bubbled with nitrogen, room air or pure oxygen, in a closed container (Figure 7 A and B respectively). The findings established that the output was reproducible, stable and linear with oxygen concentration and that the microelectrode had a longer response-time.

To explore the reliability of oxygen measurements in the complicated environment of the rat spinal cord *in vivo*, continuous measurements were taken while the cord was rendered ischaemic by the intraspinal injection of endothelin-1. The findings established that the OxyMicro probe is able to make stable recordings (Figure 8, representative graph), and to detect that the oxygen concentration was reduced to 0% in response to the injection of the vasoconstrictor. In the example shown, there was a cyclical change in blood pressure and

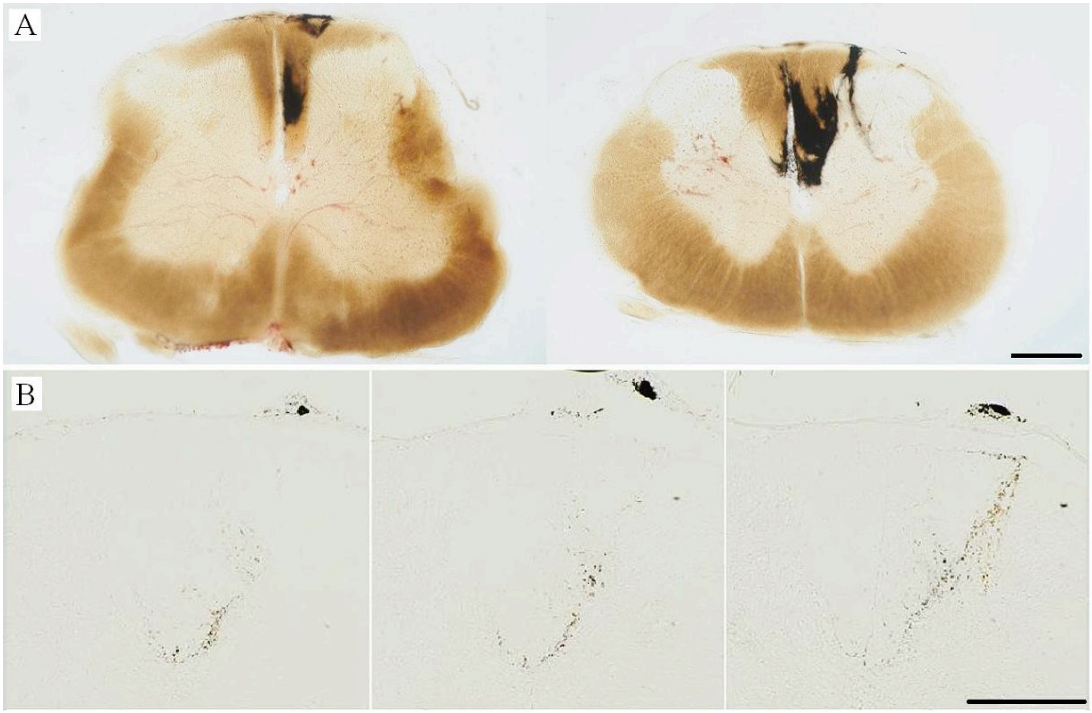

Figure 6. (A) Injection location of 0.5 and 1 µl of India ink respectively (left and right) in the rat dorsal columns, examined immediately post-injection. (B) India ink and LPS injection into rat dorsal column, examined 24 hours post-injection. At this time the India ink has been sequestered in phagocytic cells. The three pictures represent a distance of 250 micrometer through the lesion site. Scale bars represent 0.5 mm.

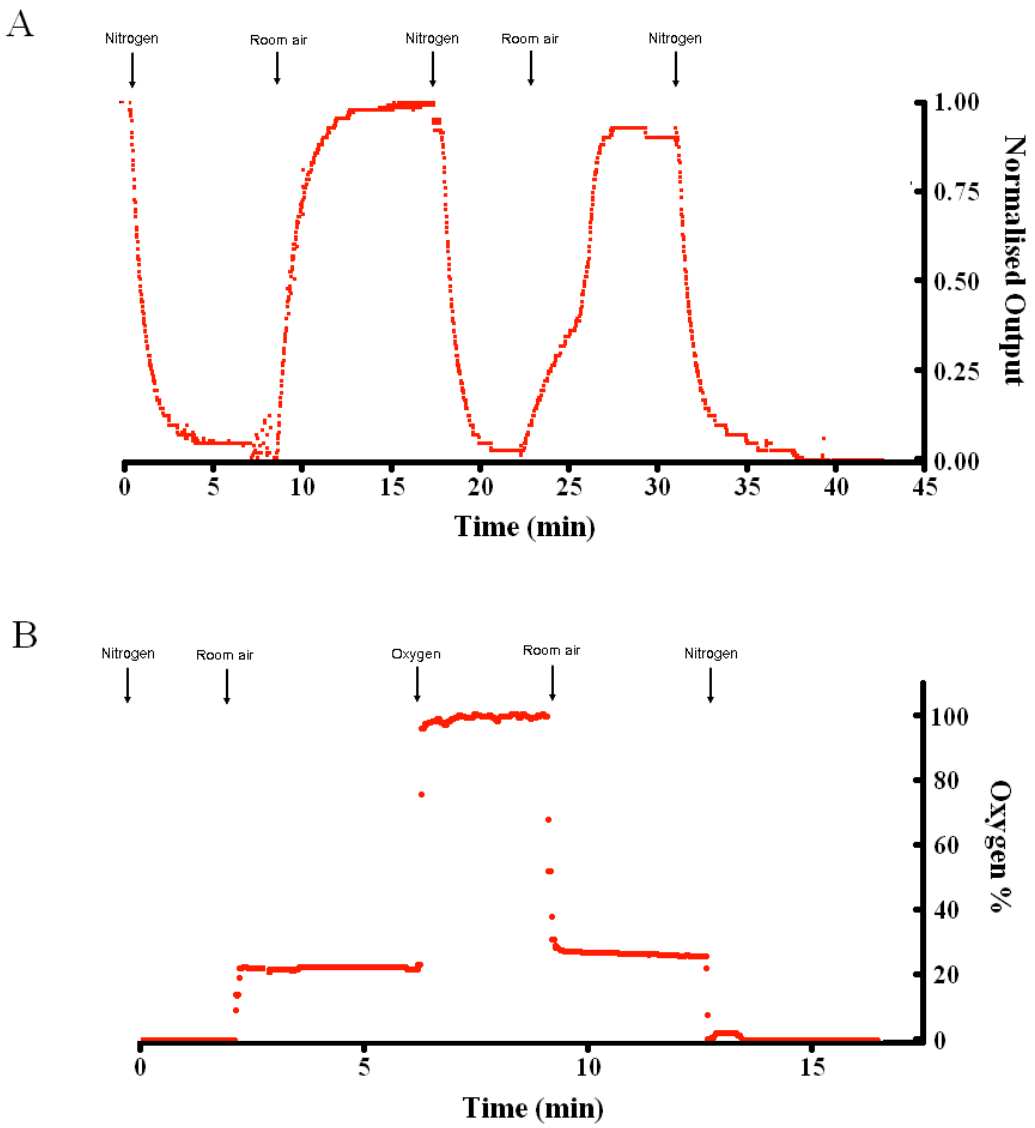

Figure 7. Calibration of an oxygen-sensitive microelectrode (A) and the OxyMicro probe (B). Both probes were (separately) recording the oxygen concentration in a Ringer's solution bubbled with nitrogen (100%), room air or oxygen (98%) at the time points indicated by arrows.

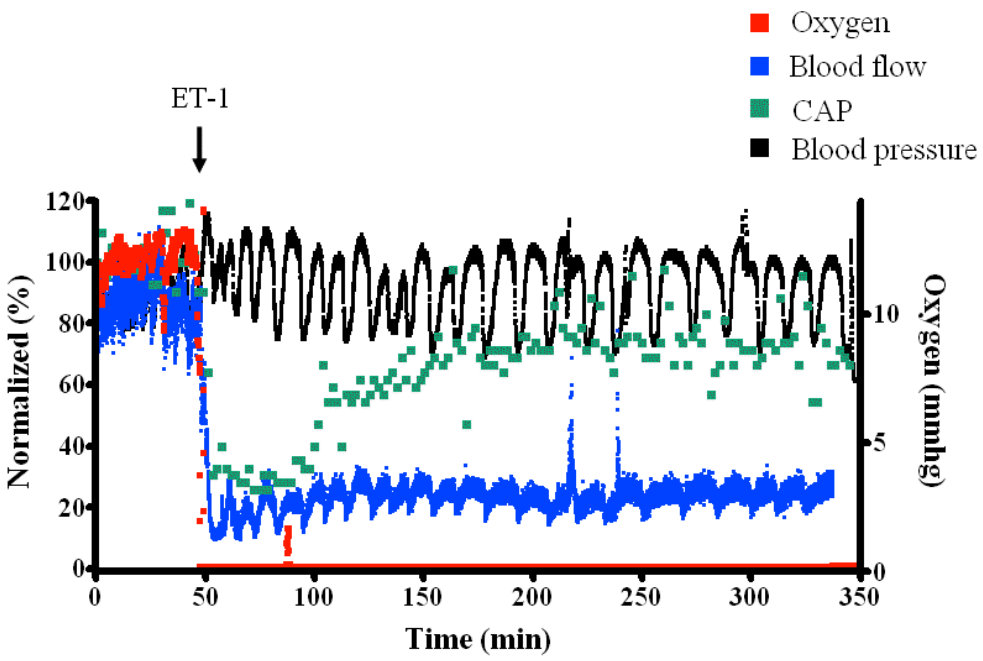

Figure 8. Graph showing how the oxygen concentration (as measured using the OxyMicro probe) within the rat dorsal column changes over time in response to ischemia induced by the intraspinal injection of ET-1 (at 45 min, indicated). Also plotted are the blood flow monitored at the lesion site, systemic blood pressure, and amplitude of the CAP conducting along the DC axons. Data were normalized to initial values and the oxygen concentration is additionally shown on the right ordinate. ET-1 injection resulted in a decrease in the oxygen concentration, blood flow and CAP (transient reduction) but not in the blood pressure.

blood flow as if resulting from the operation of an endogenous homeostatic mechanism: this phenomenon was sometimes observed. The drop in oxygen concentration correlated with a reduced compound action potential (CAP, transiently) and blood flow but did not correlate with blood pressure.

The correlation in output from the oxygen-sensitive microelectrode and the OxyMicro probe was finally examined in one experiment where both sensors were placed in close proximity in the spinal cord of a freely breathing anaesthetised rat. The animal was over time inspiring varying concentrations of oxygen (mixing of room air, oxygen and nitrogen gases) and the recordings from the two sensors synchronized with each other (the microelectrode broke before it could be re-calibrated at the end of the experiment, Figure 9). The output from both oxygen-sensors correlated well with each other, indicating that the OxyMicro probe was indeed a sensitive and qualitative oxygen sensor even though the large-diameter tip is bound to measure an average oxygen concentration instead of a site specific.

2.3.3 Effect of ROS on the oxygen-sensitive OxyMicro probe

The OxyMicro probe is supposed to be a specific oxygen sensor. However, the properties of oxygen molecules can be very similar to related molecules. Superoxide, for example, is a likely species that could distort the readings from the oxygen sensor. To confirm if such an effect could occur, the OxyMicro probe was calibrated using nitrogen vs. 98% oxygen, and then tested in a Ringer's solution bubbled with oxygen, and with oxygen in the 'headspace'. Hydrogen peroxide (H_2O_2) was then added (0.1% final concentration) to test whether the addition of ROS would affect the recordings made. The addition of the peroxide resulted in a small but significant increase of the 'oxygen' reading (4% increase, Figure 10). It appears that the OxyMicro probe will respond to the presence of ROS, in addition to oxygen.

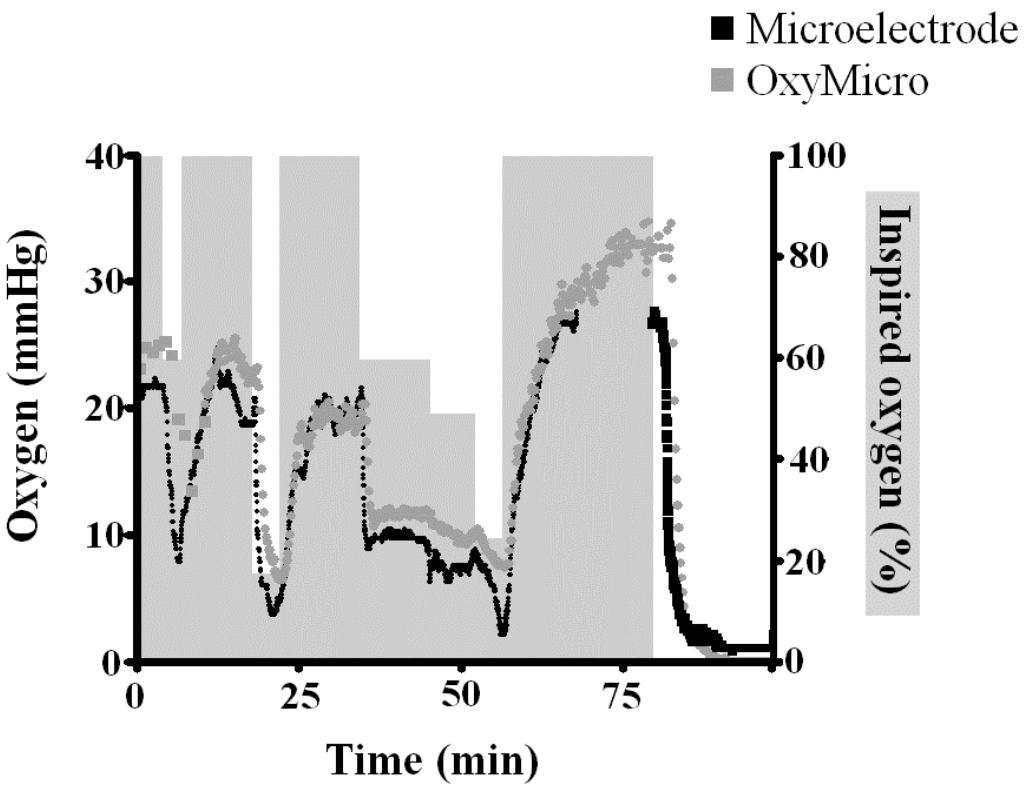

Figure 9. Plot showing the measurements obtained from an oxygen-sensitive microelectrode and the OxyMicro probe which were placed in close proximity within the rat spinal cord. The oxygen concentration of the inspired gas was varied during the experiment as shown by the grey background shading. The two curves are similar, despite the very different technical methods involved in the two types of measurement, giving confidence that the methods are capable of giving reliable results.

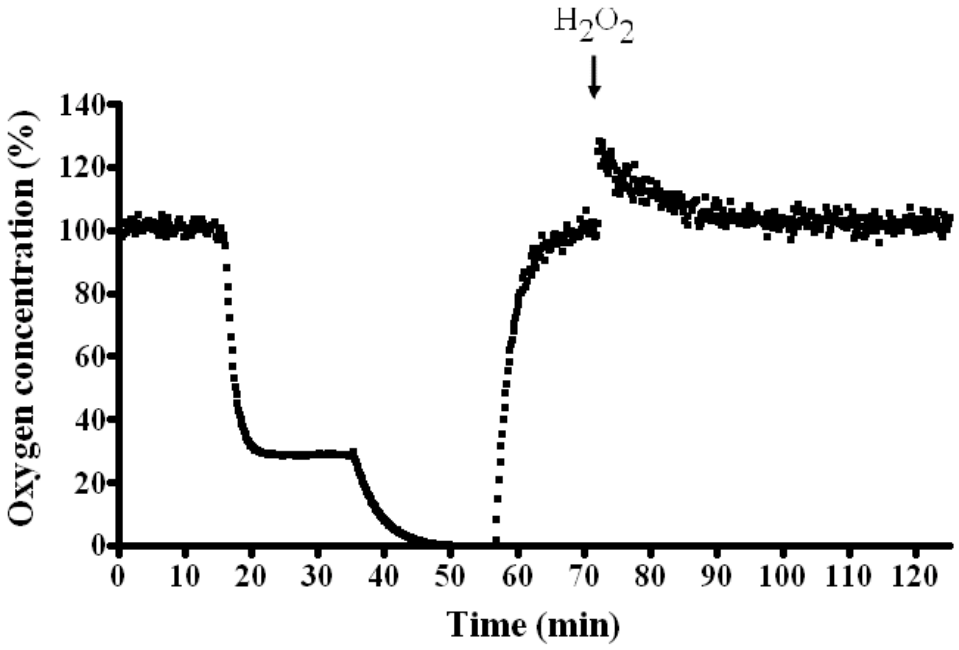

Figure 10. Effect of adding (indicated by arrow) hydrogen peroxide (H_2O_2 final concentration 0.5%) to Ringer's solution bubbled with 100% nitrogen on the readings obtained from the OxyMicro probe immersed in the same solution. The addition of the peroxide increased the reading substantially, but this declined progressively over time.

2.3.4 The oxygen concentration *in vivo* in the LPS-DC lesions

We could detect a significant increase of the oxygen concentration in the LPS-injected animals (64±7 mmHg) compared with saline-injected (35±5 mmHg, P<0.0024) and naïve animals (31±4 mmHg, P=0.0010, Figure 11). The elevation of oxygen in the LPS-injected animals extended beyond the focal lesion, to tissue several centimetres away (perilesion, 54±7 mmHg) and was significantly higher when compared with the saline perilesion (P=0.0100). All animals were monitored for fluctuations in blood pressure through cannulation of the carotid artery but no differences could be detected between the experimental groups (Figure 12A). Moreover, in a subset of experiments (N=3-5/group), blood samples were analysed for pH, pO_2, pCO_2 and haematocrit (red blood cell) content (Figure 12B) but revealed no significant differences. Finally, no differences were seen between the experimental groups when they were monitored at the foot pad with a non-invasive pulse oximeter for heart beat, blood oxygen saturation and breath rate (N=2-3/group, Figure 12C).

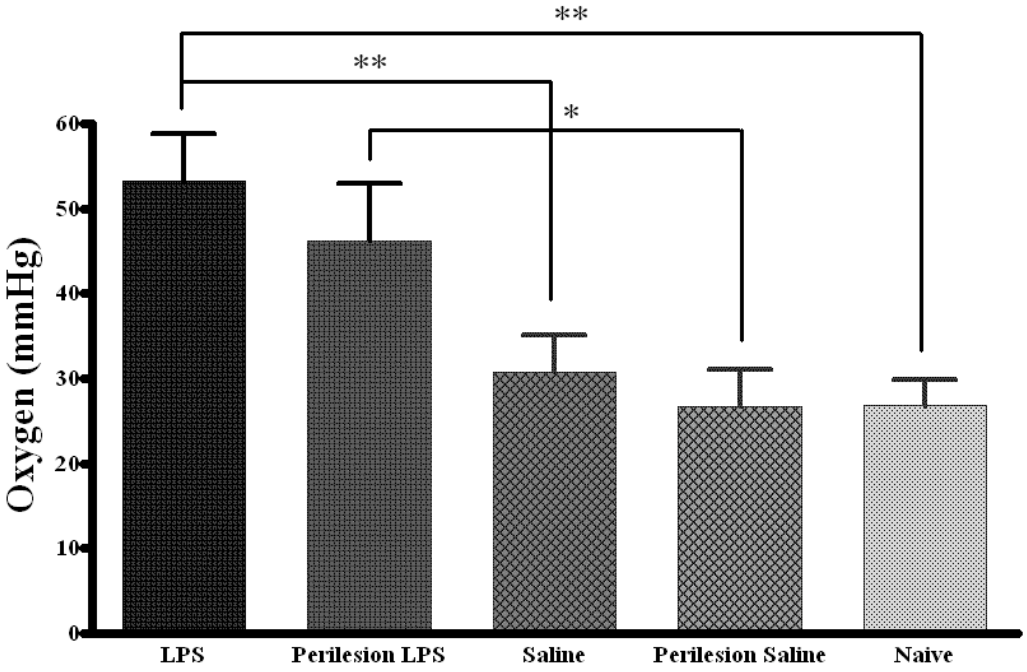

Figure 11. The oxygen concentration measured by an OxyMicro probe in the dorsal columns of LPS- or saline-injected animals as well as in naïve controls. The oxygen concentration was examined at the injection site ('LPS' and 'Saline') and in the dorsal columns several centimetres away ('Perilesion'). Animals injected with LPS had a significantly higher oxygen concentration within the lesion than saline-injected or naive animals. N=8-9/group.

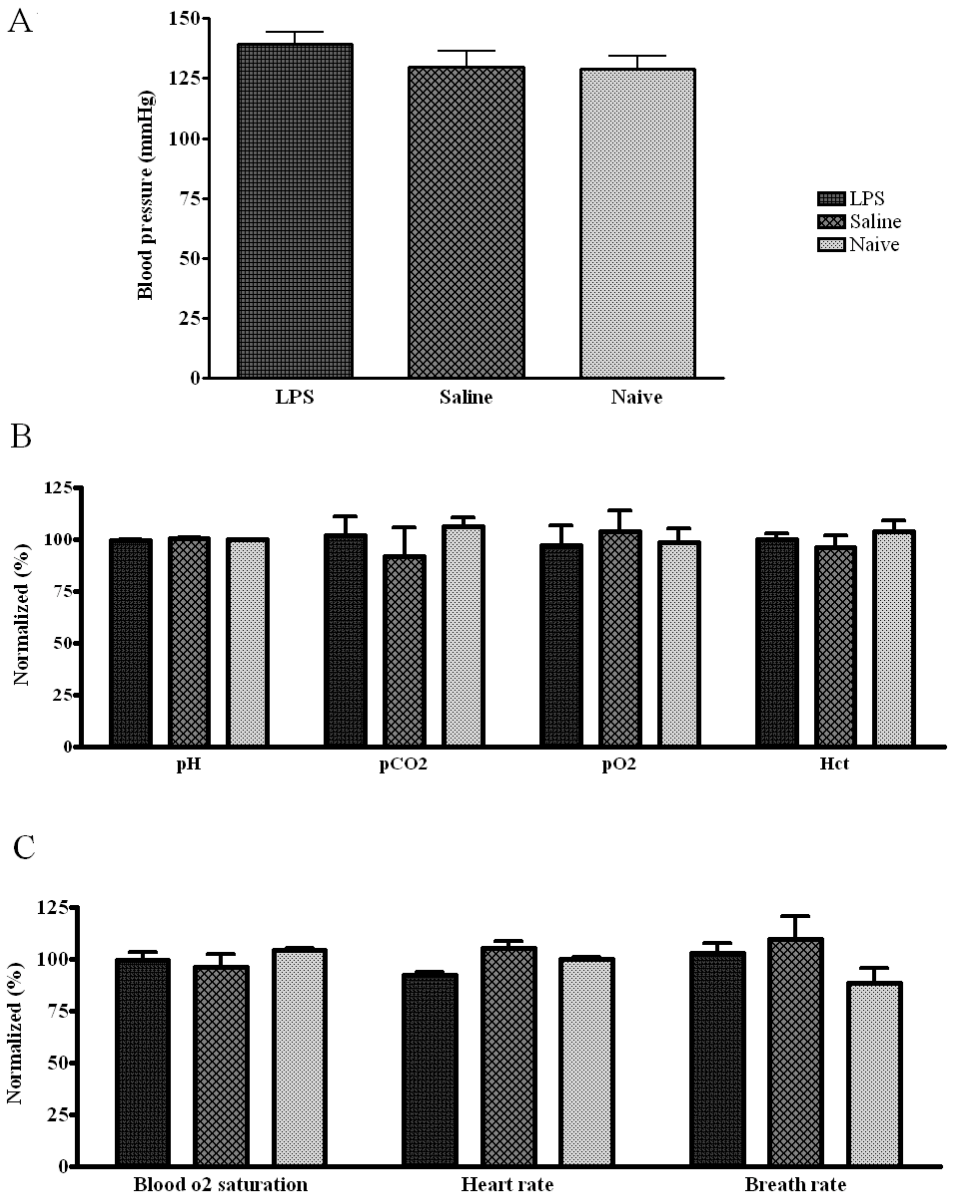

Figure 12. Experimental animals were monitored throughout the experiments to measure blood pressure through cannulation of the carotid artery (A). Blood sampling in a cohort of animals displayed no difference in blood pH, pCO_2, pO_2, hematocrit (Hct) between the experimental groups (B). Some animals were monitored with a non-invasive pulse oximeter for blood oxygen saturation, heart rate and breathing rate (C). Values from graph B and C were normalized to the average value for all animals, N=3-10/group.

2.3.5 Oxygen challenge in the LPS-DC lesion

In addition to measuring the oxygen concentration within the LPS- or saline-injected control rats, or from a similar location within naïve rats (at 700 μm depth) when the animals were breathing room air (21% oxygen), measurements were also taken during an 'oxygen challenge'. Thus different oxygen concentrations (in nitrogen) were administered to freely breathing, anaesthetised rats, and readings of the intraspinal oxygen values were obtained accordingly. Once a stable value was obtained for a minimum of 6 min in room air, the gas mixture was changed to 50%, 100%, 10%, 100% and 0% oxygen (with a minimum of seven minutes stable recording in each, except for 0% oxygen which was only for 1 minute maximum): the animals were administered room air (21% oxygen) between the different values to restore stability. An example of the raw data regarding tissue oxygen concentration is illustrated in Figure 13. The results show that the LPS-injected animals and, to a lesser degree, the saline-injected animals had been preconditioned by the injection which results in an increased response to the oxygen challenge compared with naive animals (Figure 14A). No change between the groups could be detected when the animals were allowed to breathe pure nitrogen gas, but clear changes were observed when breathing 50% or 100% oxygen. No difference in blood pressure was detected between the groups (Figure 14B). The animals initially regained the baseline oxygen concentration once they were allowed to breathe room air again but as the experiment progressed, the values within groups started to have a higher variance, especially after breathing 0% oxygen (Figure 15A). LPS- and saline-injected animals that were breathing pure oxygen increased their oxygen concentration in the tissue 6 fold compared with baseline values, while naive animals only had 1.7 times higher oxygen concentration under the same conditions (Figure 15B).

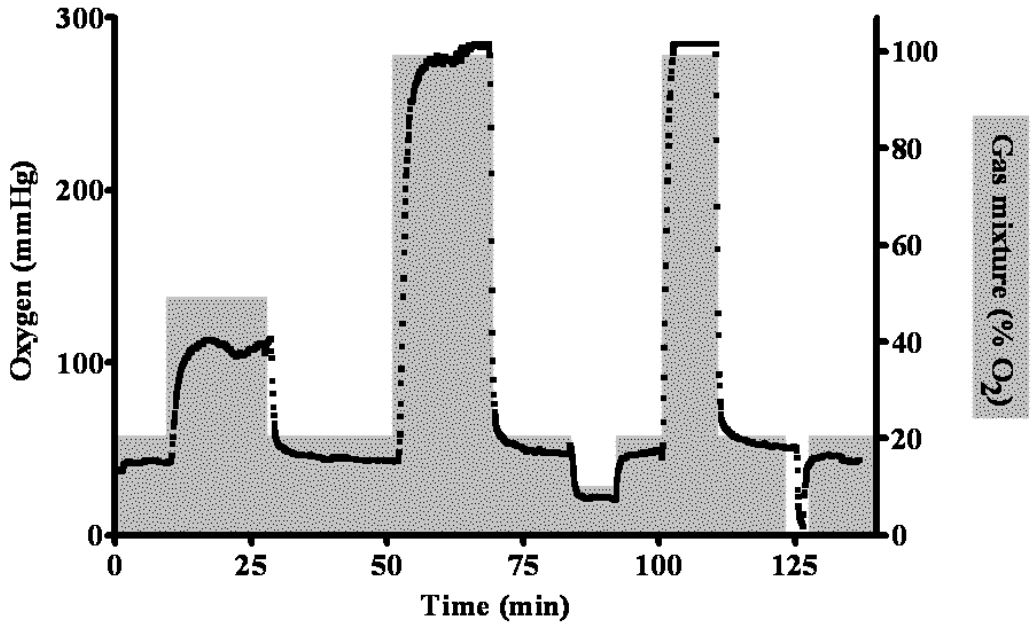

Figure 13. An example of a raw data set obtained in response of the tissue oxygen concentration to different gas concentrations (indicated by the grey background), given to freely breathing anaesthetised animals.

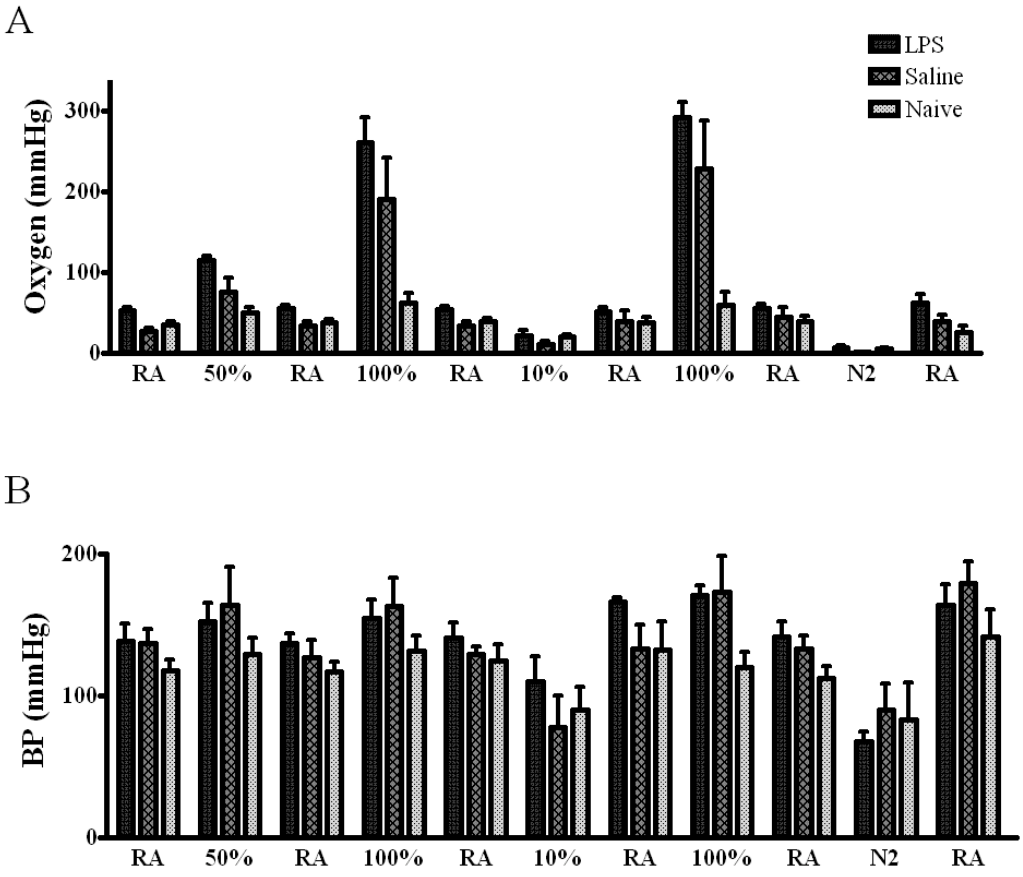

Figure 14. The response of the tissue oxygen concentration (A) and blood pressure (B) to different inspired concentrations of oxygen given to freely breathing, anaesthetised animals. Room air = RA. (A) LPS- and saline-injected animals reported much higher tissue oxygen concentrations under hyperoxic conditions than naïve animals. (B) There was no significant change in the blood pressure between the experimental groups. Values were only recorded once the output had been stable for at least 6 minutes. N=3-5/group.

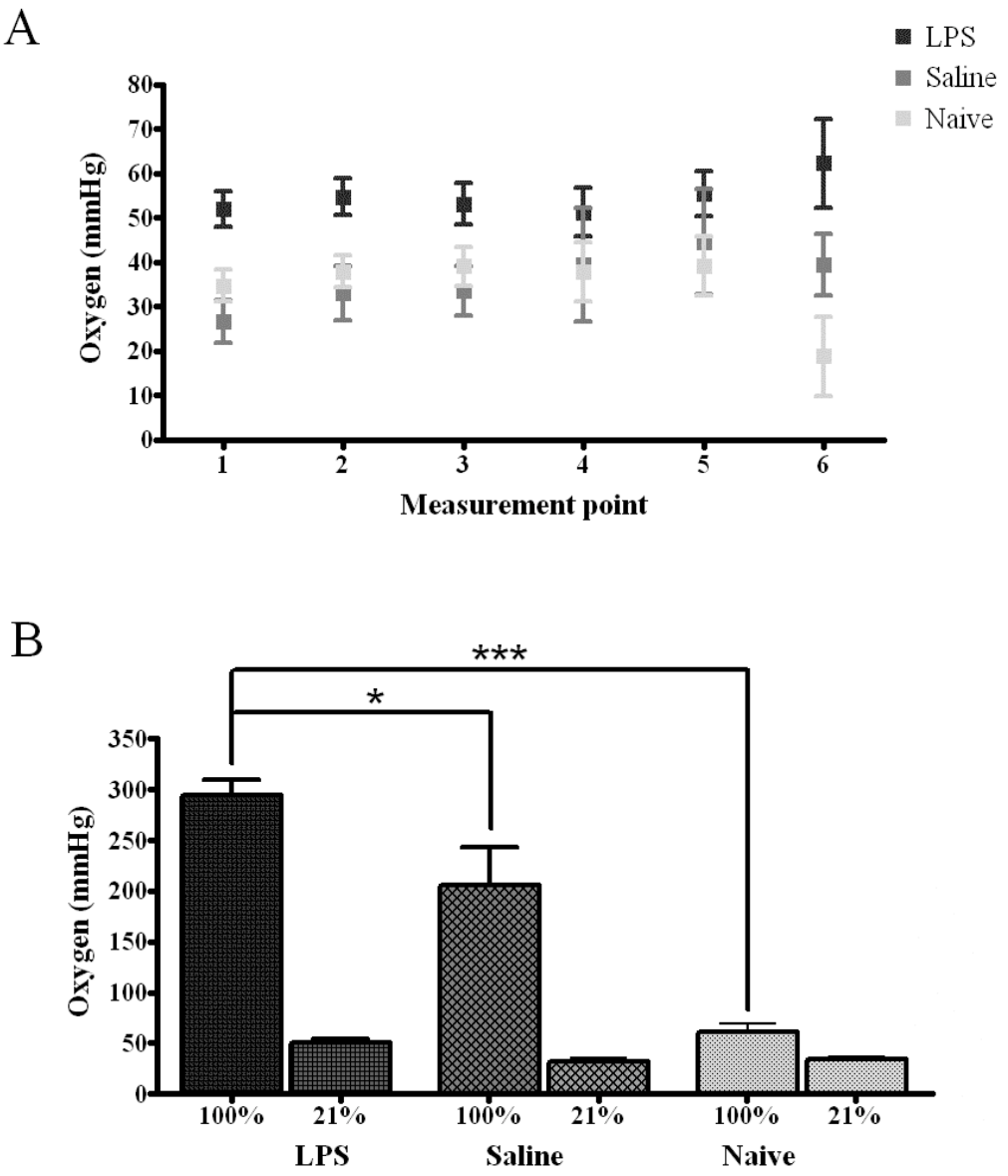

Figure 15. The response of the tissue oxygen concentration to different gas concentrations administered to freely breathing anaesthetised animals. (A) Graph showing the values obtained when breathing room air 1) at the start of the experiment, 2) after 50% oxygen, 3) after 100% oxygen, 4) after 10% oxygen, 5) after 100% oxygen and 6) after 0% oxygen. The variance within the experimental groups increased during the course of the experiment as animals were repeatedly breathing 21% oxygen between oxygen insults. (B) There was a very high significant increase in the tissue oxygen concentration when the LPS- or saline-injected animals were breathing 100% oxygen, but the increase in naïve animals was only minimally significant.

2.4 DISCUSSION

The main finding of this chapter is that the oxygen concentration in the LPS-DC lesion is significantly higher than in saline-injected control animals, or in naïve animals (Figure 11). This finding decisively supports the hypothesis that the hypoxia-like state results from poisoning of mitochondrial complexes, most likely by diffusible products of inflammation, such as nitric oxide. Such poisoning would prevent the utilization of oxygen, with a consequent rise in the oxygen concentration within the tissue. Strengthening the concept that mitochondrial inhibition may be responsible for the hypoxia-like pathology of demyelinating lesions is the occurrence of demyelinated axons in cases of carbon monoxide poisoning (Mascalchi et al., 1996, Murata et al., 2001) or cyanide poisoning (Ibrahim et al., 1963). Interestingly, in diseases where the mitochondrial genome is affected, demyelination may also be seen (Carelli et al., 2004). It is also worth noting in this respect that if, as expected, the mitochondrial production of respiratory ATP is deficient, cells will have to rely on the less efficient anaerobic metabolism with likely production of lactic acid, and indeed lactate levels are increased in MS patients (Miller et al., 1991, Simone et al., 1996).

Another result of this chapter is that the oxygen concentration of LPS-injected experimental animals was also increased in the surrounding peri-lesional tissue (Figure 11) indicating that the inflammatory consequences of LPS injection spread over a significant area.

2.4.1 Inhibition of mitochondrial respiration

LPS is a potent pro-inflammatory agent which may induce cytokine production and increase the levels of iNOS within cells (Lembo et al., 2003). This increase may be important because the most likely cause of failure of the mitochondrial electron transport chain in this study is NO-mediated inhibition of complex IV. Indeed, pro-inflammatory cytokines have been reported to be toxic to oligodendrocytes (Robbins et al., 1987), possibly through ATP depletion (Agarwal et al., 1988) caused by NO production (Tatsumi et al., 2000).

NO may affect the function of the mitochondrial enzymes in several ways, for example, NO can compete with oxygen at the active site of complex IV, reversibly remove the iron from the iron-sulphur cluster within the mitochondrial complexes, form a reversible nitrosyl adduct, or via its toxic metabolite, peroxynitrite, modify protein structure and function through nitration of tyrosine (Beltran et al., 2000, Bolanos et al., 2007, Elfering et al., 2004, Orsi et al., 2000, Davis et al., 2009).

Both LPS-mediated inflammation and MS patients display production of NO by activation of iNOS (De Groot et al., 1997, Murphy et al., 1993, Oleszak et al., 1998), although NO is also produced from within the mitochondria by the neuronal form of NOS (nNOS) (Bolaños et al., 2008). During metabolic dysfunction, the altered energy homeostasis within the cells results in a transient calcium release which may induce the activation of the constitutive form of NOS (Perez Velazquez et al., 1997).

NO has been shown to bind to cytochrome c oxidase (Cox et al., 1971) and reversibly to inhibit the activity of the complex (Brown and Cooper, 1994, Cleeter et al., 1994, Giulivi, 1998, Schweizer and Richter, 1994, Takehara et al., 1996) by forming either a Fe^{2+}-nitrosyl adduct or a Cu^{2+}-nitrate derivate (Brunori et al., 2004). Although cytochrome c oxidase has a very high affinity for oxygen and functions well at oxygen concentrations below 1 mmHg (Turrens, 1997), it has been suggested that the NO and oxygen under physiological conditions actively compete at the binding site of complex IV (Eke and Delpy, 1999). Normal tissue levels of oxygen and NO have been estimated to be approximately 30 μM and 100-500 nM respectively (Brown and Cooper, 1994). In experiments exploring the inhibition of complex IV by NO, Brown and Cooper found that at 30 μM oxygen and only 60 nM NO, there was an approximately half-maximal inhibition of the respiration taking place at cytochrome c oxidase *in vitro* (Brown and Cooper, 1994). This effect of NO on the oxygen binding to complex IV may serve to decrease the ATP production at baseline metabolism (Eke and Delpy, 1999). However, during pathological conditions, the balance between NO and oxygen may be altered, affecting the tissue energy homeostasis. Over-production of NO has, for example, been suggested to mediate mitochondrial dysfunction during septic shock (Forfia et al., 1998).

NO may also affect mitochondrial function by acting on other proteins located in the organelle. Nitration of enzymes of the citric acid cycle or miss-folding of proteins has been reported (Nakamura and Lipton, 2008), and a reversible nitration of mitochondrial proteins was detected in an ischemia-reperfusion model (Aulak et al., 2004). In addition, toxic metabolites of ROS may have more or less direct effects on mitochondrial respiration, for example, hypochlorous acid (HOCl), formed by the reaction of peroxide and chloride anions, can mediate mitochondrial dysfunction (Wei et al., 2009). Mitochondrial dysfunction may of course also be caused by insufficient substrate delivery, or depletion of the membrane potential.

Except for NO there are other mechanisms possibly responsible for mitochondrial dysfunction. One effect could be via mitochondrial DNA. The respiratory chain itself produces reactive oxygen species which may oxidise DNA residues (Turrens, 1997). Genes for the essential subunits of complexes I, III, IV and V are all harboured within the mitochondria where they are therefore at an increased risk of potential mutation compared with DNA housed within the nucleus. LPS has indeed been reported to promote DNA damage (Suliman et al., 2003). ROS produced in the mitochondria may also act directly on the protein complexes and thereby increase ROS formation by impairing electron transport (Zeevalk et al., 2005).

2.4.2 Alternative reasons for increased oxygen concentrations

Hyperoxia may also be induced in LPS-injected tissue by mechanisms not involving mitochondria by, for example, increased vasodilation. NO is a potent vasodilator and so its increased production would be expected to increase blood flow to the site of the lesion. Vasodilation is a basic mechanism of inflammation and the blood brings inflammatory cells, nutrients etc. to the site of inflammation. Changes in blood flow have been implicated in MS patients. Indeed, an increase in perfusion has been reported early in lesion development and it includes an increase in both blood volume and cerebral blood flow (Wuerfel et al., 2004, Wuerfel et al., 2007). In contrast, in *established* lesions of both relapsing-remitting, secondary progressive, and primary progressive patients the perfusion is reported to be decreased compared with controls (Adhya et al., 2006, Ge et al., 2005, Inglese et al., 2007a, Inglese et al., 2007b). Such reductions may well simply reflect the relative loss of tissue in chronically lesioned

brain, in which case the reduced flow may nonetheless be entirely sufficient for the brain tissue surviving. Alternatively, it is possible that the reduction in perfusion is a compensatory response to hyperoxia.

Another relevant factor which may affect the oxygen concentration of the LPS-injected spinal cord is pH. Inflamed tissue typically has a low pH, and although this effect was not confirmed in the current experiments, a low pH would increase the dissociation of oxygen from haemoglobin by Bohr Effect (Riggs, 1960). Angiogenesis may also have an effect on the oxygen concentration by increasing the density of blood vessels. Indeed, abnormal vasculature (Singh and Zamboni, 2009) and increased expression of many angiogenesis stimulating factors, leptin, VEGF, tumour necrosis factor (TNF) –α/β, IFN-γ, NO, lactate and endothelin-1 (Cooke and Losordo, 2002, Erwin, 1995, Hunt et al., 2007, Lingen, 2001, Salani et al., 2000), have been found in lesions of MS patients (Bo et al., 1994, De Rosa et al., 2006 , Haufschild et al., 2001, Matarese et al., 2007, Miller et al., 1991, Nath et al., 2004, Noseworthy et al., 2000, Simmons et al., 1982, Simone et al., 1996, Su et al., 2006, Proescholdt et al., 2002). Since we have examined the LPS-DC lesion within only 24 h after injection of the pro-inflammatory agent, increased vasculature may have less of an effect on the oxygen concentration found as angiogenesis typically requires a longer time period.

It may be wondered why a raised oxygen concentration would not reduce vascular supply, but in fact blood flow to the CNS is not regulated by the oxygen concentration itself, but rather by other, poorly-understood, mechanisms, which may include glucose (Tesfamariam et al., 1991), neurotransmitters (Peppiatt et al., 2006) and free radicals (Gupte and Wolin, 2008). Interestingly, the effect on the vasculature from ROS is likely to be important in the LPS-DC lesion since mitochondrial dysfunction will increase ROS production (Cacciuttolo et al., 1993, Zeevalk et al., 2005).

It is interesting to consider that if hyperoxia occurs in MS, it may be deleterious by, e.g. promoting oxidative damage. If so, therapies that dampen angiogenesis would be expected to be beneficial. In this respect it is notable that IFNβ, a common therapy in MS, has anti-angiogenic properties both *in vitro* and *in vivo* (Fidler, 2000, Lindner and Borden, 1997). Corticosteroids are also used successfully in some relapsing-

remitting MS patients and these can inhibit formation of new blood vessels in tumors (Folkman et al., 1983). Again, minocycline hydrochloride has been used successfully to ameliorate symptoms in two EAE studies, and it is a potent inhibitor of angiogenesis in vitro and in tumors (Brundula et al., 2002, Gilbertson-Beadling et al., 1995, Natalija Popovic, 2002, Tamargo et al., 1991, Weingart et al., 1995).

2.4.3 Historic therapy of MS

The research described in this chapter was aimed at distinguishing which of two hypotheses about a cause of energy insufficiency was correct, mitochondrial poisoning, or hypoxia. Although the findings have clearly supported the possibility that mitochondria are damaged, initially it seemed more likely that the tissue was hypoxic, partly based on an old treatment for MS patients used in the nineteen fifties (Horton et al., 1944, Jonez, 1952). The treatment was the intravenous administration of the vasodilating drug histamine, which was thought to act via increasing blood supply and oxygen to the brain, just as it did to the skin – which provided the nickname for the therapy as "relief by flush". It was noticeable that histamine sometimes improved symptoms, promptly but transiently, but the therapy was later abandoned since it did not change the course of the disease.

2.4.4 Consequences of hyperoxia

The consequences of hyperoxia on the brain are barely explored, perhaps because the condition is believed to be uncommon, but some studies have examined oxygen toxicity in relation to hyperbaric pressure and the effects on marine divers, or in relation to ischemia-reperfusion injury. Oxygen toxicity in divers manifests itself by convulsions, and less often, bradycardia, dyspnea or hyperventilation (Dean et al., 2003). Reperfusion injury causes microvacuolisation of the cytosol, and progressive neuronal damage (Sato et al., 1990).

From studies on hyperoxia we can conclude that oxygen exerts most of its detrimental effects through oxidative damage mediated via the production of ROS (Bitterman, 2004, Turrens et al., 1982b). Another effect of the hyperoxia is increased carbon dioxide (CO_2) concentrations in the blood stream according to the Haldane effect,

resulting from a decreased CO_2-carrying capacity of haemoglobin in the presence of high oxygen concentrations (reviewed by Siggaard-Andersen and Garby, 1973). Increased CO_2 concentration within the blood will add to the inflammation-mediated effect on blood pH, making it lower, as discussed above. Interestingly, both CO_2 and low pH may further increase the production of ROS (Rehncrona et al., 1989). Finally, increased CO_2 concentration will increase the vasodilation of the vessels, exacerbating the already high oxygen concentration.

It is indeed likely that an increased CO_2 concentration will be present in the tissue, because a mitochondrial deficiency is suspected, and this would bias cells towards anaerobic metabolism to replace the loss of ATP. Interestingly, for each pyruvate molecule catabolised anaerobically (alcohol fermentation), two ATP and one CO_2 molecule are formed, whereas, in contrast, aerobic metabolism of the same substrate would result in 15 ATP molecules but only two CO_2 molecules. Thus for an equal amount of ATP to be produced anaerobically vs. aerobically, there would be an increase in CO_2 production. It therefore seems likely that the LPS lesion may have a high CO_2 concentration, and this would be expected to result in dilation of the vasculature and a high blood flow. It is possible that such a high blood flow may contribute to the unusually high oxygen concentration detected during the oxygen challenge with 100% oxygen in the LPS-treated animals.

ROS, and/or their toxic nitrogen-containing metabolites (RNS), act on DNA, lipids and proteins by stealing electrons so as to fill their outer electron layer. By taking an electron, ROS and RNS may modify the structure and function of the electron donor. The first insult may start a chain-reaction, spreading the damage to neighbouring molecules. It is only when the molecule which holds the unpaired valence electron meets another molecule in the same state that the chain-reaction is stopped. Cells have different systems in place for protection against ROS and RNS, including the presence of high concentrations of antioxidants. Interestingly, if the current findings in a model of PPP lesions are indeed representative of the condition in early MS lesions, it seems that the condition of hyperoxia may be imposed on tissue which is unusually ill-equipped to withstand it. Thus, in MS patients, the concentrations of the endogenous antioxidants uric acid, SOD and GSH are reported to be abnormally low

(Calabrese et al., 1994, Rentzos et al., 2006, Ronquist and Frithz, 1979, Toncev et al., 2002, Zagorski et al., 1991).

A particularly damaging form of ROS-mediated damage is damage to DNA (Cadet et al., 1999). When DNA is oxidised it not only disturbs the transcription of genes, it also activates a DNA-repairing machinery consisting of more than 150 proteins (Martin et al., 2008). One molecule overseeing the repair-process is poly ADP ribose polymerase (PARP). The importance of PARP is illustrated by the fact that functional mutations in the gene for PARP results in several types of cancer (Spry et al., 2007). PARP can efficiently repair single strand breaks and exchange damaged residues for functional ones. However, if DNA damage exceeds a threshold value, PARP activation is detrimental because during the repair process it utilises large quantities of both ATP and NAD which may result in a lethal energy deficiency within the cell (Berger et al., 1986, Pieper et al., 1999, Szabo et al., 1996).

2.4.5 Antioxidant therapy

As noted in the Introduction there is substantial evidence for oxidative damage in MS lesions, and so it is interesting to consider the possible beneficial effect of antioxidants as a therapy for MS patients (reviewed by Carlson and Rose, 2006). Although antioxidants are typically considered as preventing oxidative damage to DNA and proteins etc., it is interesting to wonder whether they may also affect function. The ameliorating effect of intravenous histamine on symptoms (see above) was attributed to vasodilation, but since those early studies there have been several papers reporting that histamine has many effects, including, for example, inhibition of the ROS-producing NOX enzyme complex. Furthermore histamine can induce the production of inosine, a precursor of the major antioxidant uric acid (Betten et al., 2003, Hansson et al., 1996, Hellstrand and Hermodsson, 1986, Mellqvist et al., 2000, Obata et al., 2001). Thus it is possible that the beneficial effects of histamine were due to the suppression of oxidative damage, rather than increased oxygen delivery. During the period when histamine therapy was in vogue, additional vasodilatory substances were also tried for their potential ameliorating properties, and some were effective, including amyl nitrite, papaverine hydrochloride and nicotinic acid (Brickner, 1952). However, these agents also have antioxidant properties (Gromovaya et al., 2002, Nicolescu et al., 2004, Pedanova et al., 1983) so it would again be unreliable to conclude that the symptoms of MS were improved by increasing oxygen delivery.

2.4.6 Hyperoxic insult

The second main finding of this chapter was the consequence on the tissue oxygen concentration of breathing different oxygen concentrations, 'oxygen challenges'. By making the experimental animals inspire 0-100% oxygen instead of room air (21% oxygen) we were able to alter the local spinal cord oxygen tension dramatically (Figure 14A). LPS- and saline-injected animals increased their oxygen concentration at the lesion site six-fold (of the baseline value) when the animals inspired 100% oxygen compared with 21% oxygen (Figure 15B). Naïve animals barely increased their oxygen concentration two-fold during the same conditions. The finding in LPS- and saline-injected animals may indicate that the tissue has been preconditioned by

the intraspinal injection. Preconditioning through HIF-1α, HO-1 and heat shock proteins protect cells against further metabolic stress (D'Souza et al., 1994, Sharp and Bernaudin, 2004, Zou et al., 1998). Importantly, preconditioning can induce angiogenesis through activation of VEGF (Ren et al., 2008, Sun and Liao, 2004).

The transcription of the stress proteins is mediated through protein misfolding (Zou et al., 1998), perhaps initiated by the multitude of preconditioning activators, e.g. NO, ROS (Bellmann et al., 1995), LPS (Belosjorow et al., 1999, Hiasa et al., 2001), cytokines (Cannella and Raine, 1995, Mycko et al., 2003) and interestingly perhaps also by both ischemia (Racay et al., 2009) and hyperbaric oxygen (Wang et al., 2010). The cytokines responsible for preconditioning of tissue (Cannella and Raine, 1995, Mycko et al., 2003) and stress proteins are found in increased concentrations in both EAE and MS lesions (Schipper, 2004, Emerson and LeVine, 2000). It is likely that the intraspinal injection causes a stress response of the tissue which may contribute to increased vasodilation and hence hyperoxia.

It is interesting to consider whether the exaggerated hyperoxia in the LPS-DC lesion seen in response to 100% oxygen may arise from a mitochondrial deficiency. If there is an inhibition of the mitochondrial electron chain, for example by NO, and the mitochondria are normally inhibited to 50% of their maximal capacity, as discussed above, it would help to explain the difference between the minimal increase in naïve animals compared with LPS-injected animals. Thus the un-affected mitochondria in naïve animals will just increase their oxygen usage and hence the increase in tissue oxygen concentration will be less evident. In LPS-injected animals, mitochondria would be unable to utilise the increased oxygen, in which case the oxygen concentration would increase without compromise.

It is noteworthy that the exaggerated hyperoxia seen in LPS-injected animals was also apparent in the saline control animals. The mechanism is not clear. Perhaps the saline injection causes inflammation that we have been unable to detect (see immunohistochemical studies in Chapter 3), or, more likely, it is the consequence of the needle insertion, or pressure from the injected volume, that triggers the mechanisms responsible for preconditioning.

The oxygen concentration values measured in the oxygen insult experiments are high, with a maximum value of 300 mmHg in one experiment, whereas spinal cord tissue from naïve animals measured around 30 mmHg oxygen (Figure 11). Such high values for tissue oxygen concentration are not, however, unprecedented, and two papers have reported values of between 240-400 mmHg oxygen upon inspiration of 100% oxygen in the dog eye and cortex of the rat brain (Ivanov et al., 1999, Stefansson et al., 1989). Nonetheless, it seems wise to consider the possibility that the values measured by the OxyMicro can be inaccurate under exceptional circumstances, for example, it may be influenced by the presence of ROS. Thus it is possible that the very high oxygen concentrations measured during the oxygen challenge is partly due to an increased production of ROS in the tissue (Cacciuttolo et al., 1993, Turrens et al., 1982a, Turrens et al., 1982b). Indeed, when tested, the value reported by the OxyMicro probe immersed in Ringer's solution increased when hydrogen peroxide was added to the Ringer's, despite the fact that the solution was already equilibrated to 100% oxygen (Figure 10). The interpretation of this finding is likely complicated, but on face value it appears that the OxyMicro must detect species in addition to oxygen, arguably ROS such as peroxide or superoxide. If so, the values obtained *in vivo*, where ROS will be present, may not faithfully reflect the oxygen concentration alone.

2.4.7 Blood pressure

In the ET-1-induced ischemia model used as a validation of the OxyMicro probe reading, it is clear that the blood pressure was oscillating with variation up to 30% of its maximal value (Figure 8). This observation might raise concern regarding the validity of observations in this animal, but in fact the oscillation likely reflects a feed back loop and it has been shown to vary between 0.4 Hz in rat to 0.1 Hz in human (Brown et al., 1994, Malliani et al., 1991). Although this is the only example shown, the phenomenon was commonly observed in our experimental rats and it is believed to be due to the action of the sympathetic nervous system on the vasculature. There is no reason to believe the preparation was not healthy and physiological.

2.4.8 Oxygen sensitive OxyMicro probes versus microelectrodes

Although experiments initially were planned to be conducted with an oxygen-sensitive microelectrode there are several advantages with the fiberoptic OxyMicro probe for our *in vivo* measurements. First, in contrast to oxygen-sensitive microelectrodes, the probe does not consume oxygen during use. The probe may also equilibrate in a solution where the diffusion of oxygen is sporadic, whereas a microelectrode relies on a steady diffusion of oxygen from the medium to the reduced surface. Optimally, a microelectrode should be calibrated in the same medium as where the oxygen measurement is to take place since values may drift when moved between the calibration chamber and experiment site, but the OxyMicro probe does not have this limitation. The OxyMicro probe is also not sensitive to electrical interference, and it is re-usable. Re-calibration of the probe revealed minimal baseline drift during an experiment and a reliable readings can be assured for experiments lasting for several hours.

2.4.9 Conclusions

The injection of LPS into the dorsal column results in a hyperoxic state, both within the lesion site and in surrounding peri-lesional tissue. The increased oxygen concentration is likely caused by an accumulation of several factors, including: an inability of the mitochondria to utilise the oxygen present, perhaps due to NO-mediated mitochondrial inhibition; vasodilation by NO or through the Haldane effect; an increased release of oxygen from haemoglobin caused by low pH at the site of inflammation (Bohr effect); and a pre-conditioning of the tissue by the intraspinal injection.

3. POSSIBLE ENERGY FAILURE IN THE LPS-DC LESION

3.1 INTRODUCTION

In the previous chapter the oxygen concentration in the LPS-DC lesion was found to be significantly higher than in the control groups, indicating an inability of mitochondria to respire. Indeed, the pathology of the PPP lesion, that the LPS-DC lesion models, has been suggested to arise from an energy deficiency (Lassmann, 2003). Strengthening a concept that mitochondrial function is impaired in MS are descriptions of reduced mitochondrial gene transcripts in the motor cortex (Dutta et al., 2006), (Dutta et al., 2006), a decrease in mitochondrial complex IV expression in active lesions (Mahad et al., 2008) and oxidation of mitochondrial genes in active lesions (Lu et al., 2000). Moreover, the pathology of the model LPS-DC lesion has been suggested to be caused by an energy-deficiency, perhaps through reactive oxygen and nitrogen species (including NO) (Lassmann, 2008). Additionally, reported fragmentation and DNA condensation in the LPS-DC lesion implicates apoptosis (Marik et al., 2007) thereby strengthening the indication that the energy homeostasis of the tissue is affected.

In this chapter the aim is to explore whether the increase in oxygen concentration within the LPS-DC lesion is due to deficits in the respiratory chain. An additional aim is to examine markers of oxidative and nitrative stress, as well as to detect injury of the tissue, using histological techniques.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

The inflammatory lesion induced by the intraspinal injection of LPS into the dorsal columns was examined at different stages of lesion progression to determine the activity of mitochondrial complex II (succinate dehydrogenase (SDH)) and IV (cytochrome c oxidase (COX)). Adjacent sections were fixed and examined (immuno-) histochemically for markers for inflammation (ED1; activated macrophages and microglia), nitric oxide production (iNOS and nitrotyrosine), reactive oxygen species (oxidized DNA (8-oxo-dG), lipids (4-hydroxynonal (4-HNE)) and superoxide (dihydroethidium (DHE))) and neuronal injury (amyloid precursor protein (APP), caspase 9, NeuN and resin sections). Mitochondrial activity and immunohistochemical labelling were examined at 1, 3, 5, and 10 days post-injection with additional immunohistochemistry at 1, 9 and 14 days after intraspinal injections.

3.2.2 Induction of the model Pattern III lesion

The LPS-DC lesion was induced as described before. Briefly, SD rats were injected with 200 ng LPS from *Salmonella enterica* serotype typhimurium or abortus equi at vertebrae level Th12-13. Control animals received injections of saline alone.

3.2.3 Histochemistry

3.2.3.1 Perfusion and tissue preparation

Animals were anaesthetised with isoflurane delivered via a nose cone throughout the procedure. An incision was made to expose the peritoneal cavity and the intestines were clamped to bias the later flow of fixative to the structures of interest. The chest was opened to expose the heart, the inferior vena cava was severed, and a needle inserted into the left ventricle of the heart. A rinse solution (2000 U/l heparin, 0.025% lidocaine, 0.002% NaNO2 and 0.02M N-2-hydroxyetthylpiperazine-NO-2-ethanesulphonic acid (HEPES; pH 7.4)) was perfused through the vasculature until the blood emerging from the severed vena cava was clear. The perfusate was then

changed to paraformaldehyde (4% in a 0.15 M phosphate buffer) or glutaraldehyde (4% in 0.15 M phosphate buffer) depending on the protocol. In the case of tissue destined for the assay of mitochondrial activity, no fixative was used, only rinse. The spinal cord was dissected free and if destined for the assay of mitochondrial activity the whole spinal cord was frozen in isopentane and stored at -80^{o}C. If destined for immunohistochemistry, the cord was placed in paraformaldehyde, and if for resin sections, the cord was placed in glutaraldehyde and stored at 4^{o}C until prepared for processing and embedding.

In the cases where India ink was injected intraspinally as a verification of the injection site, the spinal cord was either dissected free immediately, or 24 hours later. The location of the ink was observed in 0.5 mm segments taken through the injection site, following dehydration and 'clearing' in methyl salicylate.

3.2.3.2 Resin sections

The relevant tissue destined for resin sectioning was cut into 0.5 mm thick sections and a small incision was made on the lateral, non-injected side of the cord for orientation. The blocks were post-fixed in osmium tetroxide (1.5% OsO_4 in 0.15M phosphate buffer, pH 7.4) for 2 hrs. The samples were then dehydrated in a series of increasing ethanol concentrations (30%, 50%, 70% and 90%, 15 min each), with 3x100% ethanol (20 min each) followed by 2x100% propylene oxide (30 min each) and infiltrated with resin (1:3, 1:1 and 3:1 resin:propylene oxide, 3 hrs or overnight each), then 100% resin (overnight) and polymerised in fresh resin at 60°C for 48 hrs. The blocks were sectioned (0.7 μm) on a Reichert Ultracut S ultramicrotome (Leica, Milton Keynes, UK) and the sections stained with 0.1% thionin acetate (50% ethanol, basic) and 1% aqueous basic acridine orange, and mounted in DPX (BDH, Poole, UK).

3.2.3.3 Immunohistochemistry

The spinal cords intended for immunohistochemistry were left in paraformaldehyde for one hour, transferred to a phosphate buffered saline (PBS) solution containing 30% sucrose for 24 hrs at 4^{o}C before the tissue of interest was cut into 5 mm blocks that were frozen in optimal cutting temperature (OCT) compound (Bright,

Huntingdon, UK). Transverse 12 μm thick sections were cut at -20°C (cryopstat, CM 1950, Leica, Milton Keynes, UK). The slides, each with two sections, were left at room temperature for a minimum of two hrs and then stored at -20°C for later usage.

To reveal antibody binding the slides were once again dried at room temperature (RT) for a minimum of two hrs after removal from the freezer. The area around the sections was marked with a block pen to minimise volume of reagents. Sections were washed with PBS containing 0.2% Triton-X (PBST), 3x 5 min and pre-treated using the following solutions for antigen retrieval and inhibition of endogenous peroxidase activity:

ED1, iNOS and nNOS: 0.2% sodium azide and 10% peroxide in PBST, 20 min RT and 70% cold methanol for 15 min at RT.
NeuN, nitrotyrosine and 4-hydroxynonal (4-HNE): citrate buffer, 8 mM, heat buffer until boiling and heat the sections for 1 min in the microwave, let cool for 5 min and then repeat heating twice.
Caspase 9: EDTA, 1mM, pH 8.0, heat solution until boiling and heat the sections for 1 min in the microwave, let cool for 5 min and then repeat heating twice.
8-oxo-dG : RNase buffer, 100 μg/ml, 150 mM NaCl and 15 mM sodium citrate for 15 min at 37°C, 2 N HCl for 5 min RT, 1 M Tris base for 5 min RT.
Amyloid precurson protein (APP): 0.3% peroxide in PBST for 20 min RT, 100% cold methanol for 20 min RT, 1 mg/ml $NaBH_4$ in PBST for max 5 min.

After the pre-treatment, slides were blocked (20 min RT) with either normal goat serum or normal horse serum depending upon the antibody used (2% in PBST). Sections were incubated with the first antibody overnight at 4°C. A summary of the relevant antibodies and their dilutions are shown in Table 1. The following day, biotin-coupled second

Antibody	Source	Dilution	Company	Cat. No	Lot/Batch
ED1	Mouse	1:200	Serotec	MCA341R	060509
iNOS	Rabbit	1:200	BD Bioscience	610333	54151
nNOS	Rabbit	1:300	Abcam	ab1376	N/A
eNOS	Rabbit	1:300	Santa Cruz	Sc-653	L036
Nitrotyrosine	Rabbit	1:50	Millipore	MAB5409	LV1434073
HIF-1α	Rabbit	1:500	Millipore	07628	DAM1655269
NeuN	Mouse	1:500	Millipore	MAB377	LV1573084
APP	Mouse	1:1000	Millipore	MAB248	LV1504177
Casp 9	Rabbit	1:100	Cell signalling	D353	3/05/2009
8-oxo-dG	Mouse	1:50	Trevigen	4354MC050	17674A9
4-HNE	Mouse	1:50	Abcam	ABA48506	607569
IgG	Rat	1:100	Vector	BA-4001	F0105

Table 1. Properties of the antibodies used.

antibodies (Goat anti Rabbit (BA1000) or horse anti mouse (BA2001) depending upon the species in which the first antibody was produced, Vector labs, Peterborough, UK) was applied to the slides for 60 min at RT (1:200 dilution). The Vectastain® ABC Elite kit (30 min RT, Vector labs, Peterborough, UK) was used to enhance the signal. Between steps the sections were washed three times with PBST. Labelling was developed using 3,3-diaminobenzidine (2-5 minutes, DAB, Vector labs, Peterborough, UK). The reaction was stopped with cold tap water.

Disruption of the BBB was investigated using a rat-specific antibody to immunoglobulin (IgG) coupled to biotin. The sections were washed in PBS, the antibody applied overnight at 4^{o}C and, after three consecutive washes with PBS, the antibody was visualised with DAB.

General histology was examined using a combination of luxol fast blue (LFB), periodic acid-Schiff (PAS) and haematoxylin staining as follows: PBS washed sections were brought successively to 100% ethanol by bathing the slides on a rocker (70, 90 and 100% ethanol, 5 minutes each). Slides were incubated overnight at 50^{o}C in LFB solution (0.1% in 95% ethanol and 0.05% glacial acetic acid). The following day sections were washed with running tap water. Excess LFB staining was removed from non-myelinated tissue by cycling the slides through saturated lithium carbonate (30 seconds), washing in running tap water, ethanol (70% for 30 seconds), washing again and repeating until satisfactory staining remained. Next, slides were oxidized with periodic acid (1% for 20 min, RT), washed again and incubated with neat Schiff's reagent (20 minutes, RT). After the subsequent wash, slides were dipped into haematoxylin (3 min), washed again, and differentiated until clear in acidic alcohol (1% HCl in 10% ethanol). After a final wash, sections were processed together with the immunohistochemical sections as described below.

The presence of iron deposits was studied using Perl's stain. Sections were washed twice in PBST and incubated in the dark with equal amounts of FeCN (4% in dH_2O) and HCl (4% in dH_2O) for 30 min at room temperature. After three washes in PBST, sections were incubated with DAB in the dark for 30 min at room temperature. After washing in water, sections were dipped in haematoxylin for three min, cleared in

acidic acid (1% HCl in 10% ethanol) and processed with the other sections for dehydration and mounting.

All slides were finally dehydrated in increasing concentrations of ethanol (70, 90,100%), cleared in xylene (VWR, Lutterworth, UK), mounted in DPX and left to dry overnight. Sections in which the primary antibody had been omitted served as negative controls. Sections were viewed with a light microscope (Zeiss Axiophot, Germany) and pictures taken with a digital camera (D300, Nikon, Japan).

3.2.4 Assay for the presence of superoxide

Dihydroethidium (DHE, Molecular Probes, Paisley, UK) was employed as a fluorescent label to detect the presence of superoxide within lesions *in vivo* (more details attached in the appendix). Intravascular (i.v.) or intraperitoneal (i.p.) injections of 10 µl (100 mg/ml in DMSO) of DHE were performed on anaesthetised animals, 20 min to two hrs before perfusion for fixation (4% paraformaldehyde). The tissue was cryoprotected in sucrose (30% in PBS), embedded in OCT and frozen sections were cut at 20-25 µm. Sections air-dried prior to two consecutive washes in PBS to remove OCT and mounted with aqueous mounting medium (Vector laboratories, Peterborough, UK). Special care was taken not to expose the slides to light during cutting and processing. Photographs were obtained with an Axio confocal microscope (Zeiss, Welwyn Garden City, UK). Oxidized DHE was detected using 535-nm excitation and 610-nm emission.

3.2.5 Assay for Mitochondrial Activity

The mitochondrial activities for complex II (succinate dehydrogenase (SDH)) and complex IV (cytochrome c oxidase (COX)) were investigated. A 10 mm block of spinal cord, centred on the site of the lesion, was removed from the frozen spinal cord and cryoprotected overnight at 4^{o}C (576 mM Na_2HPO4, 224 mM NaH_2PO4, 5% glycerol). The segment was quickly washed in cold PBS, embedded in OCT and cut into sections 20-25 µm in thickness. Sections were air dried for 60 min and then placed in -80^{o}C or used immediately. If stored in the freezer, sections were once again air dried for 60 min and then incubated for 30 min at 37^{o}C in the reaction buffer.

The complex IV reaction buffer consisted of 100 μM cytochrome c, 4 mM diaminobenzidine tetrahydrochloride and 20 μg/ml catalase in 0.1 M phosphate buffer at pH 7.0. Complex II activity was detected using a buffer consisting of 130 mM sodium succinate, 200 mM phenazine methosulphate, 1mM sodium azide and 1.5mM nitroblue tetrazolium in 0.1 M phosphate buffer, pH 7.0. Sections were subsequently washed twice in PBS, fixed in 4% paraformaldehyde for 10 min.

Adjacent sections were labelled with antibodies for inflammation (ED1), presence of mitochondria (porin), presence of COX (subunit 1 of COX (COX1)) and neurons (NeuN). Pre-treatment consisted of permeabilisation by bathing the sections for 10 min each in the following succession of solutions; 0.2% Triton x-100 containing Tris-buffered saline (TBS), 70% MeOH, 95% MeOH,, 100% MeOH, and then through the reverse sequence. The first antibody (ED1 (1:200); porin (1:2000); COX1 (1:2000) and NeuN(1:500)) was diluted in TBS and sections incubated for 90 min at room temperature. Instead of a biotin-labelled secondary antibody, the Menapath X-Cell Plus HRP Polymer detection system, without a blocking step, was used according to manufacturer's instructions (A. Menarini Diagnostics, Wokingham, UK). DAB or the Vector® SG (Vector laboratories, Peterborough, UK) in PBS was used as the substrate for the peroxidase enzyme.

All sections were finally dehydrated in increasing concentrations of ethanol, cleared in Histoclear (Sigma) and mounted in DPX. Bright field images of the two chromogens (DAB-brown and Vector® SG-blue) were obtained using the Nuance system (CRi, Woburn, MA).

3.3 RESULTS

3.3.1 Mitochondrial activity in the LPS-DC lesion

Mitochondrial function within the spinal tissue was assessed by examining the enzymatic activity of mitochondrial complexes II and IV in transverse sections from saline control, or LPS-injected, experimental animals.

The initial plan was to examine mitochondrial function within the white matter that became demyelinated, but in practice the density of labelling in the white matter was so low that it was difficult to discern any reduction in labelling due to the lesion (Figure 16). However, in more than half of the cases, it was noticed that the grey matter adjacent to the injection site had a clear decrease in the activity of both complex II and complex IV. The decrease was apparent at day 2, more pronounced at day 3 (Figure 17), and it persisted throughout the time points investigated, up to ten days after lesion induction (Figure 17). However, the interpretation of the reduction in activity was complicated by the parallel observation that the affected region of grey matter also displayed a loss of both porin and COX1 labelling, hence indicating a loss of both mitochondria and the complex IV enzyme respectively (Figure 16). Additionally, the neuronal marker NeuN was missing in the area of lost activity (Figure 17). Together, the observations that porin, COX1 and NeuN labelling are all reduced, indicates that neurons and perhaps other cell types at the lesion site are missing, presumably due to necrosis or apoptosis. Interestingly, it was noticeable that the temporal sequence of the suppression of mitochondrial activity correlated with the presence of ED1 labelling (activated macrophages and microglia; Figure 16-Figure 17).

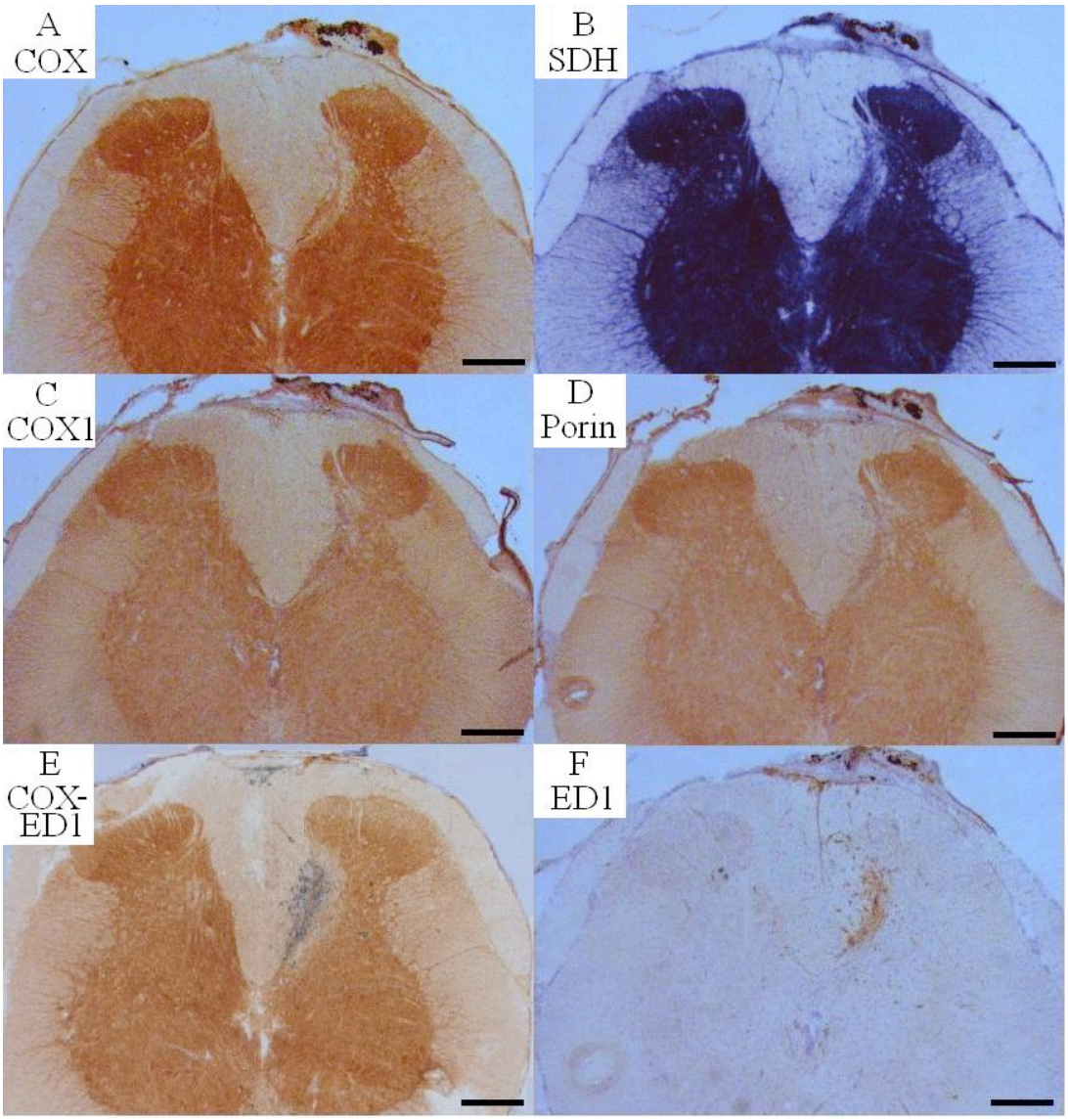

Figure 16. Transverse neighbouring sections of spinal cord from an experimental animal, 10 days following intraspinal injection with a low dose of LPS, treated to reveal the distribution of the markers indicated. (A) Labelling for the activity of complex IV reveals a reduction in such activity within the dorsal horn grey matter adjacent to the injection site. (B) Loss of activity of complex II was identical that of complex IV. (C and D) Labelling of COX1 (subunit one of complex IV) and porin indicated minor loss of tissue in the grey matter of the right (injected) dorsal horn. (E and F) The expression of ED1 was located at the site of decreased mitochondrial activity but not at the site of tissue loss (as seen by COX1 and porin). Scale bars represent 0.5 mm.

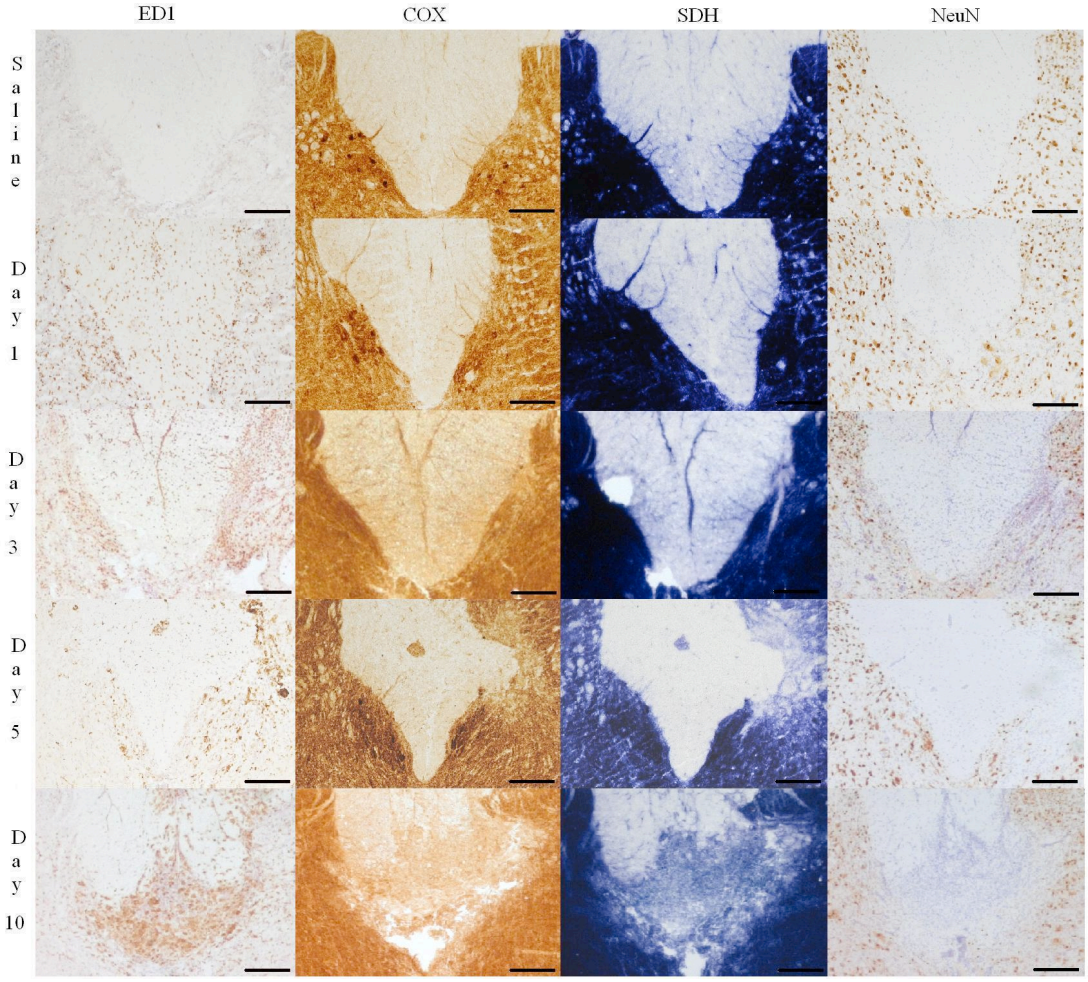

Figure 17. Transverse neighbouring spinal cord sections from five experimental animals that received intraspinal injections of either saline (top row, 3 days post-injection) or LPS (lower rows) at the different time points indicated post-injection. In the LPS-injected tissue alone, activated macrophages and microglia ($ED1^+$, first column) appeared dispersed within the tissue, but over time their distribution became more focused towards the bottom of the dorsal columns, namely the site of the developing demyelinating lesion. The mitochondrial activity of COX and SDH (second and third columns) was present in control tissue and at early time points, but over time (day 3 onwards) the activity was lost from the grey matter adjacent to the injection site. There was a similar gradual loss of the neuronal marker NeuN which occurred over the same time scale as the loss of activity, suggesting that the decrease in activity may at least partially due to tissue loss. Scale bars represent 0.25 mm.

3.3.2 Immunohistochemical examination of the LPS-DC lesion

To verify that the lesion in the dorsal columns induced by the injection of LPS from *Salmonella enterica* serotype abortus equi was exactly similar to the model described by Felts and colleagues (2005), the lesioned tissue was examined immunohistochemically in frozen sections, and at high resolution in resin sections. Histological markers for activated macrophages and microglia (ED1), iNOS, HIF-1α, neurons (NeuN), apoptosis (caspase 9), neuronal injury (amyloid precursor protein; APP), and general histology (luxol fast blue (LFB) and periodic acid-Schiff (PAS) staining) were studied at the level of the lesion. A typical section stained with LFB/PAS is illustrated in Figure 18, and a box marks the location at the junction of the dorsal columns and dorsal horn at which the illustrations in Figure 19 and Figure 21 were taken.

At one day post-LPS injection, inflammation (diffuse ED1 labelling marking activated macrophages and microglia) was spread throughout the spinal cord at the level of the lesion (Figure 19a): saline-injected controls showed only minor labelling of cells on the surface of the spinal cords near the injection side but did have some disruption to the BBB (presence of IgG), most likely caused by the needle insertion. LPS-injected tissue displayed more BBB leakage at the same time point. HIF-1α was found in the grey matter on the injected site of the cord. Minor loss of the neuronal marker NeuN was detected in the LPS-injected tissue, and neuronal damage was indicated by APP labelling. Furthermore, labelling for caspase 9 was evident in the white and grey matter around the injection site, indicating apoptosis. No myelin loss was detected in the white matter by LFB but the integrity of the grey matter seemed to be affected as shown by PAS staining. Resin sections of LPS-injected tissue resembled controls.

Nine days post-LPS injection, labelling for ED1 revealed that the activated macrophages/microglia as well as HIF-1α had aggregated more tightly at the injection site (Figure 19 b). BBB leakage was minimal. Demyelination at the ventral portion of the dorsal columns was evident by LFB. The expression of caspase 9 and APP was decreased overall, but more focused at the injection site. Labelling for NeuN had diminished, such that a region devoid of labelling was present in the grey matter adjacent to the dorsal column on the injected side. Some demyelinated axons were identifiable in the resin sections.

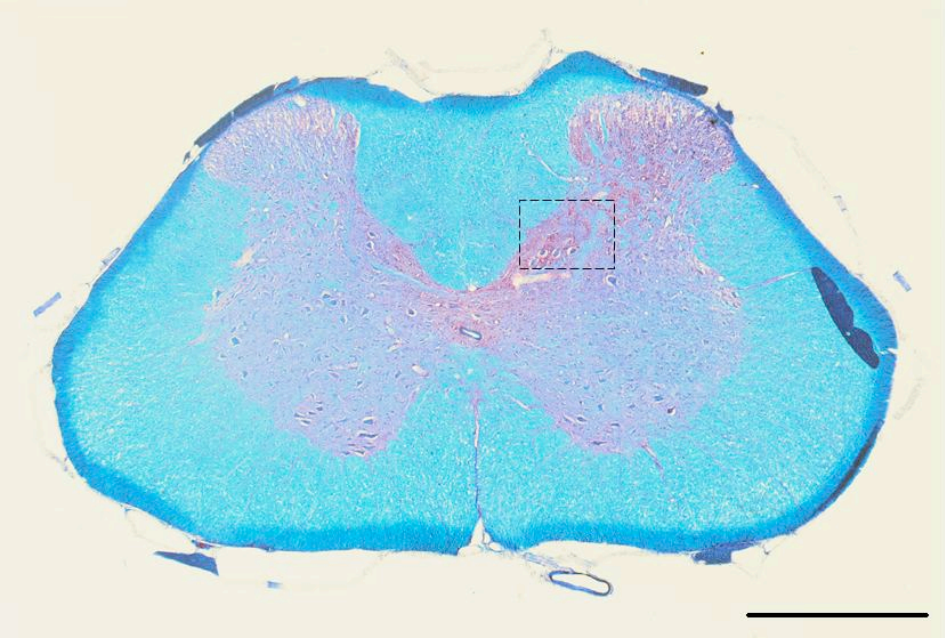

Figure 18. Low power photomicrograph of a transverse section through the spinal cord of a control (saline-injected) rat illustrating the site chosen for the high power immunohistochemical photographs shown in Figure 19 and Figure 21.

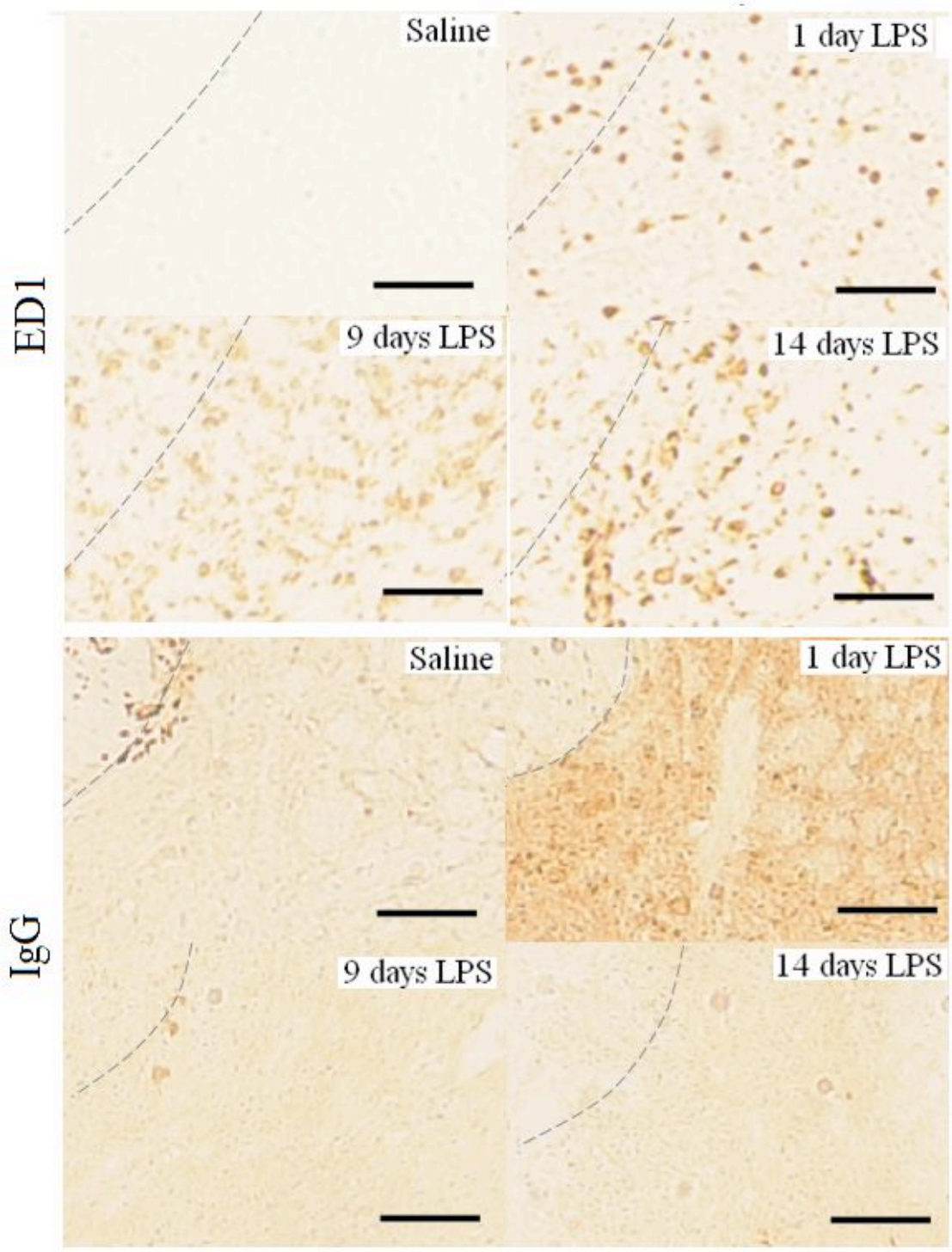

Figure 19 a. Inflammation (ED1) was found throughout the lesion and the BBB was disrupted (IgG) both in the saline and LPS-injected animals at one day after injection. Scale bars represent 0.1 mm.

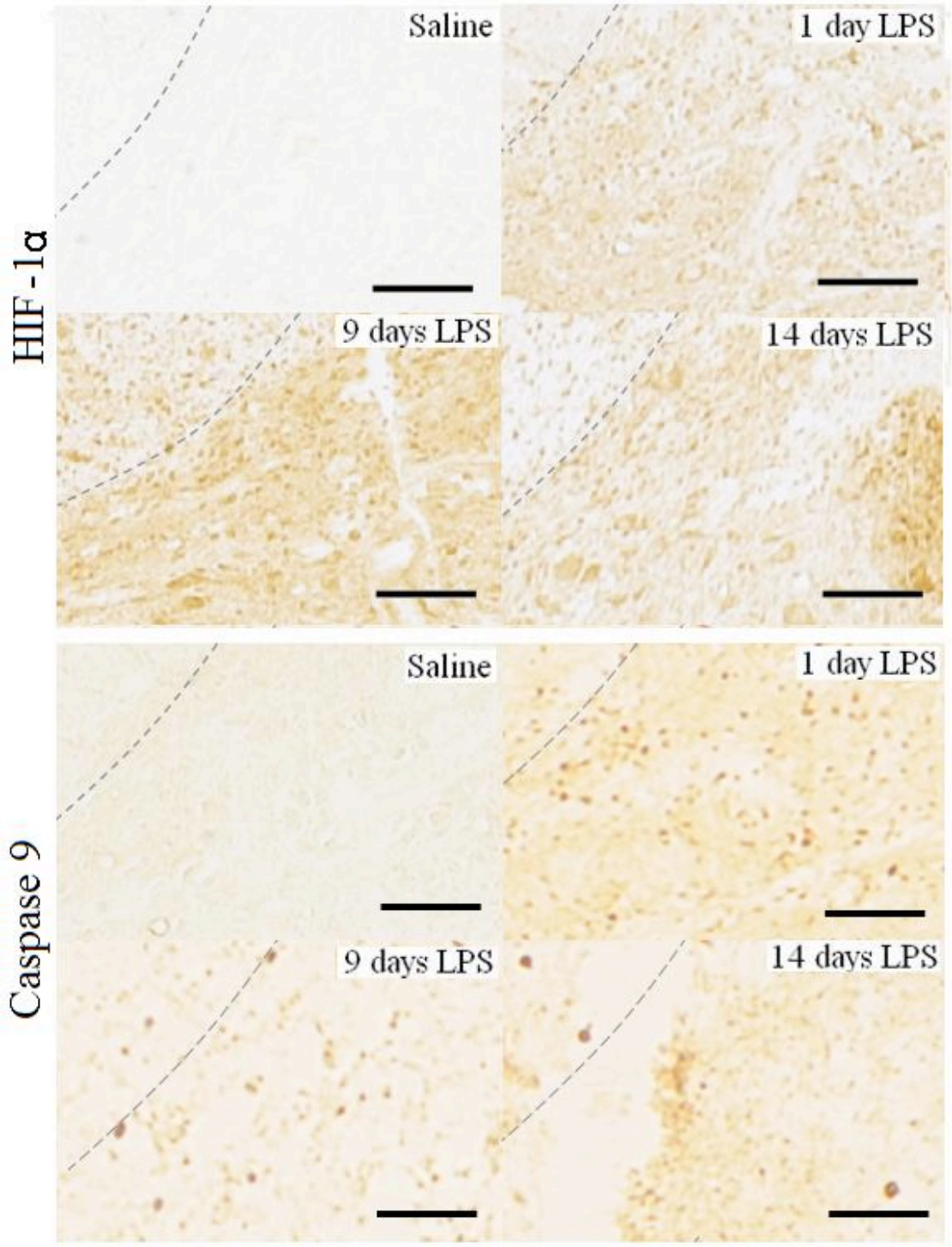

Fig 19b. HIF-1α was found predominantly in the grey matter or at the site of demyelination. Tissue injury was indicated by the presence of caspase 9. Scale bars represent 0.1 mm.

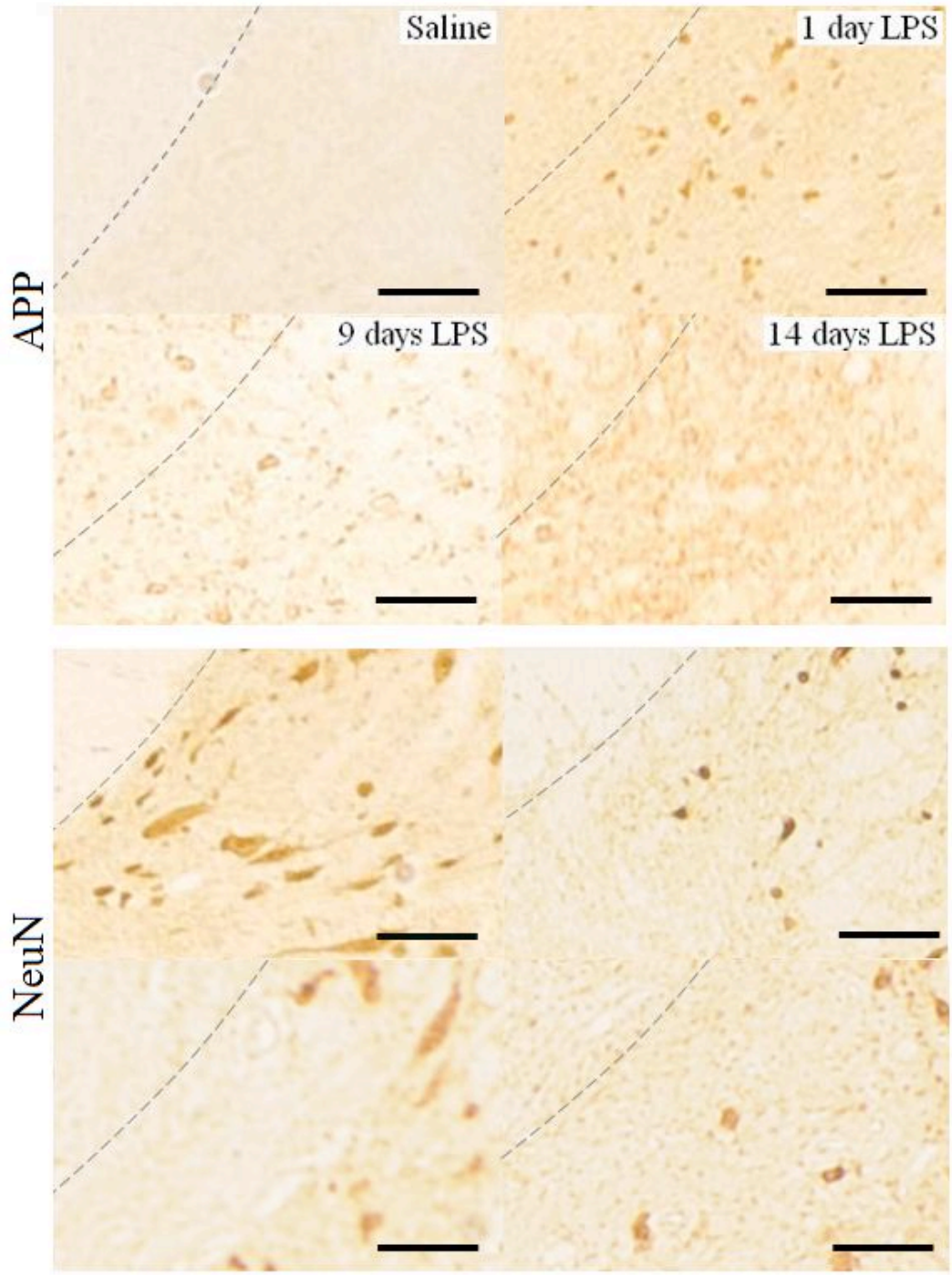

Figure 19c. Tissue injury was further indicated by APP labelling, as well as an absence of the neuronal marker NeuN. Scale bars represent 0.1 mm.

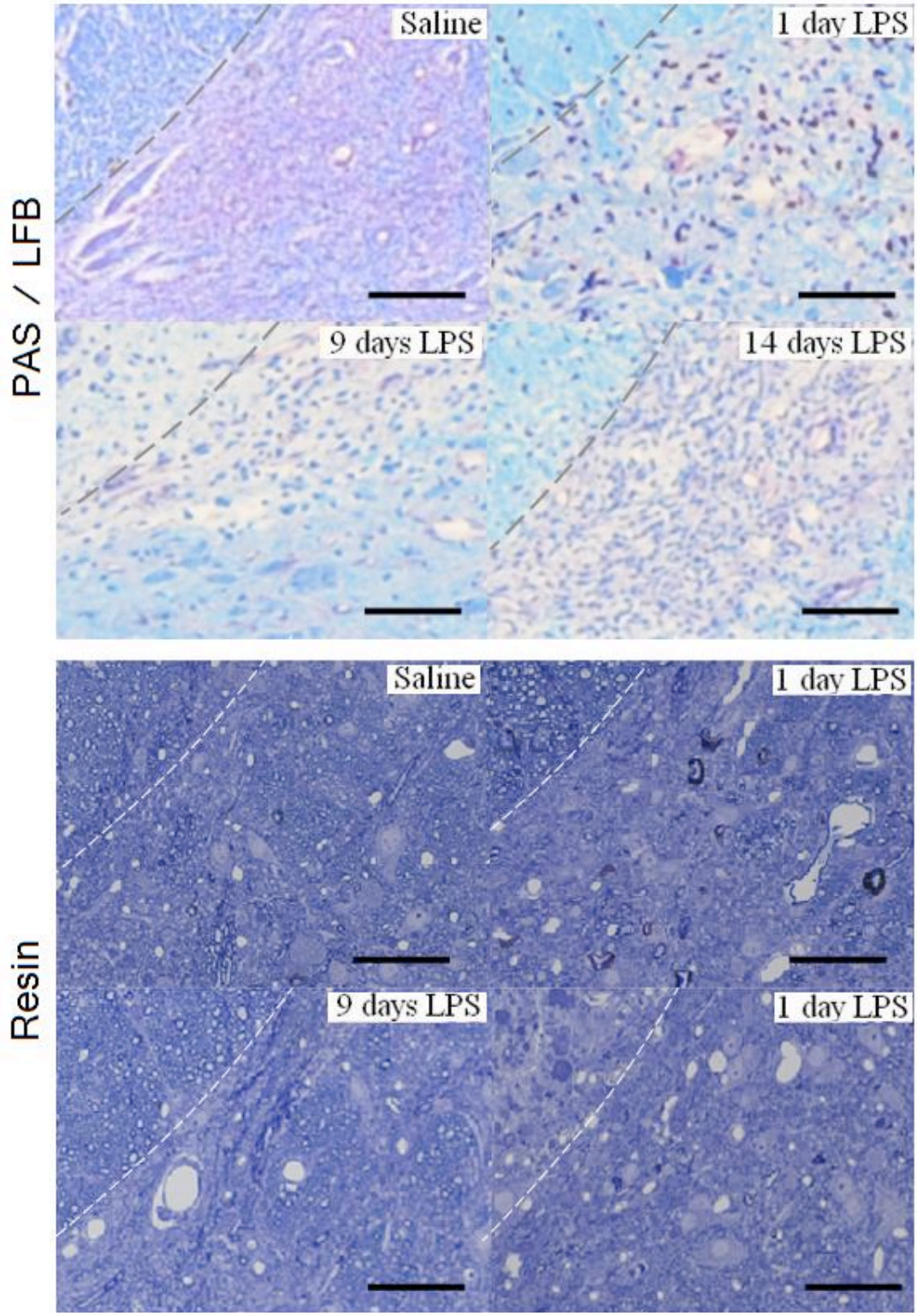

Figure 19d. Demyelination (LFB/PAS and resin sections) was seen at 9 and 14 days after induction of the lesion. Scale bars represent 0.1 mm.

At day 14, activated macrophages (ED1 positive cells) were localised to the injected side and bottom of the dorsal columns (Figure 19a). No IgG-labelling was found. HIF-1α expression was decreased compared with day nine but was still evident in the grey matter and the area of the demyelination. Myelin loss was less apparent at this time point, suggesting that repair of demyelinated axons by remyelination may have been established. However, the integrity of the grey matter adjacent to the injection site was more severely affected. Resin sections revealed the continued presence of some demyelinated axons, and a possible increase in the diameter of some vessels. The neuronal label NeuN revealed a decreased density of labelled cells compared with the appearance at nine days, and neuronal damage was marked by APP and caspase-9 labelling, localised to the inflamed region.

3.3.3 A low dose LPS-DC lesion

The LPS lesion induced within the dorsal columns displays a considerable amount neuro/axonal damage (Figure 19c & d). To study the effect of inflammation on the energy homeostasis of the CNS cells, we considered that a less severe model would be advantageous and an alternative LPS was examined. LPS from another E-coli strain; typhimurium, has a lower endotoxin level compared with serotype abortus equi (0.6 endotoxin units (eu)/mg cf. 3.0 eu/mg) and this strain was used at the same concentration as in previous experiments (100 ng/µl). Tissue collected one day after saline or LPS (tryphimurium) injection was examined by immunohistochemical techniques for markers of inflammation and neuro/axonal damage. The LPS-injected tissue displayed activated macrophages and microglia (ED1) in a disperse pattern which was mimicked by iNOS in an even stronger labelling indicating NO production in the tissue (Figure 20a). The saline-injected control animals displayed none of the markers except for some BBB leakage (IgG). APP and caspase 9 labelling was only found in minute amounts which implies that the neuro/axonal injury is decreased compared to high dose (LPS from abortus equi, Figure 19c). No apparent neuronal loss as detected by the neuronal marker NeuN was found. The staining for PAS and LFB indicated no demyelination at the one day time point examined but indicated some affect on the integrity of the grey matter adjacent to the injection site. The low dose was used for both the mitochondrial activity assay (Figure 16 and Figure 17) and in the in vivo oxygen measurements (Figure 11).

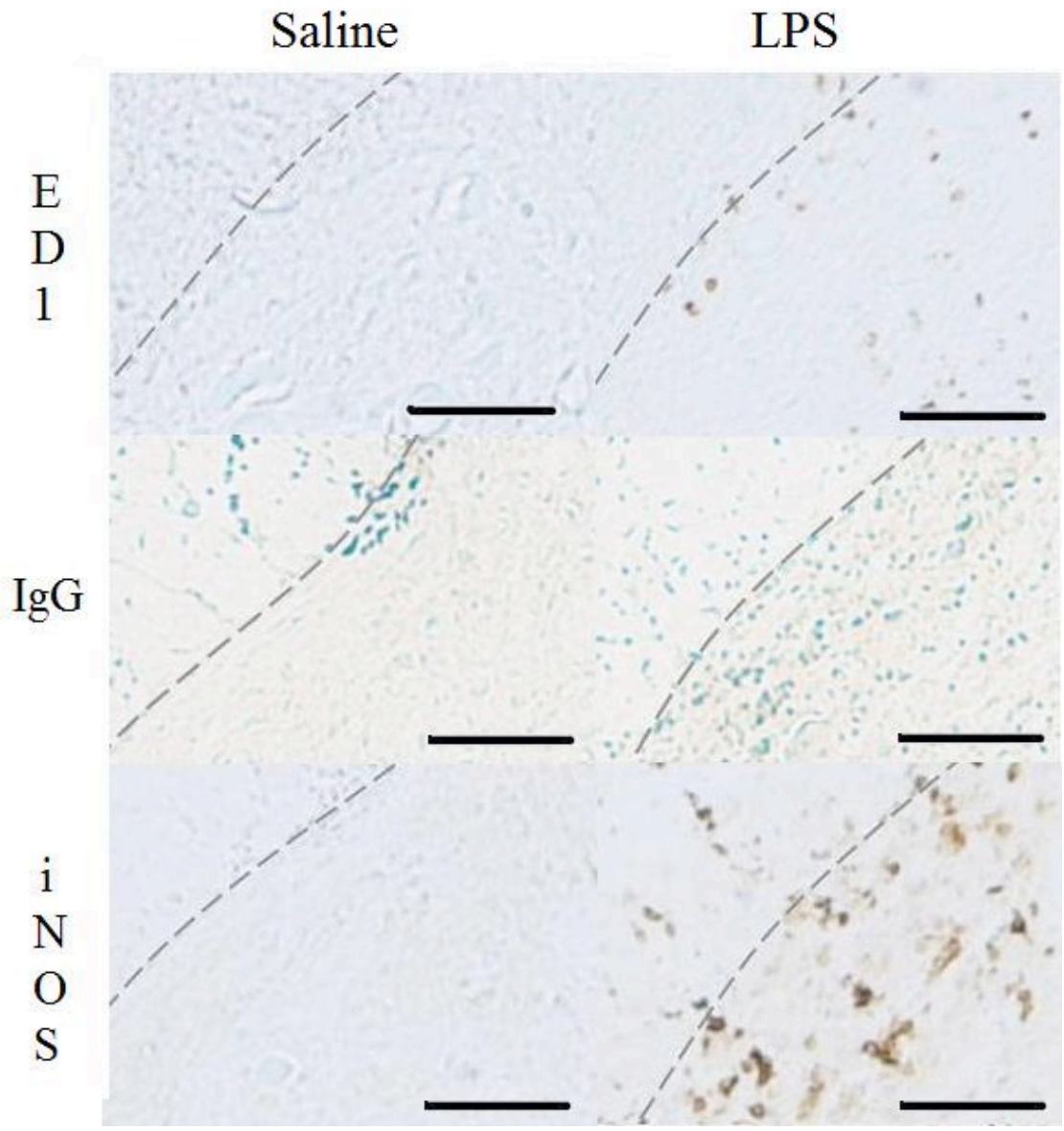

Figure 20a. A montage of photomicrographs displaying the bottom of the dorsal column on the injected side (saline or low dose LPS) in tissue collected at one day after intraspinal injections. The dashed lines mark the margin between the dorsal columns and dorsal horn grey matter. Sparse inflammation (ED1), with disruption to the BBB (IgG) and NO-producing components (iNOS) was found predominantly in the grey matter of LPS-injected experimental animals. Some IgG labelling was found in the saline-injected control animals. Scale bars represent 0.1 mm.

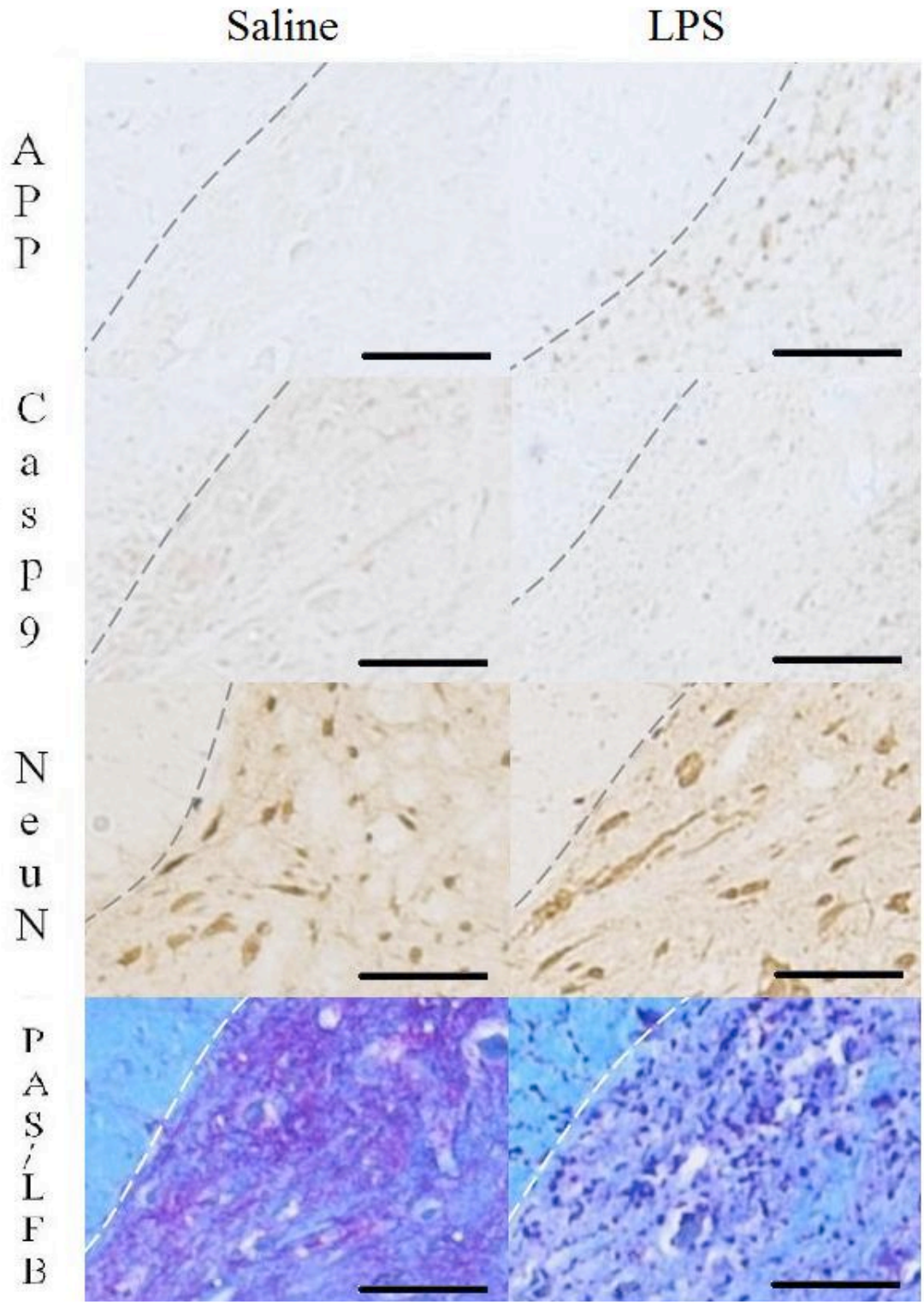

Fig 20b. Minor APP and caspase 9 labelling indicated that cells were not dying in the same extent as in high-dose LPS-injected tissue (Figure 19c). LFB/PAS staining and NeuN labelling in the LPS-injected rats was similar to that in saline-treated animals except for presence of inflammatory cells. Scale bars represent 0.1 mm.

3.3.4 Reactive oxygen and nitrogen species (ROS/RNS)

As shown above (Figure 19 and Figure 20), LPS induces inflammation and neuronal damage. However, the mechanisms underlying the tissue damage remain unclear. To explore the potential role of ROS and RNS, the tissue was examined immunohistochemically for the presence of appropriate markers. Thus adjacent sections to those previously studied (Figure 19, high dose LPS) were examined for the production of NO (e.g. iNOS and nitrotyrosine), oxidative damage (8-oxo-dG and 4-HNE) and for iron deposits. Furthermore, the LPS lesions were studied for the *in vivo* production of superoxide by i.v. or i.p. administration of the fluorescent molecule dihydroethidium (DHE).

3.3.4.1 Immunohistochemical markers of ROS and RNS

At day one after the injection of LPS, punctuate iNOS labelling was found, mainly in the grey matter adjacent to the injection site (Figure 21). Labelling for nitrotyrosine along the grey/white margin of the injected side indicated the evanescent presence of peroxynitrite, formed by the combination of NO with superoxide: nitrotyrosine is therefore also indicative of the presence of superoxide in the tissue. Additional oxidative damage to the DNA (8-oxo-dG) and lipids (4-HNE) was found, predominantly in the white and grey matter around the injection site but also on the whole right lateral side of the cord, when compared with the left side. Iron staining was found sporadically on the injected side of the dorsal columns. It seemed to preferentially stain the margin between the white and grey matter but not always at the site chosen for representation (Figure 18).

At day nine iNOS labelling was almost absent (Figure 20). Nitrotyrosine was still evident and localised to the bottom of the dorsal column. Residues of oxidized deoxyguanosine, as revealed by 8-oxo-dG labelling, together with 4-HNE and iron, was less pronounced, but found in the same area as the inflammatory marker ED1 (Figure 19a). Two weeks after the injection of LPS, no iNOS labelling was detectable, but weaker labelling of nitrotyrosine was still found (Figure 21). Oxidative damage, as seen by 8-oxo- dG was detected in smaller amounts compared to the earlier time points. 4-HNE labelling was consistent with day nine. Iron deposits were still found in small quantities.

Figure 21

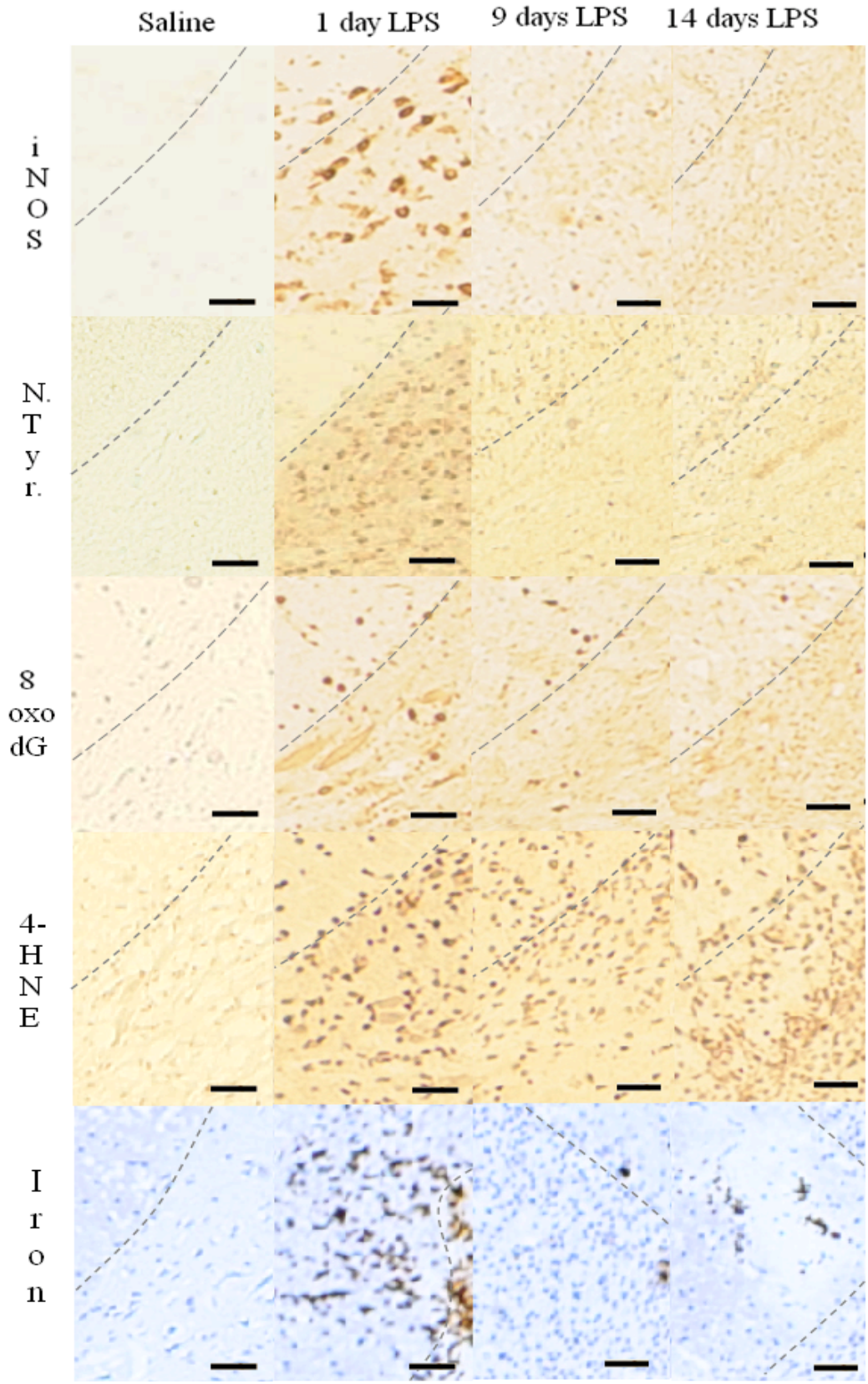

Figure 21. As in Figure 19 and Figure 20, the margin between the dorsal column and the dorsal horn grey matter is indicated by a dashed line. NO production was indicated by the presence of iNOS and nitrotyrosine (N.Tyr.). Oxidative damage to DNA (8-oxo-dG) and lipids (4-HNE) was found, primarily at one day after LPS injection. Iron deposits were sporadically found along the edge of the white to grey matter. Scale bar represents 0.1 mm.

3.3.4.2 Superoxide production in the LPS-DC lesion

In the high-dose LPS injections, DHE was injected i.p. and although there was no labelling at one day post-injection, there was clear labelling at 9 and 14 days after the injection of LPS (Figure 22), including in sections adjacent to those studied by immunohistochemistry (Figure 21). Given that the labelling observed with i.p. injections was quite low, and that the labelling in low-dose LPS lesions was expected to be lower than with the high dose, we explored a different method of administration of the DHE, *viz*. by intravenous administration instead of intraperitoneal. The alternative route of delivery resulted in a higher background signal, mostly from cell bodies, indicating a continuous endogenous production of superoxide, likely taking place in the mitochondria (Figure 23). However, in the LPS-injected animals, the signal was visibly enhanced at the injection site, most notably in the grey matter adjacent to the dorsal column, when compared with saline-injected animals. The larger magnification photograph in the figure was taken in collaboration with Prof. Michael Duchen, Ph.D., and was used to illustrate the un-reacted DHE (blue, excitation 355 nm / emission 420 nm) although the validity of this method was not confirmed.

At all time points examined, increased labelling of DHE was located at the margin between the white and grey matter, hence correlating with the aggregation of inflammatory cells (Figure 22).

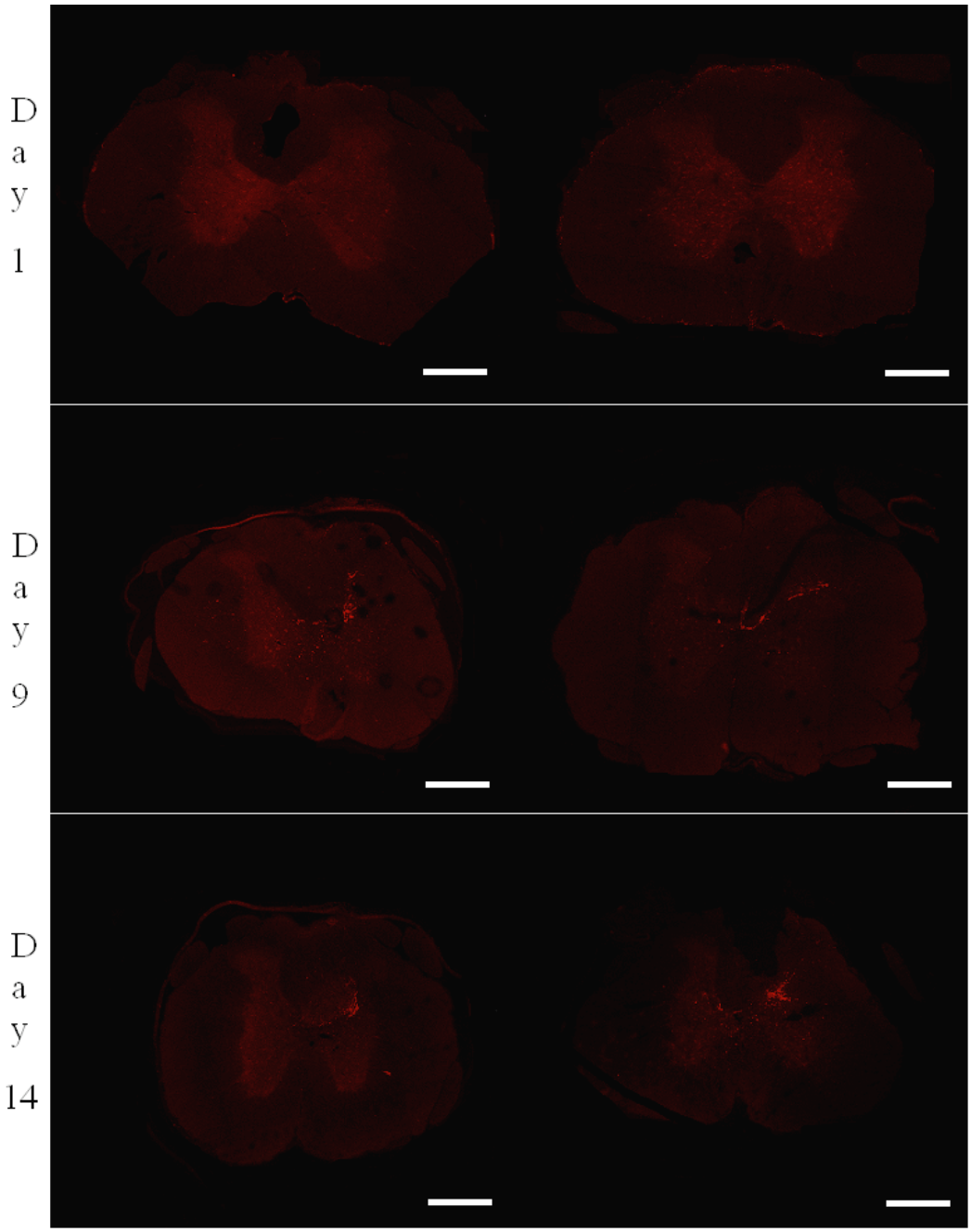

Figure 22. A montage of photographs of transverse spinal cord sections (taken by a confocal microscopy), displaying the presence of superoxide at different time points following LPS-injection. The panels illustrate two examples from 1, 9 and 14 days post-injection respectively. DHE labelling was visibly increased at day 9 and 14 compared to day 1 and seemed to coincide with the inflammation as seen in Figure 19. Scale bars represent 0.5 mm.

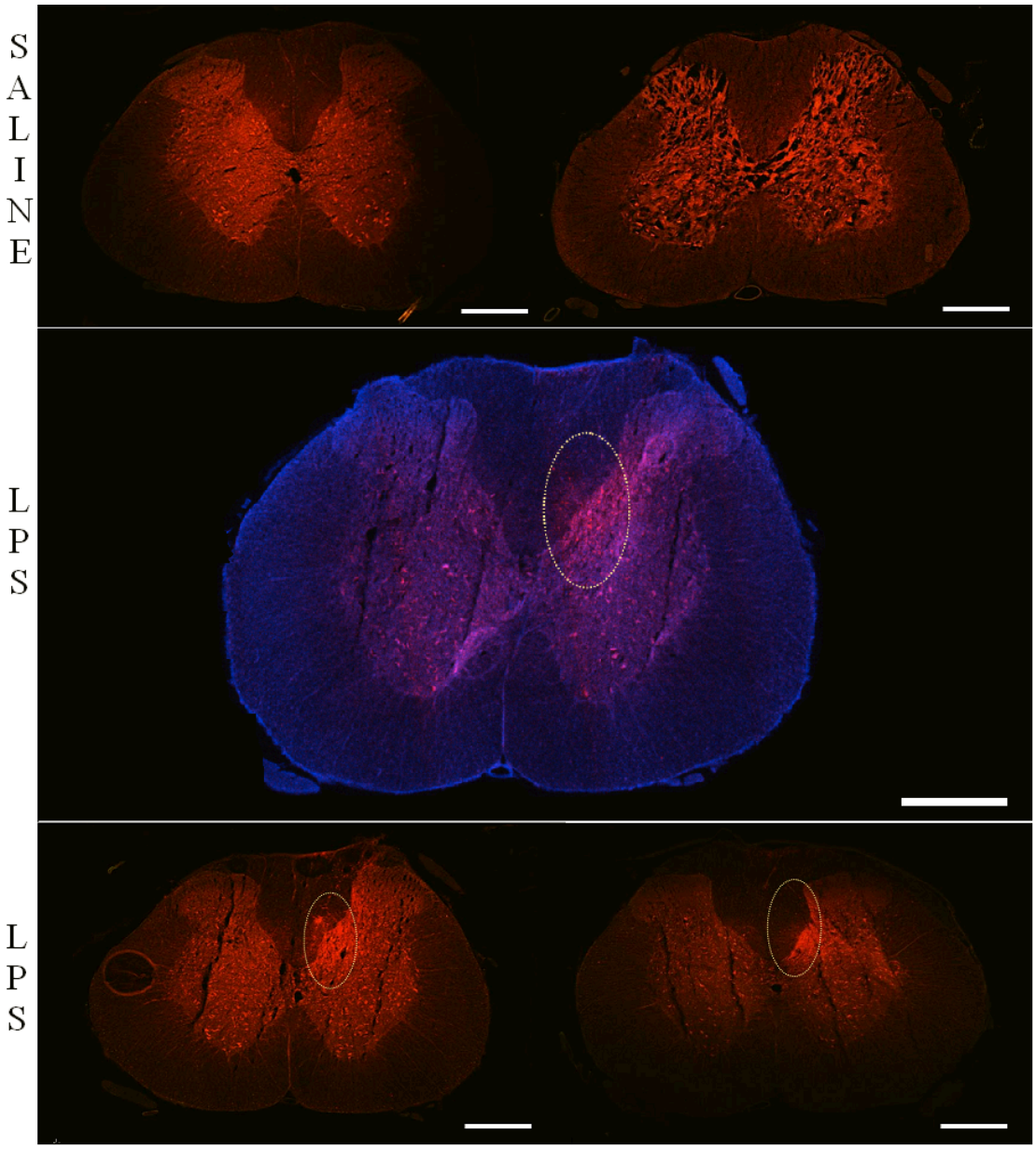

Figure 23. Superoxide production in tissue taken 24 hrs after low-dose LPS-injections. The top panel illustrates two saline injected animals, followed by three examples of LPS-injected experimental animals. The higher magnification photograph in the middle shows oxidised DHE in red and un-reacted DHE in blue. Scale bars represent 0.5 mm.

3.4 DISCUSSION

In this chapter the activity of mitochondrial complexes II and IV was investigated in the LPS-DC lesions. Initially, the aim was to measure a possible reduction in axonal/oligodendroglial activity in the belief that demyelination may be caused by an energy deficiency within the axons or oligodendrocytes. However, the mitochondrial activity of axonal and oligodendroglial cells could not easily be examined because the sensitivity of the method was insufficient to distinguish the low activity of the mitochondrial enzymes in the normal appearing white matter. The large difference in grey to white matter activity may reflect the fact that the metabolic rate of the grey matter is three to four time higher than in the white matter (Buxton, 2002, Hasegawa and Ravens, 1968).

However, we have in this chapter described how LPS-injections into the dorsal column of experimental animals resulted in a decreased activity of mitochondrial enzymes SDH and COX in the dorsal horn grey matter (Figure 16 and Figure 17), reducing the oxygen utilisation of the tissue. This finding is consistent with an interpretation that the hypoxia-like pathology described in the LPS-DC lesion is caused by mitochondrial deficiency. The high oxygen tension reported in chapter two is hence most likely caused by a mismatch between oxygen availability and cellular demand.

The results show that the lowered activity of complex II and IV was at least partially due to the loss of tissue, because not only was there a loss of subunit one of COX (COX1), but also loss of a mitochondrial marker (porin, Figure 16) and the neuronal marker NeuN (Figure 17). However, the area of decreased activity of complexes II and IV extended beyond the region of clear tissue loss, indicating that LPS did not only lead to cell death but also had an inhibitory effect on the mitochondrial enzymes. Indeed, LPS is commonly used in models of sepsis, where it has been reported to decrease mitochondrial activity and increase the oxygen concentration within affected tissues (Rosser et al., 1995, Trumbeckaite et al., 2001, Crouser et al., 2002, Schenkman et al., 2007, Chuang et al., 2002).

3.4.1 Mitochondrial alternations within the LPS-DC lesion

As described in the previous chapter, the LPS-DC lesion is hyperoxic, probably caused by an inflammatory-mediated deficiency of the mitochondria. Such an inhibition could be caused by several factors (discussed in chapter two). An important note to make is that the assay for enzymatic activity of complex II and IV in the current study was performed *in vitro*, and so many of the effects discussed in chapter two, e.g. NO competition with oxygen at complex IV, will not be applicable here. Thus although NO may have an inhibitory effect on mitochondrial activity *in vivo*, other modification to the enzymes must be responsible for the reduction in mitochondrial activity reported here (Figure 16 and Figure 17). The most likely effects have been mentioned before, e.g. post-translational modification of complexes by oxidation or nitration, or the mutation of DNA.

Mitochondrial deficiency is expected to lead to an insufficiency of ATP, and the dramatic and prompt consequences of such a shortage in the CNS is aptly illustrated by the fact that a person will faint if the brain is deprived of blood for a mere ten seconds (Sife, 1998). For this and other reasons it is safe to believe that CNS neurons are highly susceptible to ATP loss and mitochondrial deficiency. Even though the brain has backup mechanisms for energy deficiency, they are all short-term, and ATP shortage will eventually lead to apoptosis or necrosis of the cells: apart from neurons, oligodendrocytes seem especially sensitive to hypoxia (Petito et al., 1998), which is interesting from the standpoint of demyelinating disease. Deficiency in the aerobic production of ATP will activate the alternative pathways for anaerobic metabolism, resulting in raised concentrations of lactic acid in the tissue. Interestingly, increased lactate levels have been found in EAE (Simmons et al., 1982b) and MS patients (Arnold et al., 1990, Miller et al., 1991, Simone et al., 1996).

3.4.2 Inflammation and degeneration

The LPS-DC lesions have been described as inflammatory and demyelinating lesions displaying partial remyelination (Felts et al., 2005), and these observations were confirmed here (Figure 19). To the existing markers described by Felts, Marik and colleagues (2005, 2007) we have added an examination of the expression of IgG, HIF-

1α, caspase 9, NeuN, nitrotyrosine, 8-oxo-dG, 4-HNE and Perl's stain for iron. The apoptosis marker caspase 9 had a maximal expression at one day after LPS injection, but it was also evident at the lesion site throughout the time course examined (14 days, Figure 19c). We have also shown how the labelling of the neuronal marker NeuN is absent in the grey matter adjacent to the local injection site. The loss in expression of NeuN and the labelling of caspase 9 and amyloid precursor protein (APP) illustrates the 'toxic' consequences of LPS in higher doses. It should however be noted that loss of NeuN does not necessarily indicate neuronal loss, but may be due to metabolic disturbances (Ünal-Çevik et al., 2004).

The cellular damage that has been confirmed by both APP, caspase 9, and partial absence of NeuN labelling and PAS/LFB staining, could be brought on by an energy deficiency but it could alternatively be evidence of collateral damage from the respiratory burst of the inflammatory cells (Markesbery, 1997, Mirshafiey and Mohsenzadegan, 2008). To discern between these two possibilities, lesioned animals could be treated with an inhibitor of the phagocyte's ROS producing enzyme complex NOX2, with for example diphenyleneiodonium (DPI), apocynin or '4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride' (AEBSF) (Sorce and Krause, 2009). The effect of reactive oxygen and nitrogen species could also be investigated by treating experimental animals with either an antioxidant, or the LPS-DC lesions could be tested in genetically modified animals (-/- NOX2) to see if the same pathology will, or not, occur. Interestingly, the myelin stain LFB is also missing in the Pattern III lesions and in white matter ischemia, and appear there to correlate with the loss of MAG (Aboul-Enein et al., 2003). LFB loss is also found within the 'prephagocytotic' lesions (Barnett et al., 2009).

HIF-1α was found in the high-dose LPS-injected animals. This oxygen-sensitive transcription factor has earned the title 'master regulator' of hypoxically-activated transcriptional systems (Firth et al., 1995, Ebert et al., 1995). It is down-regulated under normoxic conditions but upon low oxygen concentrations it binds to the hypoxic response elements (HREs) and thereby induces transcription of glucose transporters, glycolytic enzymes and vascular endothelial growth factor (VEGF), amongst a host of other factors. There are non-hypoxic mediators of HIF-1α stabilization, including hormones such as insulin, growth factors, coagulation factors,

vasoactive peptides, cytokines, ischemia and LPS (Blouin et al., 2004, Fukuda et al., 2002, Hellwig-Burgel et al., 1999, Kietzmann et al., 2003, Stiehl et al., 2002, Racay et al., 2009), but, interestingly, also hyperbaric oxygen (Ren et al., 2008). HIF-1α is, as described before, a preconditioner and can hence control angiogenesis, glycolysis, mitochondrial function, cell growth and survival (Webb et al., 2009). The stabilisation of HIF-1α is, as mentioned before, ROS-dependent (Chandel et al., 1998) and it may be through ROS that most of the above mentioned mediators function.

3.4.3 A low-dose LPS-DC lesion

There is extended neuronal loss as indicated by the reduced NeuN labelling and the presence of APP- and caspase 9-expression. To find the mechanisms responsible for the hypoxia-like pathology we reasoned that a milder model of experimental neuroinflammation would be more suitable. We settled for decreasing the endotoxin level by using LPS from a different serotype; typhimurium instead of abortus equi, lowering the endotoxin activity to one fifth of that used initially. Immunohistochemical examination of the low-dose LPS-DC lesion revealed inflammation as judged by the markers ED1 and iNOS, but the labelling for NeuN remained as strong as in saline injected animals (Figure 20a). Atrophy and neuronal damage seemed to be minimal since APP or the apoptosis marker caspase 9 was found in minute levels compared with the high-dose LPS-DC lesion. The low-dose LPS-DC model was used for both the *in vivo* oxygen concentration measurements in chapter two and for the assay of mitochondrial activity above.

3.4.4 ROS and RNS damage in the LPS-DC lesion

The studies of Felts and Marik (2005, 2007) revealed the presence of demyelination and axonal loss resulting from LPS injection, but the mechanisms remained unclear. In the current study we have illuminated the possible mechanisms by finding evidence for the production of superoxide and the presence of oxidative damage at the site of aggregated inflammatory cells (Figure 21-Figure23). Superoxide is, as described in chapter one, primarily produced either in the mitochondria (Boveris and Chance, 1973), or as part of the respiratory burst of inflammatory cells mediated by the enzyme complex NADPH oxidase or NOX. In the LPS lesion, superoxide is probably

produced by both of these actions; inflammation is profound (Figure 19a) and the mitochondria compromised (which may increase ROS production, Figure 21). In addition to the observed labelling with DHE (Figure 22 and Figure 23), we also detected the immunohistochemical labelling of the NO producing enzyme iNOS, which was strongly expressed 24 hrs after lesion induction (Figure 21). Apart from acting individually, the superoxide and NO can combine to form the strong oxidising and nitrating agent peroxynitrite, which can decompose to products that nitrate the ortho position of the tyrosine residues of proteins (Ischiropoulos et al., 1992). Thus oxidised DNA (8-oxo-dG), lipids (4-HNE) and nitrated proteins (nitrotyrosine) are found at the lesion site (Figure 21). iNOS and NO have also been found in high concentrations within MS lesions and normal appearing white matter (Oleszak et al., 1998, De Groot et al., 1997). As discussed in chapter two, ROS and RNS may cause functional deficits to lipids, DNA and proteins. The effect of DNA-mutations has been mentioned before, but lipid peroxidation and protein nitration are also important for cellular functions (Beckman et al., 1994). Peroxidation of lipids is a common feature of oxidative damage since cell membranes and myelin are full of easily oxidized unsaturated lipids, and they have poor anti-oxidant defences (Catalá, 2009). Lipid peroxidation results in both structural and functional changes with effects on membrane enzyme activity and membrane functionality (Sikka, 2001, Girotti, 1998, Guajardo et al., 1999). Nitration of the protein tyrosine residues by peroxynitrite ($ONOO^-$) may also result in functional and structural changes (Berlett et al., 1996, Frears et al., 1996, Francescutti et al., 1996, Gow et al., 1996).

Iron deposits were found in the high-dose LPS-DC lesion. Iron is normally present only in bound form in protein complexes like ferritin, because free-iron is a good catalyst for the formation of ROS via the Fenton reaction (below)(Zeevalk et al., 2005).

$$H_2O_2 + Fe^{(II)} \rightarrow OH^{\bullet} + OH^- + Fe^{(III)}$$

Iron is present in increased concentrations in the brain of MS patients as seen by T2 hypointensity on MRI scans (Drayer et al., 1987, Bakshi et al., 2002, Mohit et al., 2009, LeVine, 1997). Iron has also been found in models of the disease (Forge et al., 1998, Levine and Chakrabarty, 2004). Interestingly, mice fed on a low iron diet did not develop EAE (Grant et al., 2003), and the neurological deficit of mice with EAE

improved upon administration of the iron-chelator deferiprone (Mitchell et al., 2007). That iron deposits are detrimental is unquestioned, but why it accumulates in MS patients and in the LPS-DC lesion is not known. However, a role for superoxide could be suspected, as it appears to be involved in iron-acquisition in human bronchial epithelial cells (Ghio et al., 2003).

Evidence for breakdown of the BBB was indicated by the expression of IgG at the injection site in both the high and low-does LPS-injections, and in saline-injected control animals. Such breakdown shows that the injection and/or needle insertion is sufficient to cause pathophysiological change to the tissue. The IgG labelling seen in saline-injected control animals is therefore interesting with respect to the raised oxygen concentration upon the oxygen challenge studied in the previous chapter.

Dihydroethidium (DHE) is a fluorescent label for the presence of superoxide. DHE freely diffuses into cells where upon oxidation it is cleaved to the fluorescent ethidium bromide. The oxidized product intercalates into nucleic acids, and probably mitochondrial genes, which greatly enhances its fluorescence intensity. It is supposedly specifically oxidized by superoxide, but peroxidases (Patsoukis et al., 2005) and cytochrome c (Benov et al., 1998) have also been shown to influence the oxidative status of DHE. However, cytochrome c oxidation of DHE only results in minute amounts of the fluorescent ethidium as an end product (Benov et al., 1998), and other oxygen species result in non-fluorescent derivatives of DHE.

3.4.5 Cell death in the LPS-DC lesion

As described in the previous chapter (Figure 6), experiments with India ink as a marker show that there is bulk flow of the injected solution to involved the grey matter of the ventral horn, and the presence of LPS in this site is consistent with the results of this chapter. It may be noted that although LPS was administered at a lower endotoxin concentration for the mitochondrial activity assay, this pro-inflammatory agent seems to be more potent in the grey matter than in the white matter since in the grey it both affects the labelling of cellular markers in the dorsal horn, and promotes the aggregation of ED1+ cells.

Related to both the loss of tissue and the production of ROS is the apoptotic cascade, activated by the mitochondria (Zamzami et al., 1995b), and up-regulated in the LPS-induced lesion (Figure 19b, capase 9 labelling). Apoptosis is, as well known, an energy-driven process of cell death, designed to retain the essential building blocks of the cells for the surrounding tissue to use. Faulty or unnecessary cells may be induced to undergo apoptosis by immune cells or by an array of intrinsic mechanisms, or to remove damaged or infected cells (Lane, 2005). The loss of the mitochondrial membrane potential and the subsequent release of reactive oxygen species are the necessary mediators for the initiation of the cascade leading to apoptosis (Zamzami et al., 1995a). The cascade consists of the activation of a series of caspases that lyse the contents of the cells. The fact that caspase 9 labelling is found in our lesions (Figure 19 b) is indicative of a loss of the mitochondrial membrane potential and a deficiency of the energy producing machinery. However, apoptosis may also be up-regulated by immune cells, hence making the link between caspase 9 labelling and mitochondrial deficiency difficult to judge.

3.4.6 Critical evaluation

The results displayed in this chapter show that the intraspinal injection of LPS impairs the activity of neuronal mitochondria, and the histological findings suggest that the impairment may be caused by ROS and RNS. The results presented are novel and they have not yet been verified by others and thus it is important to highlight some potential artefacts. For instance, the mitochondrial activity was assayed *in vitro,* on histological slides, and so the observations may not reflect the state of the mitochondria *in vivo*. Furthermore, the tissue will only reflect the mitochondrial properties at the precise moment that the rat was perfusion-rinsed. However, the methods employed are believed faithfully to reflect the function of mitochondria in normal tissue. It is recognized that the technique of immunohistology is prone to artefacts and thus the results should be repeatable when employing variations to the adopted method, and they should also be reproducible in other laboratories. Time will establish whether these conditions are met. As a further control, quantification of labelling should be confirmed on a larger cohort of samples where the area of positive labelling can be a better way to present the data instead of representative photomicrographs.

3.4.6 Conclusions

The injection of LPS into the dorsal column results in a deficiency of the mitochondrial complexes II and IV (i.e. the enzymes succinate dehydrogenase and cytochrome c oxidase). The reduced mitochondrial activity is the most likely cause of the hyperoxia reported in Chapter two, and it is also a plausible cause of the hypoxia-like pathology of the demyelinated PPP lesions of MS.

High concentrations of the pro-inflammatory agent LPS injected into the dorsal column induces the production of nitric oxide and superoxide, and neuronal damage in the adjacent grey matter. The possibility that the ROS and RNS are involved in the damage is supported by the observation of oxidative damage to DNA, lipids and proteins. Indeed, the cause of the mitochondrial deficiency may be oxidation or nitration of the complexes by the reactive oxygen and nitrogen species found in the lesion.

4. TRANSIENT DEFICITS IN MOTOR NEURONS

4.1 INTRODUCTION

The model of PPP MS lesions that was studied in the first two chapters of this thesis results in a histologically detectable lesion with a pathology that raised several interesting questions regarding ROS-mediated damage, and the possibility that mitochondrial function was compromised. However, the LPS-DC lesion is not suited to detailed examination of a correlation between mitochondrial activity and electrophysiological function and so we sought to develop an alternative model lesion. In view of the fact that the LPS-DC lesion had incidental, although obvious, effects on the grey matter, we chose to inject the LPS into the grey matter deliberately. This strategy also allowed the development of a model that could illuminate the consequences of inflammation within the grey matter, the relevance of which to MS is increasingly appreciated (Filippi and Rocca, 2009, Vogt et al., 2009). In order to be able serially to monitor the effects of the inflammation on neuronal function we sought to involve motor neurons, because these neurons send axons to the periphery where function can be monitored using standard electrophysiological techniques. To facilitate the electrophysiological studies we decided to target the lesion to the level of the spinal cord containing the motor neurons projecting to the muscles in the hindfoot. Additionally, by focusing on motor neurons, we have been able to use technical improvements in the assay for mitochondrial activity of complex IV to study activity in relation to the presence of mitochondria and protein at a single cell level. Also, this strategy has allowed us to correlate changes in mitochondrial function determined histologically with changes in the excitability of the same population of neurons. The observations have allowed a greater understanding of how inflammation may play a role in the neurological symptoms displayed by patients (Moreau et al., 1996, Youl et al., 1991), and hence an explanation of how "..inflammation alone may be sufficient to cause significant clinical deficits without demyelination" (Bitsch et al., 1999b), including a new potential role for nitric oxide.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

As described in the previous chapter, LPS-injections into the dorsal column results in, also result in some LPS passing into the adjacent grey matter. In this location the endotoxin was found to be far more reactive than in the white matter as assessed by ED1, NeuN, caspase 9, APP-labelling, and in PAS/LFB-stained sections. It was hence reasonable to examine the effect of LPS deliberately injected into the grey. Since the mitochondrial-activity studies in Chapter 3 indicated a loss of activity in neurons, we monitored the function of motor neurons affected by the lesion.

Dark agouti (DA) rats were injected with the pro-inflammatory agent LPS, inducing an inflammatory response involving the ventral horn motor neuron trajecting to the hind feet, which were serially examined by electrophysiology. More precisely, the sciatic nerve was stimulated at the hip notch and recordings taken from the foot dorsum. H reflexes, and F- and M wave responses were collected pre-injection, and at days 1-4, 21 and 28. The mitochondrial activity of complex IV was assessed at 1-3, 5, 7, 14 and 28 days after the LPS injection and correlated with the presence of mitochondria and the complex IV protein. Furthermore, the ventral horn grey matter was studied by immunohistochemistry for markers of inflammation, nitric oxide production, reactive oxygen and nitrogen species and neuronal injury. The general histology was studied in PAS/LFB stained tissue and in resin sections.

Most of the methods utilised in this chapter has been described previously. Only new techniques are introduced below.

4.2.2 Localization of the spinal motor neurons projecting to the foot

The motor neurons are localised in the ventral horn of the spinal cord and project their axons externally to control muscles. A tracer was used to find at which vertebral level motor neurons project to the hind-limb foot muscles. The historically successful

tracer wheat germ agglutinin (WGA) coupled to horseradish peroxidise (HRP) was used (Molander and Grant, 1985, Gonatas et al., 1979).

A dark agouti (DA) rat (193 g) was anaesthetised with isoflurane, injected with 10 µl of the painkiller Vetergesic, and the site of operation shaved and sterilised with iodine. An incision in the skin of the left ankle exposed approximately 8 mm the tibial nerve leading to the foot muscles. A suture was placed at the caudal end of the exposed nerve and the nerve severed distally. A fitted resin tube (approximately 5 mm) was pulled onto the nerve and filled with ca 10 µl of the tracer (40 mg/ml, Vector laboratories, Peterborough, UK), the tube was sealed with Vaseline and the wound sutured. At day two, the animal was anaesthetised again, administered the painkiller and the *right* hind leg was shaved and sterilized. The sciatic nerve was exposed, the surface scraped with a needle and an incised resin tube (approximately 10 mm) fitted around the nerve. WGA-HRP (10 µl) was injected into the tube which was subsequently sealed with Vaseline. The wound was closed and the animal returned to its cage after recovering from the anaesthesia.

At day five the animal was anaesthetised for the final time and perfused with rinse and fixative (4% paraformaldehyde) as previously described. The cord was divided into 5 mm segments as illustrated in Figure 24 for identification of vertebral levels. The tissue was post-fixed for six hrs and then cryprotected (PBS with 30% sucrose) overnight at 4^{o}C. OCT embedded tissue was cut and processed for immunohistochemical detection of WGA as described in the previous chapter. Pre-treatment was 0.2% sodium azide, 30% H_2O_2 in PBST for 20 min. The first antibody (goat anti-WGA (Vector laboratories,

Peterborough, UK) was diluted 1:500 and left on the sections for 2 hrs at 37^{o}C and then overnight at 4^{o}C. The secondary antibody, rabbit anti-goat (1:200, Vector laboratories,
Peterborough, UK) was left on the sections for four hours at room temperature. Labelling was detected as usual with the ABC detection kit and developed with DAB for 5 min.

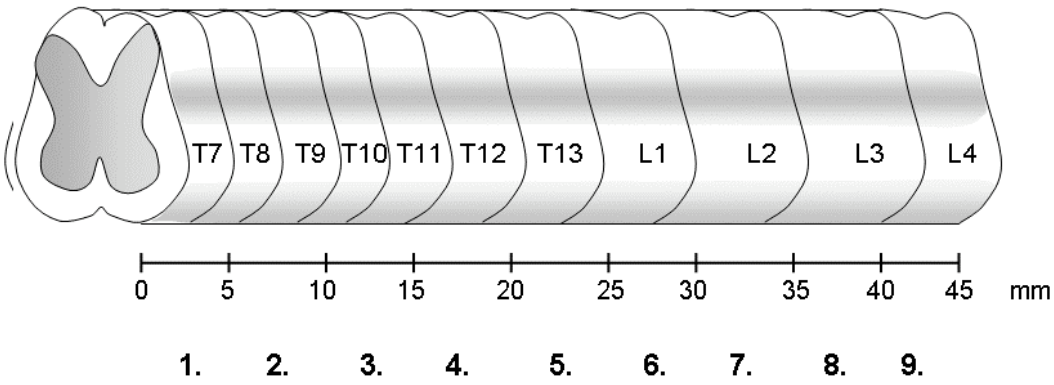

Figure 24. Spinal cord segmentation for detection of the tracer WGA-HRP. The spinal cord was sectioned into 9 segments (5 mm each) and placed in OCT for immunohistochemical processing.

4.2.3 Induction of the ventral horn LPS model

Adult female DA rats (160-180 g) were anaesthetised with xylacare/ketaset (1.1/6.4 mg per 100 gram animal weight, Animal Care Ltd, York, UK / Fort Dodge, Southampton, UK) by i.p. injection (for injections done in conjuncture with EMG recordings) or with isoflurane administered via a nose cone (for histological examination of the tissue). The site of operation was shaved and sterilised with iodine. A quarter laminectomy was performed between vertebrae T13 and L1 or between L1 and L2. A small incision was made in the dura and underlying pia on one or both lateral sides of the central vein. LPS (from *Salmonella enterica* serotype typhimurium, 2x 0.5 µl, 40ng/ml) was injected on the right lateral side of the central vein at 1100 and 1300 µm depths. In most cases (not all EMG experiments), sterile saline was injected on the left side (2x 0.5 µl) and control animals received sterile saline only on the right side (2x 0.5 µl).

In the case where xylacare/ketaset was used as anaesthesia, no Vetergesic was used since xylacare/ketaset also functions as an analgesic. In the animals anaesthetised with isoflurane, 10 µl of Vetergesic was administered intramuscularly.

4.2.4 Mitochondrial activity at single cell level

The assay for determining the COX activity was done as described in the previous chapter. However, the method was altered by subjecting sections, stained for COX activity, to immunohistochemical detection of porin (presence of mitochondria) or COX1 (presence of the protein (complex IV subunit I (COX1)). The binding of DAB (COX activity) blocks the binding of immunohistochemical markers by its bulk. Hence, where the antibody (porin or COX1) binds, there is a loss of activity. Bright field images of the two chromogens (DAB-brown and Vector® SG-blue) were obtained using the Nuance system (CRi, Woburn, MA), which uses liquid crystal tuneable filters to acquire multi-spectral images, and deconvolution methods to separate the original double-labelled image to reveal images of each chromogen (Mansfield et al., 2008). The unmixed images may then be used to quantitate each chromogen, which can be pseudo-coloured and combined into a composite that appears like an immunofluorescently-labelled image (Mahad et al., 2009). This

method enables the determination of COX activity, related to the actual amount of mitochondria or protein at a single cell layer.

4.2.5 Electromyographic techniques

Animals were sedated with xylacare / ketaset (1.1 / 6.4 mg per 100 gram weight, Animalcare Ltd, York, UK; Fort Dodge, Southampton, UK respectively) by i.p. injection. Intramuscular EMG was recorded with needle electrodes by stimulation of the sciatic nerve at the sciatic notch and recording the compound muscle axon potential at the foot dorsum (Figure 25A). Ten records (0.1 Hz), were recorded by a oscilloscope (DPO2024, Tektronix, Bracknell, UK) and raw data collected with Microsof Excel. Graphs and analysis were performed with GraphPad Prism 4.

Stimulation of a motor neuron gives rise to an action potential and a compound muscle action potential (CmAP) is the sum from all nerve fibres that are stimulated. In these experiments, H reflex, M and F waves were recorded. The Hoffmann (H) reflex is a reflex reaction to electrical stimulation of Ia sensory afferents (Figure 25B). The action potential arising in Ia afferents proceeds into the dorsal column and up to the brain, but will also, like a knee-jerk reaction, directly activate the motor neurons which gives rise to the long latency muscle response detected. The H response will hence involve the synapses and may provide information of their function. H reflexes are recorded at low stimulation voltages since higher intensities will give rise to a direct activation of the motor axons, resulting in the orthodromic short latency M wave. F waves are the backwards activation of motor neurons, caused by an antidromic action potential travelling from the stimulation site, and upon reaching the CNS, back-firing some of the motor neurons to give a second long latency compound action potential. Both H reflex and F waves are prone to intra-individual changes at different times, temperatures, needle electrode placements etc., to counteract these effects and minimise the variances, both H reflex and F wave values were plotted in relation to the M wave response which is much more consistent and reproducible.

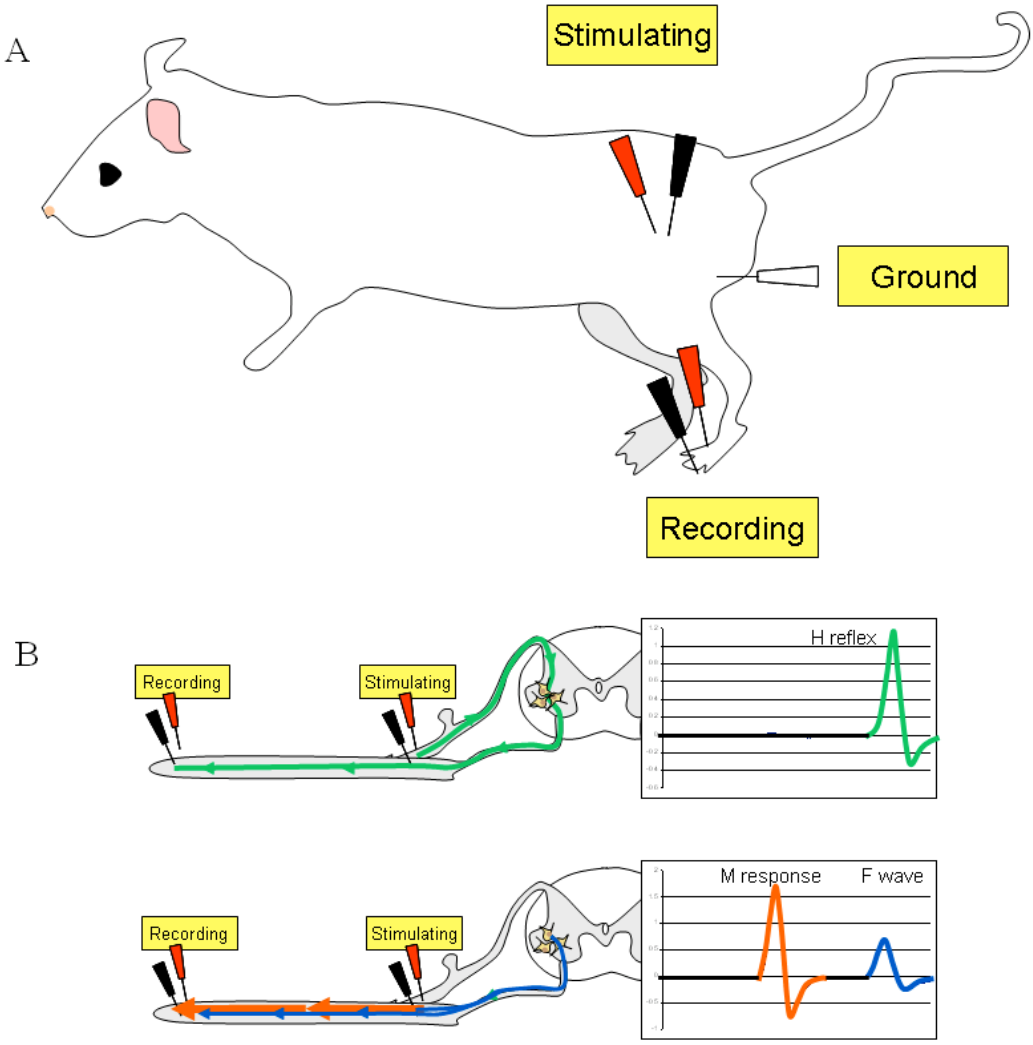

Figure 25. EMG recordings from the foot dorsum in response to stimulation of the sciatic nerve at the sciatic notch. (A) positioning of needle electrodes. (B) Schematic figure of how the pathways responsible for H reflexes and F- and M-waves.

4.3 RESULTS

4.3.1 Validation of injection site in the rat spinal cord

India ink was injected as a marker to ensure that solutions were administered to the correct location in the central grey matter. The spinal cord was immediately fixed with paraformaldehyde and then removed and 0.5 mm segments of the injection site examined as whole mounts. We could detect the marker in the central grey matter on the injected side (Figure 26), located just dorsal to the ventral horn motor neurons.

4.3.2 Localization of motor neurons projecting to the foot

To ensure that the inflammatory lesion was placed at the correct vertebral level, namely where the motor neurons are located that project to the foot muscles, WGA-HRP was applied to the tibial nerve at the ankle on one side, and to the sciatic nerve on the contralateral side, so that the marker was retrogradely transported to label the motor neurons in the spinal cord. The tracer was allowed five days to travel from the tibial nerve, and three days from the sciatic nerve, to the spinal cord. The tracer was detected from the caudal end of vertebra T13 to the middle of vertebra L2 on the right side, i.e. from the sciatic administration of the tracer (Figure 27). The left side (motor neurons projecting specifically to the distal tibial nerve) of the spinal cord, only labelled for WGA at the junction between vertebrae L1 and L2.

The location of the ventral horn motor neurons was also confirmed electrophysiologically, by making antidromic recordings of the CAP within the spinal cord resulting from electrical stimulation applied at the foot dorsum. Maximum CAP amplitude was obtained at the junction between vertebrae L1 and L2 (Figure 28, black colour), in agreement with the tracing experiment. To confirm that the signal was from the L4 and L5 ventral roots (which are known to project to the hindleg), these roots were severed and the recordings repeated (Figure 28, grey colour). The results of the histological and electrophysiological methods are in agreement, and verify that the motor neurons projecting to the foot muscles are located at the junction between vertebrae L1 and L2 in DA rats.

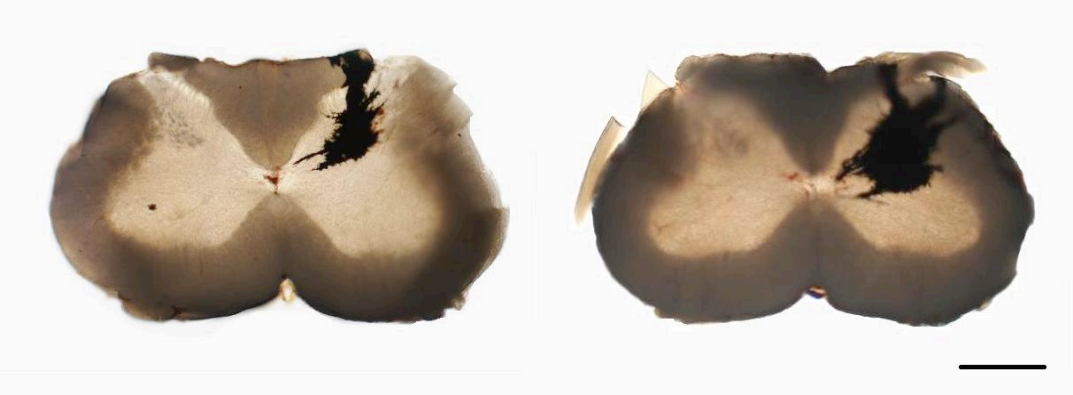

Figure 26. Injection location of 0.5 and 1 µl of India ink respectively (left and right) in the rat lateral grey matter, examined immediately post-injection. Scale bar represents 0.5 mm.

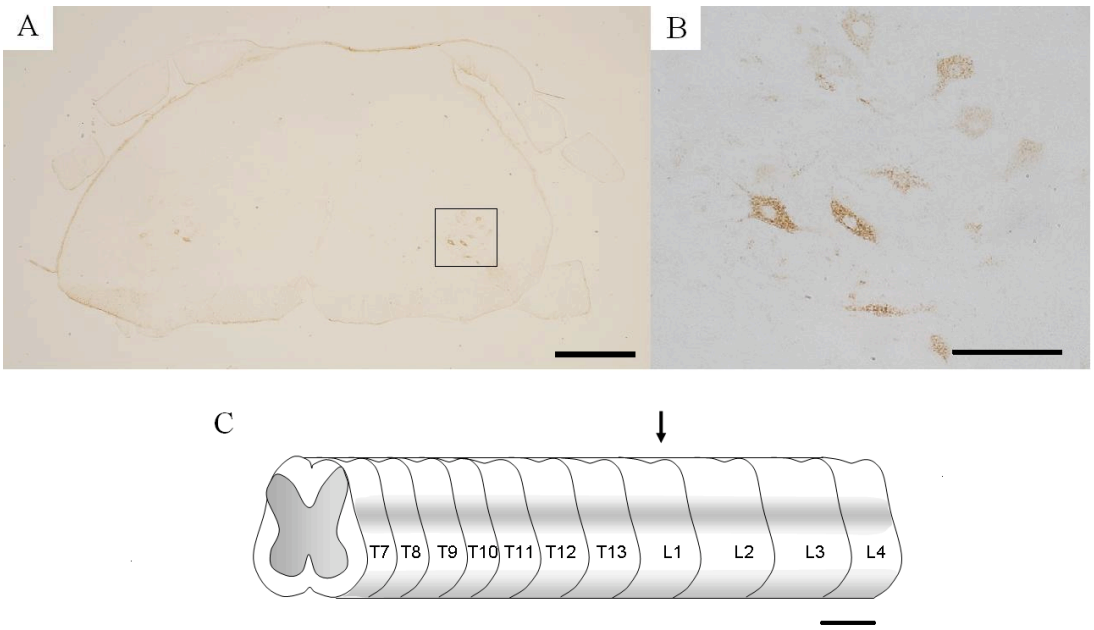

Figure 27. Location of the motor neurons projecting to the foot muscles. (A) The HRP-WGA tracer was found at vertebral levels L1 to L2 for both the foot (left ventral horn) and sciatic nerve (right ventral horn, marked in a square). Scale bar represents 0.5 mm. (B) Higher magnification photomicrograph of the motor neurons indicated in (A). Scale bar represents 0.1 mm. (C) A representative diagram of the spinal vertebral segments in the rat, an arrow indicates where the motor neurons projecting to the foot were found. Scale bar represents 0.5 mm.

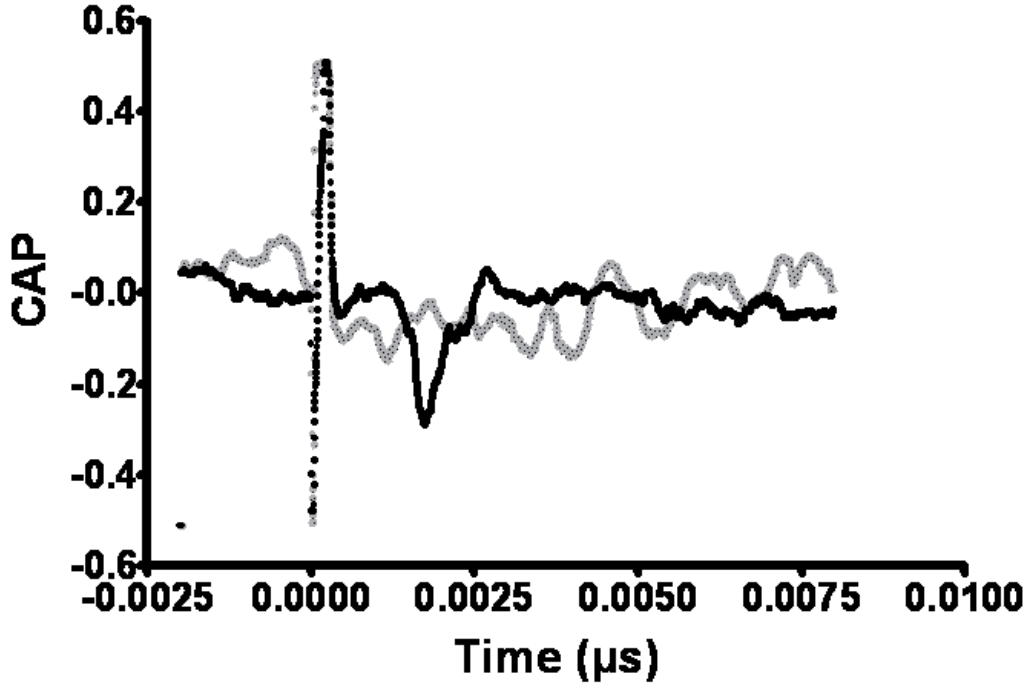

Figure 28. CAP as recorded at the junction of vertebrae L1 and L2 in response to stimulation of the foot dorsum. The max negative amplitude at the site (black) disappeared in response when the ventral roots L4 and L5 were severed (grey).

4.3.3 Histological examination of the lesion

The grey matter was examined for a range of immunohistochemical markers in LPS-injected (40 ng/µl) rats at days 1, 3, 5, 7, 14 and 28 post-lesion, as well as saline-injected controls at 1, 3 and 7 days post-injection. This series of experiments was conducted prior to the finding that day two was especially informative and so an additional series of LPS injections (100 ng/µl) was performed at 1, 2 and 3 days post-injection.

Inflammation, as evidenced by ED1-labelling (activated macrophages and microglia), was minimal in saline-injected animals, but it was prominent over a large area in the LPS-injected experimental animals from 24 hrs after injection
Figure 29). The ED1-labelling in the LPS-injected cords decreased slowly over time and was only present at the focal injection site at day 28. NeuN, the antibody of neuronal cell bodies, displayed a loss of labelling 24 hrs after LPS-injection but had returned at days 14 and 28 indicating a transient impairment of the cells. A loss in integrity of the grey matter could also be detected with PAS/LFB-staining, consistent with the loss of NeuN.

Damage revealed by PAS/LFB was thus only found at the local injection site and it had largely recovered at later times; indeed, the staining from both 14 and 28 days post LPS-injection was comparable to the saline-injected tissue. Damage to neurons was additionally indicated by caspase 9 and APP-expression. Interestingly, expression of the neuronal marker NeuN returns once the caspase 9 marker is absent (as seen at day 14 and 28). HIF-1α-expression was found at day one post-injection in the LPS-injected experimental animals, and less noticeable at days three to five, indicating an acute period of cellular stress.

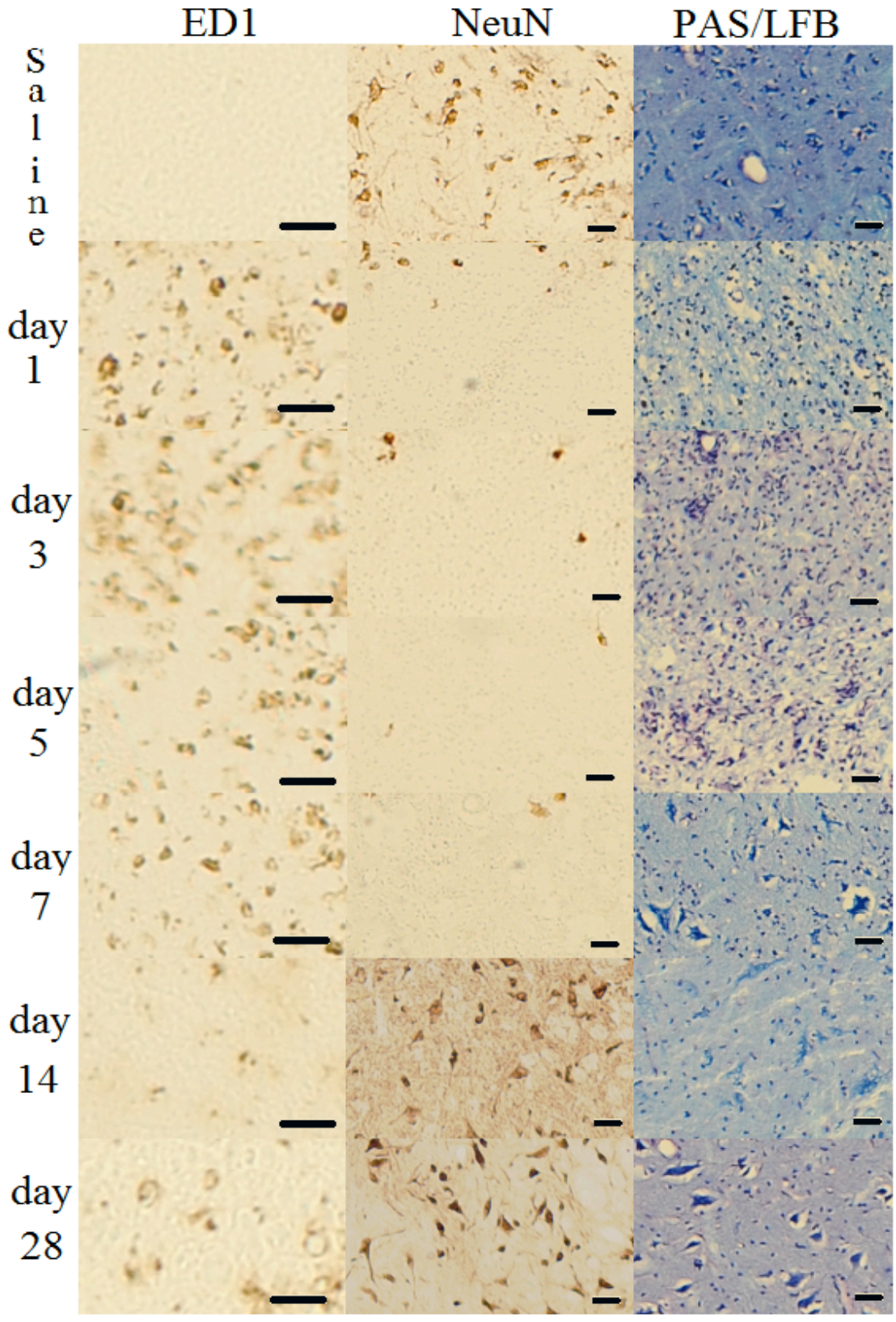

Figure 29a. Photomicrographs displaying the expression of immunohistochemical markers of inflammation (ED1), general pathology (NeuN and PAS/LFB). Scale bars represent 0.1 mm.

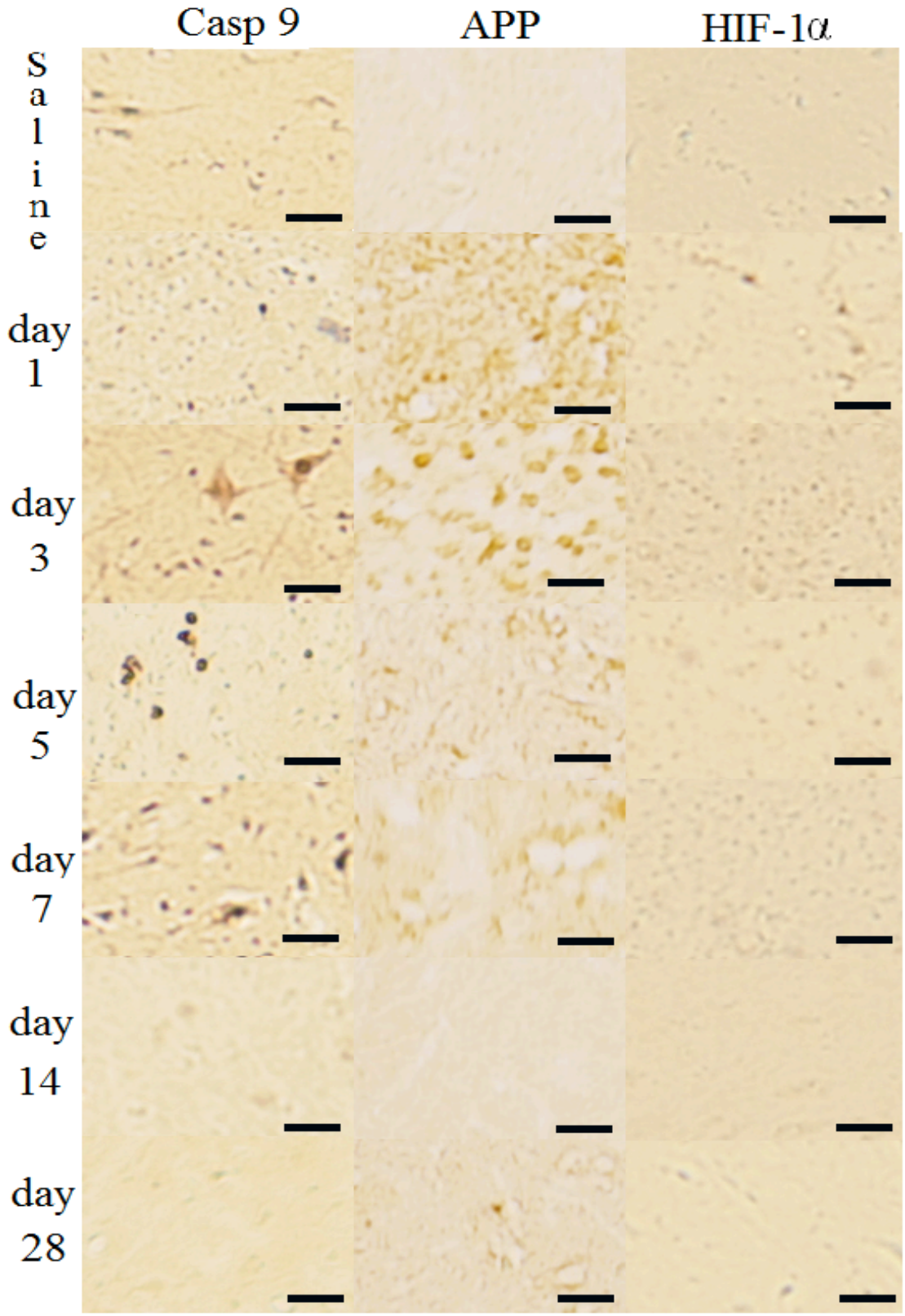

Figure 29b. Neuronal damage (APP and caspase 9) and cellular stress (HIF-1α), in saline-injected (one day post-injection) and LPS-injected animals (1to 28 days post-injection). Scale bars represent 0.05 mm.

Adjacent sections to those studied before (Figure 29) were examined for oxidative damage as well as inflammation-mediated leakage of the blood brain barrier (Figure 30). Nitric oxide production appeared to be absent in the saline-injected control animals, but in the LPS-injected experimental animals there was an expression of both iNOS (at day one only), and the neuronal form of NOS (nNOS), which appeared later and persisted for longer. The presence of the two NOS isoforms raised the suspicion of RNS-mediated damage to the cells, but no nitrotyrosine-expression could be detected (not shown). Oxidative damage was indicated by the display of 8-oxo-dG (DNA damage by superoxide), but only minimal oxidation of lipids (4-HNE) was found.

The earlier time-points investigated displayed some BBB leakage (presence of extravascular IgG) on the injected side of the cord (Figure 29). Such evidence was restricted to the needle track in saline-injected controls (not shown). Iron-deposits, as visualized by Perl's stain, were detected in LPS lesions, and occasionally in the saline-injected cords (not shown), and were found to mark the needle-injection track between three to seven days post-injection.

4.3.4 Immunohistochemistry of the LPS-DC lesion (days 1-3)

An addition to the time-series investigated above, a set of experiments was conducted where tissue was collected at days one, two and three post-injection of saline and LPS (100ng/µl, Figure 31). The decrease in 8-oxo-dG-labelling (superoxide-mediated DNA damage) may be due to the simultaneous indicated increased production of NO, because superoxide will readily react with NO to form peroxynitrite which indeed is indicated by the new found presence of nitrotyrosine-expression. The increased detrimental effect on the tissue by the high-dose LPS injection is further verified by the increased expression of 4-HNE on the injected side of the cord.

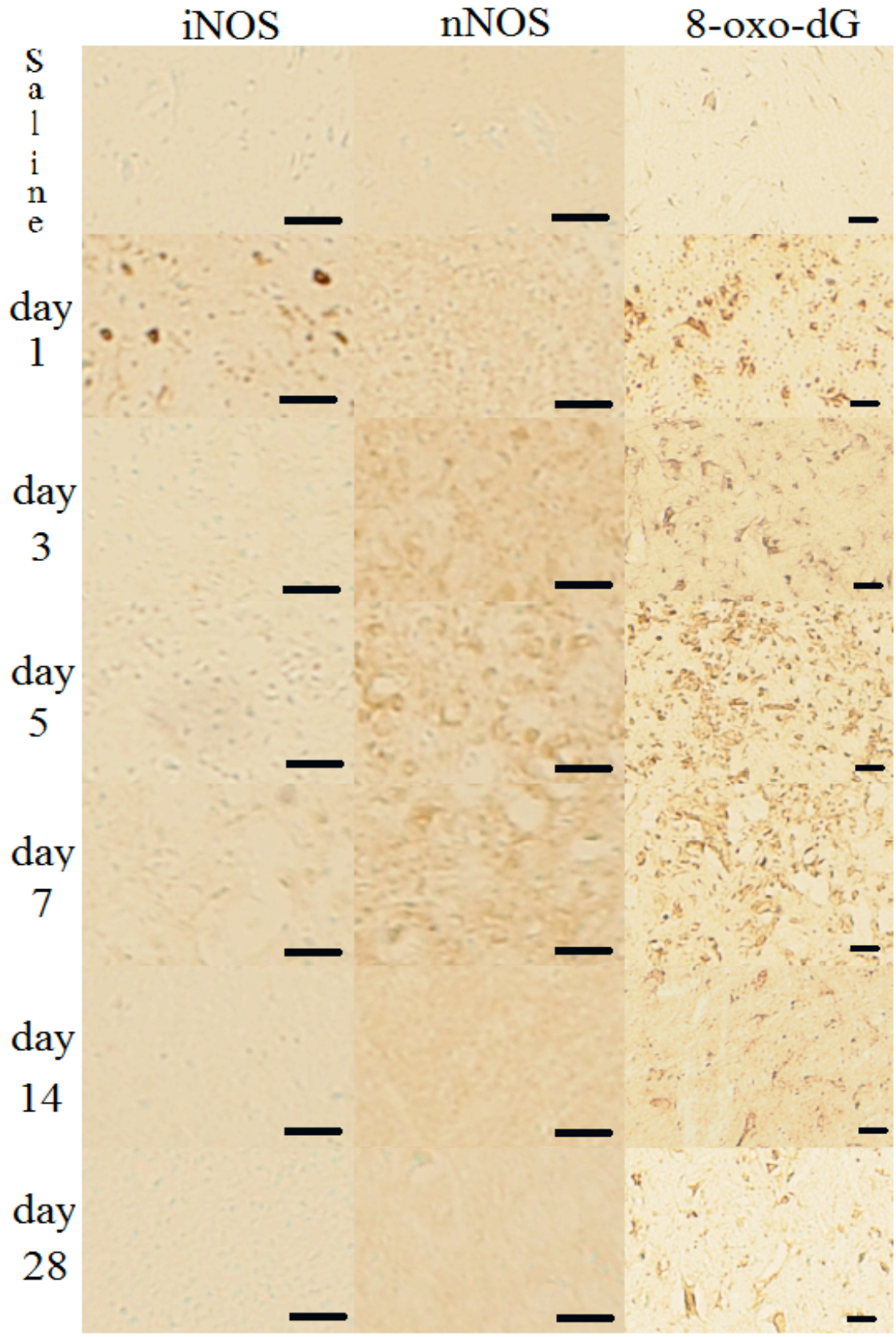

Figure 30. Montage of photomicrographs from tissue labelled with markers for oxidative damage and BBB leakage. Experimental animals were injected with saline (control, first row, 3 days post-injection) or LPS (rows 2-7, 1-28 days post-injection). Nitric oxide production was indicated in the LPS-injected animals by the transient expression of iNOS and nNOS. Oxidative damage to DNA (8-oxo-dG) was found on the injected side of the cord. Scale bars represent 0.1 mm.

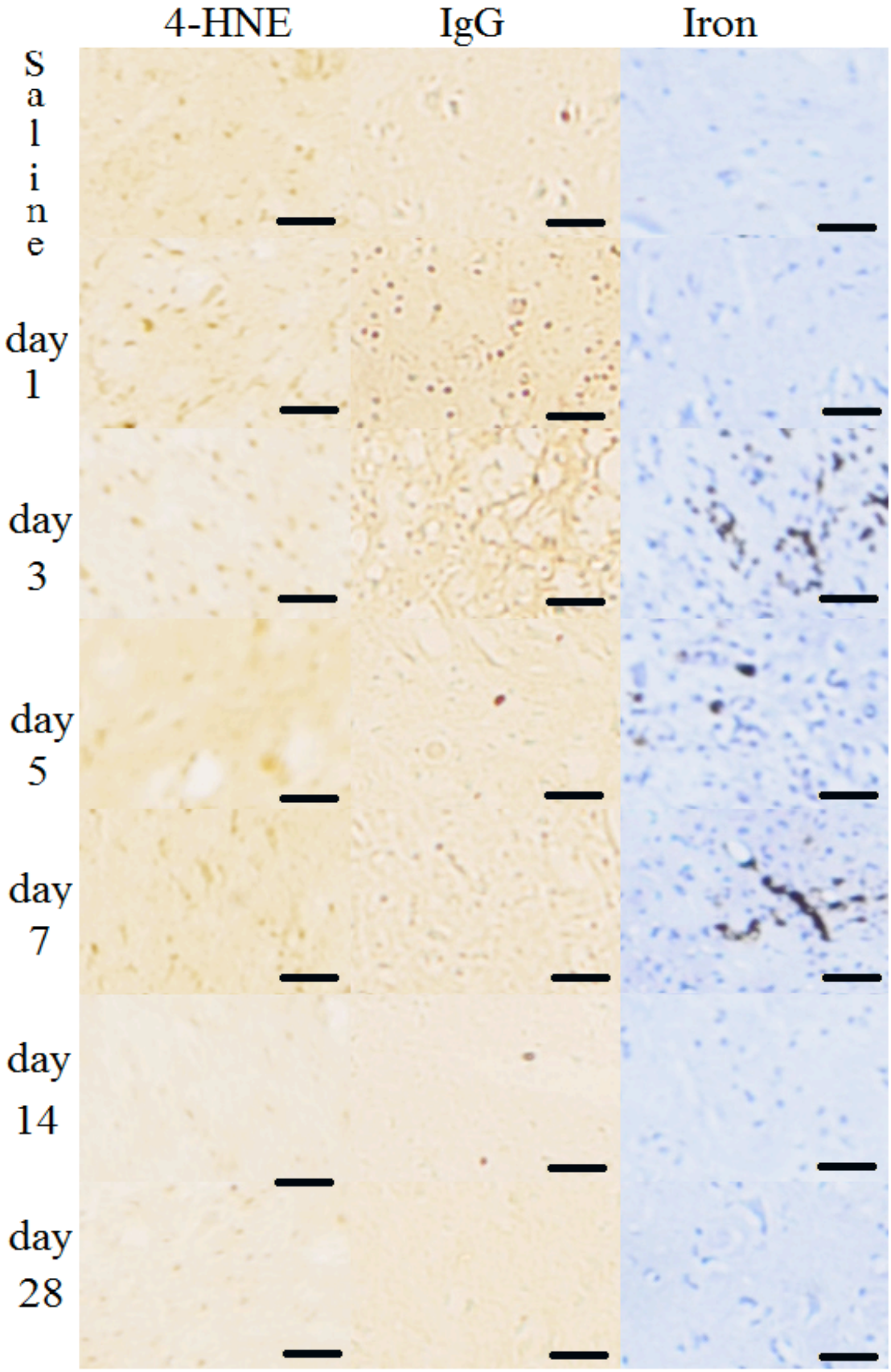

Figure 30b. Oxidative damage to lipids (4-HNE) was found. The earlier time-points investigated displayed some BBB leakage at day one to three post-injection. Needle tracks could be distinguished by iron labelling. Scale bars represent 0.05 mm.

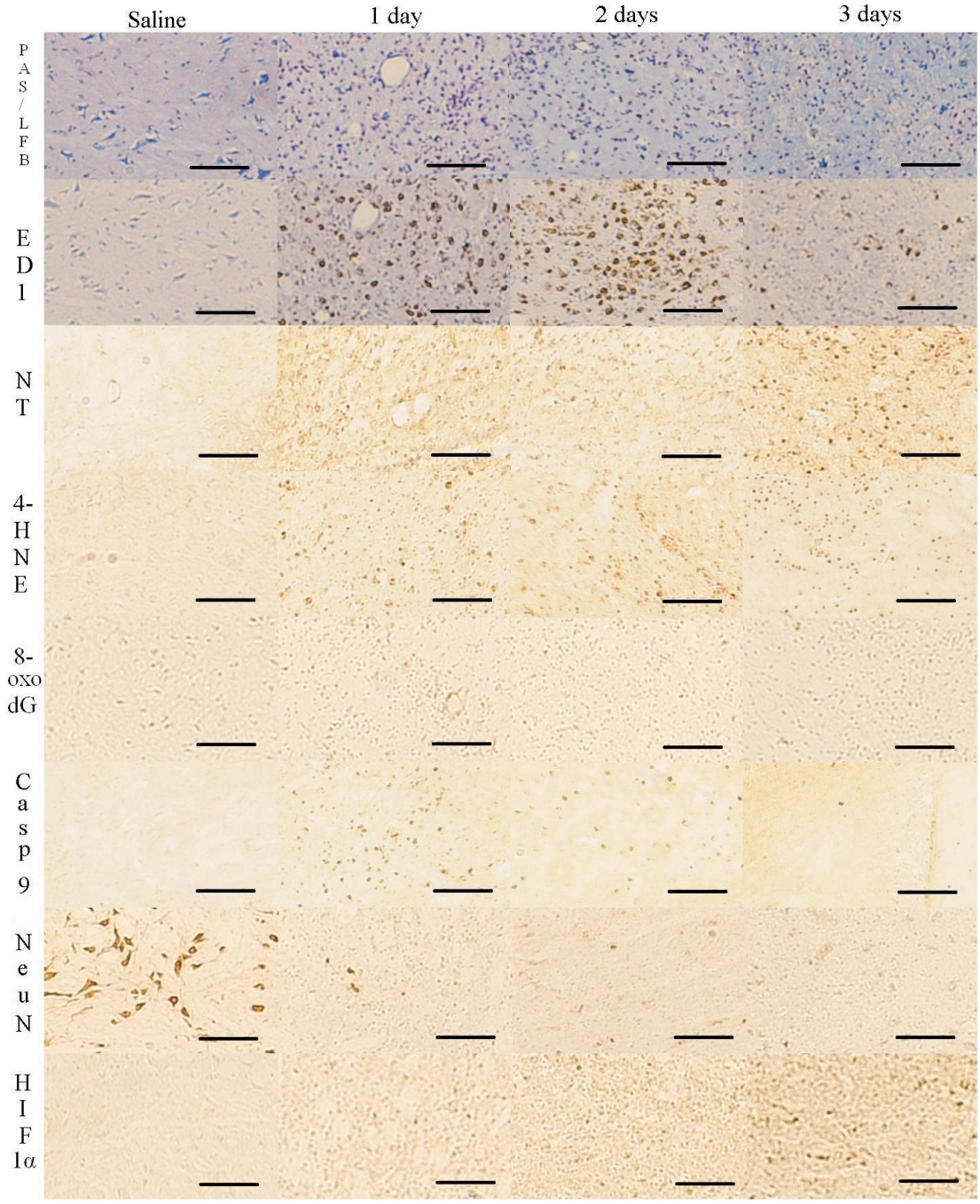

Figure 31. A montage of photomicrographs from the lateral grey matter of tissue injected with saline (two days post-injection) or LPS (day one-three post-injection). Saline-injected animals (1st column) displayed no marker of inflammation or degeneration and showed no loss of the neuronal marker NeuN. LPS-injected tissue (columns two to four) expressed evidence of inflammation (ED1 (with background stain of haematoxylin), NO production (nitrotyrosine) and some cellular damage (caspase 9 and loss of NeuN). Scale bars represent 0.1 mm.

The apoptotic marker caspase 9 was found in the LPS-injected tissue, maximally at one day post-injection but detectable at all time points examined (Figure 31). The neuronal marker NeuN was absent in the local injection site in all the LPS-injected animals but not in the saline-injected tissue. Additional cellular stress was indicated by the expression of HIF-1α on the injected side of the cord.

4.3.5 Resin sections

Resin sections were studied for the effect of the injection on the motor neurons (Figure 32). No loss of motor neurons was found but the grey matter on the right side of the cord (the injected side) appeared to have a loss of cellular integrity in LPS but not saline-injected animals. Additionally, vessels appeared rounder and dilated in the LPS-injected tissue when compared with the saline control.

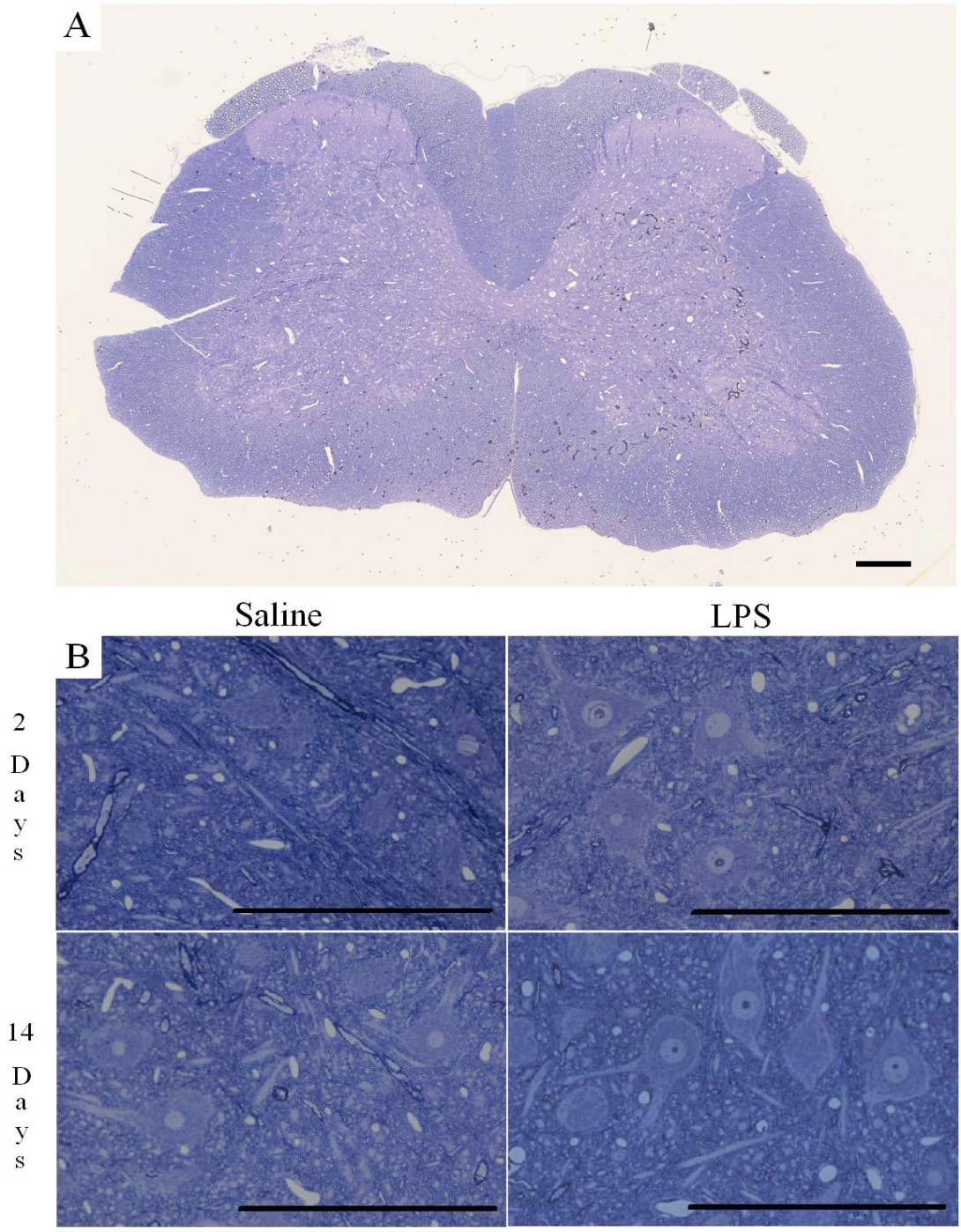

Figure 32. Photomicrographs of transverse spinal cord tissue, taken at two and 14 days after saline or LPS injection into the lateral grey matter of the spinal cord (right side). The non-injected lateral column was 'nicked' for orientation. (A) A saline injected spinal cord 2 days post-injection. (B) There was no noticeable difference between the LPS-injected and the saline-injected control animals at either of the two time-points investigated (2 and 14 days-post0injection). Scale bars represent 200 µm.

4.3.6 The activity of the mitochondria is decreased in motor neurons

We studied the activity of the mitochondrial enzyme cytochrome c oxidase (COX) in the saline and LPS-injected rats and found that the ventral horn motor neurons had a transient decrease in the activity of the mitochondrial complex IV in LPS-injected but not in saline-injected control animals (Figure 33). The loss of activity was present 24 hrs after the LPS injection but reached a significant decrease at day 2 both in relation to the amount of mitochondria (COX:porin, $P<0.001$) and protein (COX:COX1, $P=0.0021$) compared to controls. The loss of activity of complex IV in the motor neurons was regained between day three (porin, $P=0.0132$) and five (COX1, $P=0.0396$) and stayed elevated up to 28 days after the LPS insult. Interestingly, the activity of COX in relation to the amount of mitochondria present seemed to increase over the initial values at day 28 ($P=0.0274$). This is indicative of an increased expression of the COX protein per mitochondria.

4.3.7 Electrophysiology

The activity of complex IV in motor neurons is transiently decreased in the LPS-injected experimental animals raising the possibility that the function of the motor neurons is impaired. The excitability of the motor neurons was therefore examined over time in saline and LPS-injected animals, immediately before and after injection, and at 1, 2, 3, 4, 21 and 28 days after injection. The maximum M wave amplitude (out of ten recordings taken at each time point) were not significantly altered by saline or LPS-injections over the time-course investigated (Figure 34A). However, a transient decrease in both the H reflex (Figure 34B) and F wave (Figure 34C) amplitude was detected at two days after LPS-injection. To minimise the effect of needle placement, temperature, time of day and other artefacts, the H reflex and F wave amplitudes were plotted as a ratio to the M wave response (Figure 35A and B respectively). The H:M ratio was decreased already at 24 h after injection (not significantly different) and reached a maximum reduction at day two ($P=0.0114$, compared to pre-injection values) which was returned to baseline levels at day three.

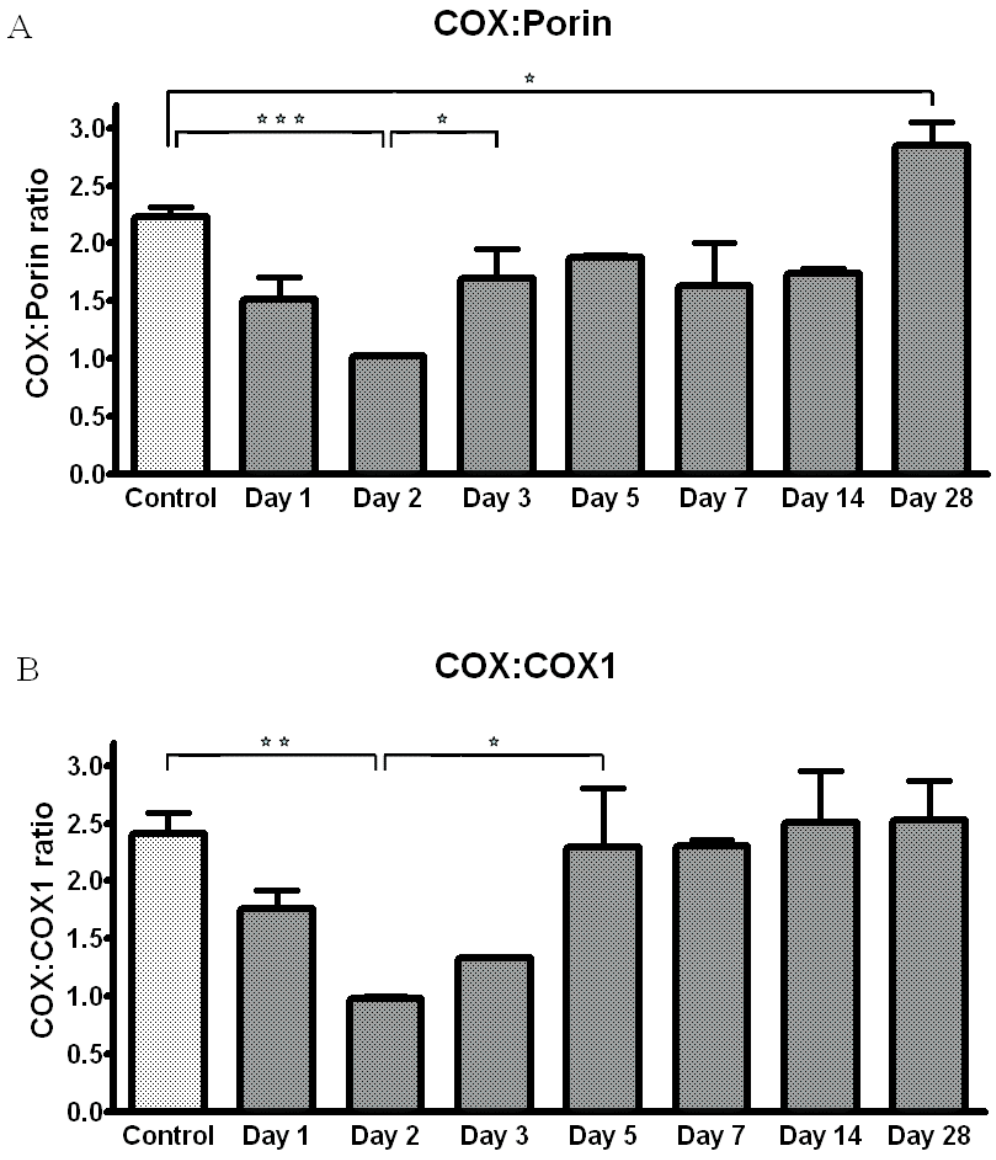

Figure 33. Activity of mitochondrial complex IV in relation to the amount of mitochondria (A, porin) or protein present (B, COX1). N=2-4.

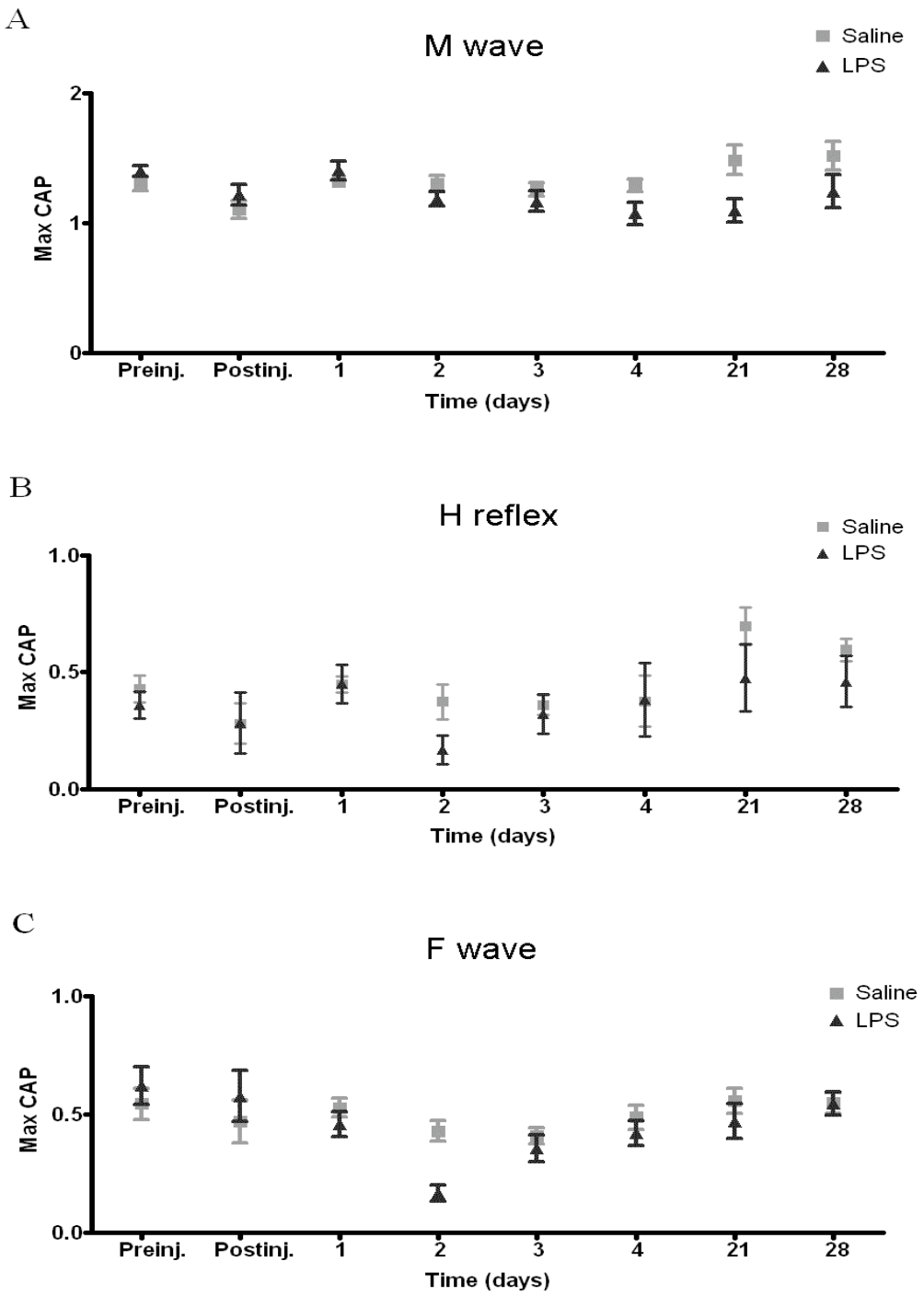

Figure 34. Raw data from EMG recordings in LPS and saline-injected rats. (A) The M response was relatively consistent throughout the time course examined in both saline and LPS-injected experimental animals. (B) Compared with saline controls, LPS-injected animals had a temporary decrease of motor neuron excitability with maximum reduction at day two. (C) The F waves from LPS but not saline-injected animals were decreased at day two. N=5-15.

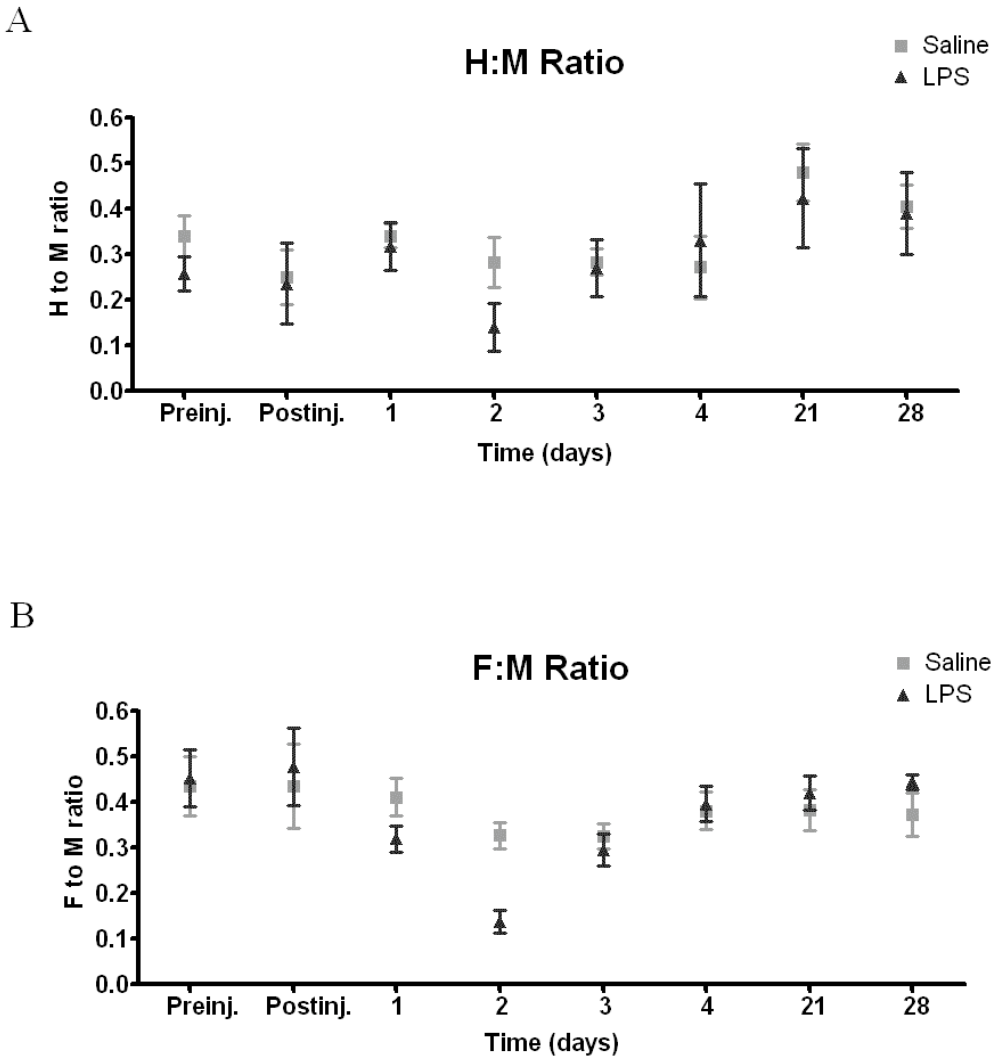

Figure 35. H reflex and F wave responses were plotted as a ratio to the M wave recordings to minimise the effect of artefacts. (A) The H to M ratio displayed a significant reduction of the CAP at day two in LPS injected versus saline-injected animals. (B) The F to M ratio was transiently decreased at day one and two. N=5-15.

The F:M ratio was significantly reduced at day two (P=0.0001) when compared with both pre-injection amplitude and saline controls. Since both H and F responses are affected by the LPS-injection to the same extent, the deficiency can most likely be attributed to a reduced excitability of the motor neurons.
If the effect had been more pronounced in the H reflex, a synaptic dysfunction would have been the likely cause. Both the excitability of the motor neurons and the activity of complex IV of mitochondria were maximally decreased at day two, suggesting that the reduced excitability was a consequence of an energy deficiency resulting from mitochondrial dysfunction. As a test of this hypothesis, the complex IV activity was plotted against the F:M and H:M ratios to determine whether a correlation existed (Figure 36).). The correlation coefficient was high (R^2 values were 0.8399 and 0.5949 for F:M (p=0.0102) and H:M respectively), implying that inflammation-induced mitochondrial dysfunction can indeed cause functional deficits.

4.3.8 Effect of TNF-α on the excitability of the motor neurons

As shown above, the LPS has a transient detrimental effect on the H reflex and F wave response indicating a deficit in the excitability of the motor neurons. LPS is an endotoxin and its toxicity is believed to be mediated via the cytokine TNF-α (Li et al., 2008). To determine whether TNF-α could substitute for LPS, the effect of the intraspinal injection of TNF- α on motor neuron excitability was investigated. The results from a preliminary study revealed that TNF- α did have a significant effect on the H:M ratio, with a temporary suppression that was maximally expressed four days post-injection (P<0.001), but the F:M ratio was not significantly affected (Figure 37). This finding, although only in two animals, suggests a specific effect of TNF-α on synaptic transmission.

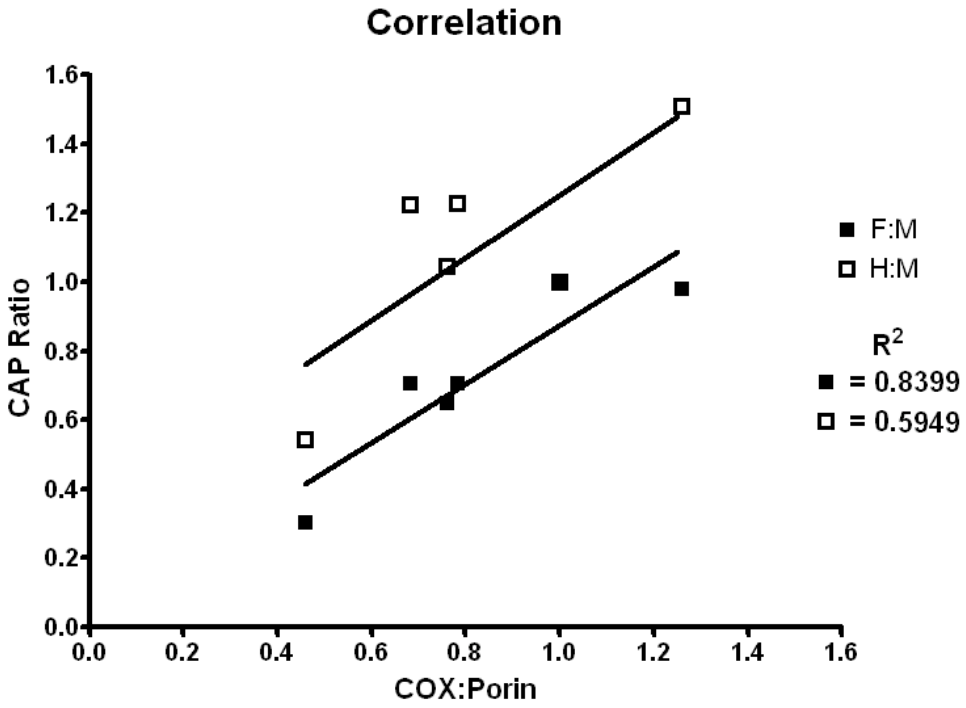

Figure 36. Correlation between the activity of mitochondrial complex IV (to porin ratio), and the compound action potential (CAP) of H reflexes and F waves (in ratio to the M wave response). Values from controls (for COX activity) or pre-injection (for CAP amplitudes) and data from day 1, 2, 3, 14 and 28 days post-injection were normalised (so that the pre-injection values were adjusted to unity) and plotted.

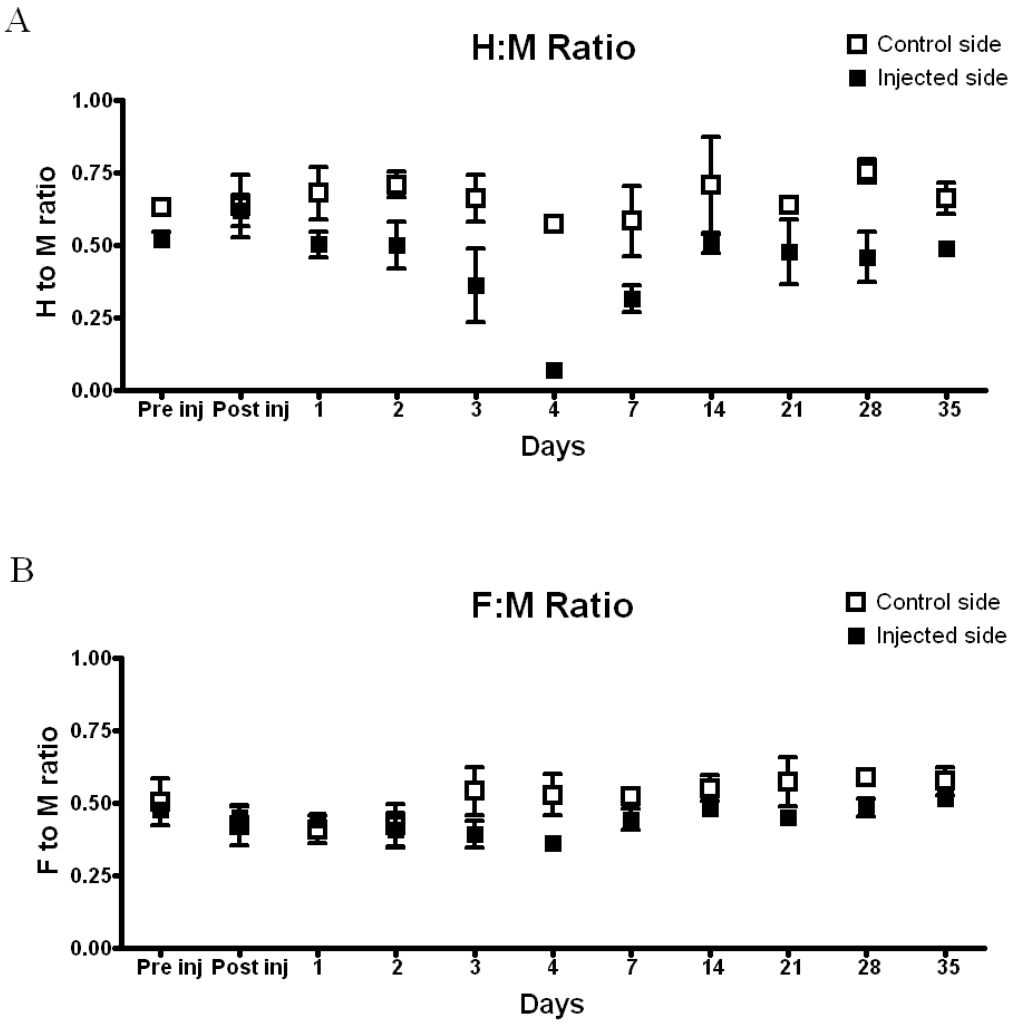

Figure 37. The effect of TNF-α on the excitability of motor neurons. (A) On the injected side alone, the H:M ratio was temporarily decreased between 24 hrs to seven days post-injection. (B) The effect of TNF-α on the F:M ratio was more subtle, but there was still a decrease four days after injection. N=2.

4.4 DISCUSSION

This chapter has explored the effect of inflammation on motor neurons by examining both the activity of their mitochondrial complex IV, and their electrophysiological function. We found that the mitochondrial activity was decreased during the first three days post-injection, with maximal reduction at day two. The data show that the activity was decreased both per mitochondrion (when expressed as a ratio with the mitochondrial marker porin), and per enzyme (ratio with subunit COX1), suggesting that although the enzyme is still present, it is damaged (or inhibited). Notably, the reduction in motor neuron mitochondrial activity correlated precisely with a transient reduction in the excitability of the *same population* of motor neurons as assessed by the amplitudes of the F wave and H reflex recorded from the foot dorsum. Thus the F:M and H:M ratios were maximally reduced at day two, but regained full amplitude at day four. A significant correlation was observed when mitochondrial activity was plotted against the F:M ratio (R^2=0.8399, P=0.0102), but not against the H:M ratio (R^2=0.5949, P=0.0725).

4.4.1 Functional deficits

The cause of the mitochondrial deficiency is not known, but possible causes have been discussed in the previous two chapters: it seems likely that NO may play an important role. In this chapter it has been shown that inflammation may have a temporary effect in suppressing mitochondrial activity, and such a reversible effect cannot easily be explained solely by either the transient inhibition of complex IV by nitric oxide, or the permanent deficiency caused by nitration of complex IV. Alternative mechanisms for a temporary effect include a down-regulation of mitochondrial complex I, which has been suggested to occur during hypoxia where the complex goes into a dormant state, mediated by NO, which decreases the formation of ROS during ischemia and reperfusion (Galkin et al., 2009). Another mechanism may involve S-nitrosation of complex I. Such nitrosation can be reversed by reduced glutathione, however when the glutathione pool of the mitochondria is oxidised, the inhibition of complex I persists (Dahm et al., 2006). Hyperoxia induced by LPS could maintain glutathione in an oxidised state, perpetuating the nitrosation of complex I. Reports also suggest that NO and/or peroxynitrite may also deplete the

glutathione pool of the mitochondria, thus both depriving them from antioxidant defences and also decreasing the capacity of glutathione to reverse the S-nitrosation (Bolaños et al., 1996, Clementi et al., 1998, Radi et al., 1991, Jorge and Oscar, 2009). Such S-nitrosation of the mitochondrial complexes may explain the temporary suppression of mitochondrial activity and motor neuron excitability observed here. This hypothesis is consistent with the observation in MS lesions that glutathione concentrations are low within the GM, as assessed by MRI techniques (Srinivasan et al., 2010).

4.4.2 Histochemical mitochondrial activity assay

The ability to study *in the same section* the results of the immunohistochemical labelling together with the assay for activity of mitochondrial complexes enabled the determination of the ratio between activity and either the presence of mitochondria (via porin immunolabelling), or the presence of the protein (via labelling of complex IV subunit I (COX1)), at the level of individual cells. This is a new method established by Mahad and colleagues, including the author, and which may prove to be a very useful tool to examine the metabolism of individual cells (Mahad et al., 2009).

4.4.3 Effect of inflammation on neuronal function

Although the neurological symptoms expressed in MS patients have traditionally been regarded to be a consequence of demyelination, several authors now report that inflammation alone may also impair neuronal function (Bitsch et al., 1999b, Moreau et al., 1996, Youl et al., 1991). Nitric oxide is often associated with inflammation, and it has been found reversibly to inhibit axonal conduction (Redford et al., 1997, Shrager et al., 1998), especially in demyelinated axons (Redford et al., 1997, Kapoor et al., 1999). Indeed, if the axons are electrically active while exposed to nitric oxide, they become especially prone to suffer irreversible axonal injury and destruction (Smith et al., 2001). Nitric oxide therefore provides a potential mechanism by which inflammation can cause neurological deficits.

There is evidence for the production of nitric oxide in the LPS-VH lesion, both from the expression of iNOS and nNOS, and also from the labelling of nitrotyrosine (Figure 31). iNOS and NO have also been found in high concentrations within MS lesions and normal appearing white matter (NAWM) (Oleszak et al., 1998, De Groot et al., 1997, reviewed by Smith and Lassmann, 2002). Traditionally, the inducible form of NOS is often regarded as being the most detrimental since the NO produced by the constitutive forms of NOS is used by the cells as signal transducers, and it also tends to be in lower concentration. The LPS-VH lesion displays an increasing expression of the neuronal form of NOS over time, indicating that it is up-regulated by LPS-induced cellular stress. Indeed, the theory that nNOS expression is induced as a response to stress is consistent with the increased labelling of nNOS during neuronal degeneration in a transgenic SOD mice model (Sasaki et al., 2002). Interestingly, nNOS is the isoform found in mitochondria (Bolaños et al., 2008). However, whether nNOS made an important contribution to the mitochondrial dysfunction described here seems unlikely, because the nNOS was found to be maximally expressed at day three to seven, i.e. mainly after the period of dysfunction (Figure 30). The expression of iNOS, on the other hand, did correlate with the temporary reduction in motor neuron mitochondrial activity and excitability (Figure 30).

4.4.4 Oxidative stress to tissue

Damage due to oxidative stress was also observed in the LPS-VH lesion, as evidenced by the detection of nitrotyrsine, 8-oxo-dG and 4-HNE. This finding indicates that the temporary function deficit of the motor neurons may be due, in part at least, by ROS and RNS. The reversibility of the effect may be due to an up-regulation of the antioxidant defences of the cells, repair or replacement of damaged proteins. Similar results have been reported in MS patients, where, for example, an increased expression of oxidised DNA was found within lesions (Lu et al., 2000, Vladimirova et al., 1998), together with an increased concentration of lipid peroxidation products within serum (Koch et al., 2006). Interestingly, patients with MS have significantly lower uric acid levels than controls, which may have a major impact on oxidative stress because uric acid is reported to comprise about 50% of the body's normal ROS defence (Rentzos et al., 2006, Toncev et al., 2002). Furthermore, the concentration of antioxidants glutathione (in red blood cells) and SOD (in cerebrospinal fluid) have

been reported to be significantly reduced in MS patients (Calabrese et al., 1994, Langemann et al., 1992, Zagorski et al., 1991, Ronquist and Frithz, 1979). The hypothesis that MS tissue is damaged by ROS is strengthened by the fact that SOD and glutathione peroxidase gene transcripts are increased 14-20 times in lesions compared with the levels found in NAWM (Tajouri et al., 2003).

The commonly used animal model of MS, EAE, also shows evidence of oxidative stress, and indeed antioxidant therapies have repeatedly been shown to be beneficial with regard to the expression of neurological deficit. Both lutein (Lutskii and Esaulenko, 2007, Hendriks et al., 2005), uric acid (Hooper et al., 2000), flavonoids (Hendriks et al., 2004), catalase (Ruuls et al., 1995), N-acetyl-L-cysteine (Lehmann et al., 1994), bilirubin EAE (Liu et al., 2003), and alpha lipoic acid (Marracci et al., 2004, Morini et al., 2004, Schreibelt et al., 2006) have all been shown to improve the disease. Together these data indicate that ROS and RNS may contribute to the pathology of neuroinflammatory lesions, including those expressed in MS.

4.4.5 Critical evaluation

This chapter has described the transient effect of LPS on mitochondrial complex IV in relation to the abundance of both mitochondria, as measured by the marker porin, and of the complex IV enzyme. The results are convincing since both parameters are affected at the same time interval. However, possible miss-interpretations might arise if LPS can selectively affect the porin expression. Another point is that although NO is a likely contributor to the effect seen, as supported by earlier observations on neuronal conduction (Redford et al., 1997, Kapoor et al., 1999) and as indicated in the results obtained, there are many other inflammatory mediators that could affect or stress the neurons and/or mitochondria, such as interferon gamma, TNF-alpha and interleukins. Finally, results may be miss-interpretated if the pro-inflammatory inducer LPS does indeed kill the local neurons, and that later the mitochondrial activity, neuronal function, and histology are seen to recover due to migration or plasticity of adjacent neurons. Such an occurrence is considered so unlikely as to be unbelievable.

GENERAL DISCUSSION

5.1 OVERALL RESULTS

This thesis describes how the pro-inflammatory agent LPS induces decreased activity of mitochondrial complexes II and IV, or alternative decreased presence of dorsal horn cells. The LPS-DC lesion also displayed increased oxygen concentrations, likely due to several mechanisms, including mitochondrial deficiency, inflammation-induced vasodilation, and the Bohr effect. When LPS was intentionally injected into the ventral horn in a lower dose to involve the motor neurons, we found a temporary reducing effect on complex IV activity in relation to the presence of mitochondria and enzyme in individual motor neurons. This temporary effect was exactly paralleled by a reduction in motor neuron excitability. Immunohistochemical techniques revealed LPS-induced oxidative stress in both the dorsal column and ventral horn, including increased NO production.

5.2 EFFECT OF INFLAMMATION

The findings show that LPS promotes prominent inflammation in the tissue and that the response is dependant on the dose, with effects ranging from demyelination in the dorsal columns, to neuronal death, to only transient effects on neuronal functionality.

5.2.1 Cause of the hypoxia-like pathology

This project's initial focus was to decide determine whether there was an alteration to the oxygen concentration in the LPS-DC model. The results were to be coupled back to the hypoxia-like pathology found within the PPP lesions of MS and hence to discern whether the pathology arose in response to *true* hypoxia, or by *histotoxic* hypoxia, since the first case would result in low oxygen concentrations, and the second, in high concentrations through mitochondrial inhibition. We found high oxygen concentrations within the LPS-DC lesion which implied that there was a deficiency to the mitochondrial electron chain. We proceeded to investigate the mitochondrial activity of the lesion tissue with *in vitro* techniques by collaboration with Dr Mahad of Newcastle University. The results from the LPS-DC lesion showed

that although the sensitivity of the technique was not high enough to distinguish a reduced activity of the mitochondrial complexes in the white matter, we did find that LPS had a potent effect on the neurons in the adjacent grey matter. The activity of complex II and IV was decreased from day two post-injection and was most likely due to a loss of cells because both porin and COX1 labelling were also diminished.

5.2.2 Inflammation affects the motor neurons

Injection of LPS into the ventral horn revealed that the mitochondrial activity of the ventral horn motor neurons was temporarily decreased, from day one to day four, with a maximal reduction at day two. This reduction coincided precisely with a deficiency of the motor neuron excitability in the same model. Because of the correlation between the two functional studies it is highly likely that the reduction in motor neuron excitability was due to an energy deficiency caused by mitochondrial dysfunction. Also in this model we found markers of oxidative stress expressed in the tissue, including increased NO production. NO has previously been reported to inhibit axonal conduction (Redford et al., 1997, Shrager et al., 1998) and demyelinated axons seem particularly sensitive (Redford et al., 1997, reviewed by Smith and Hall, 2001). It may be that NO mediates conduction deficits by interfering with mitochondrial ATP production, which is essential for signal transduction. The temporary effect may be due to S-nitrosation of mitochondrial complexes which can be reversed by glutathione.

5.2.3 ROS and RNS

We have shown that LPS may promote oxidative stress through the production of superoxide and peroxynitrite. The source of ROS may be from infiltrating inflammatory cells releasing ROS through respiratory burst, or from the mitochondrial electron chain. The mitochondria will increase their ROS release if their activity is compromised (Zeevalk et al., 2005). When labelling the tissue for superoxide with the fluorescent marker DHE we found that the superoxide production was increased at the injection site at 24 hours post-injection and seemed to co-localize with inflammatory cells at later time-points. We suggest that the early time point coincides

with mitochondrial inhibition and that the labelling found is from these intracellular organelles. Double fluorescence studies may discern whether this is the case.

Immunohistochemical examination of the tissue revealed modification of DNA, lipids and proteins from oxidative stress. These structural changes may impair function and hence cause complications to cellular compartments which does not only involve the direct target but also causes a strain on the cell since they will have to replace or repair the damage, or function less efficiently. Stress caused by ROS may impair cells to the degree where they undergo apoptosis, and indeed, we could detect caspase 9 at the lesion site, indicating that cells are dying. Additionally, although not verified in our models, it is likely that cells damaged by ROS-mediated changes, or by energy deficiency, will be targeted by phagocytotic cells and thus engulfed for removal.

5.2.4 NO-mediated energy deficiency

The LPS-injected tissue showed several signs of cellular stress, and with higher LPS concentrations, cellular death. The fact that we could detect a temporary effect on the activity of the mitochondrial complex IV in motor neurons indicates that the inflammatory cells release mediators which cause a transient effect on their function. Our prime suspect for such a temporary effect is NO. Not only have we shown that NO is most likely produced in high quantities around day one after LPS-injection but we have also detected protein nitration, most likely mediated by peroxynitrite. The expression of iNOS and nitrotyrosine implies that NO is produced in high concentration at 24 hours after injection of LPS and may be partially the cause of the mitochondrial inhibition through competitive inhibition with oxygen at the reaction site of complex IV or by nitration or S-nitrosation of the mitochondrial complexes.

5.2.5 Models of MS

Two LPS models have been utilized in this thesis, and as these are quite new to MS research it is reasonable to discuss their relevance and merits in comparison with existing models, primarily EAE. The LPS–DC model has been advanced (Felts et al, 2005; Marik et al, 2007) as a quite accurate model of Pattern III (Luccinetti et al, 2000) MS lesions, a type of lesion that otherwise has no other animal model: EAE is usually

considered to model Patterns I or II MS lesions. Apart from uniquely modelling the Pattern III lesion, the LPS-DC model also has several advantages over EAE, although it is also true that there are many variants of EAE and so it is difficult to generalize. Nonetheless, from an experimental viewpoint, it is notable that the LPS-DC is much more reliable than EAE, resulting in a lesion in almost 100% of cases, whereas EAE is notoriously variable, both between batches, and between animals within a particular batch. The reproducibility of the LPS-DC lesion is also much more reliable than with EAE: it is common in EAE to have some animals that may fail to get a deficit, while other members may get the disease so severely they have to be euthanized. The LPS-DC lesion also has a precise and known location, whereas the lesions in EAE tend to have an unpredictable location. These problems with EAE make the trials cumbersome, with more animals immunized in order to ensure that sufficient numbers with a suitable severity are available for study. On the other hand, EAE, being more varied, might better be considered to reflect the human disease, although the advantages of an unpredictable model are difficult to defend. Finally, it is known that therapeutic predictions based on observations in EAE are unreliable, although whether the LPS-DC lesion will prove to be more reliable has yet to be tested. The advantages outlined for the LPS-DC model also apply to the new model introduced here, the LPS-VH lesion. This lesion also has the clear advantage of primarily affecting the grey matter, which tends to be an overlooked, but important, target in MS. It is clear that EAE falls somewhat short of being an ideal model for MS, and so it is important to develop new models that may offer different perspectives of MS, and we believe that the LPS models are very good candidates.

5.3 CONCLUSIONS

Mitochondrial deficiency has been suggested before as a cause both of the LPS-DC lesion and MS (Aboul-Enein and Lassmann, 2005, Andrews et al., 2005, Dutta et al., 2006, Lu et al., 2000, Mahad et al., 2008, Su et al., 2009, Vyshkina et al., 2005). The results presented here strengthen the hypothesis that an energy deficiency may be an early event in the formation and progression of MS lesions. Furthermore, we suggest that there may be an increased production of ROS in MS, as has been suggested previously (Gilgun-Sherki et al., 2004, LeVine, 1992, Lu et al., 2000), and that the ROS may promote structural changes to proteins, lipids and DNA, inducing an additional strain on the cells. An effect from the structural changes caused by the oxidative stress may be an activation of the autoimmune system, targeted at the faulty lipids or proteins, and capable of recognising endogenous molecules, as previously have been described for CO poisoning (Thom et al., 2004). Finally, we believe that the models characterised here may prove beneficial for testing new therapeutics and that the focus of MS research should be directed at a possible inflammation-mediated energy deficiency. Indeed, preliminary studies in which I participated at the end of my PhD research encourage a view that such an approach is not only possible in experimental models *in vivo*, but also that it is effective.

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6. APPENDIX

7.1 CHEMICALS

7.1.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. LPS is a amphiphilic, extremely heat-stable molecule consisting of a lipid (the lipid A) which anchors the molecule in the outer membrane and a saccharide part joined by a covalent bond (Alexander and Rietschel, 2001, Holst et al., 1996). The lipid A part of LPS is the immunostimulatory or the 'endotoxic' component and interacts with macrophage, polymorphonuclear leukocytes, platelets, and vascular endothelial cells (Galanos, 1975). LPS is through these cells able to activate a multitude of endogenous immunomodulatory or inflammatory compounds such as cytokines, interleukins, tumour necrosis factor-α and eicosanoids (Acosta, 2001, Galanos, 1975). LPS may hence induce inflammation in most tissues by local injection, causing infiltration of the tissue by neutrophils and monocytes from surrounding bloodstreams within minutes (Issekutz, 1981, Cybulsky et al., 1988). LPS can also act as a non-hypoxic stimulus to increase the expression of hypoxia-related genes (Blouin et al., 2004). In small amounts, LPS can be beneficial by activation of the immune system, but in higher doses may lead to pathological reactions as that of septic shock (Alexander and Rietschel, 2001).

7.1.2 Dihydroethidium

Dihydroethidium (DHE) is a fluorescence label for the presence of superoxide. It is supposedly specifically oxidized by superoxide, but peroxidases (Patsoukis et al., 2005) and cytochrome c (Benov et al., 1998) has also been shown to also influence the output oxidative status of DHE. However, cytochrome c oxidation of DHE only result in minute amounts of the fluorescent ethidium as an end product (Benov et al., 1998) or other oxygen species. DHE freely diffuses into cells where upon oxidation, DHE is cleaved to the ethidium bromide. The oxidized product intercalates into nucleic acids, and probably mitochondrial genes, which greatly enhances its fluorescence.

7.2 OXYGEN CONCENTRATION CALCULATIONS

The concentration of oxygen in normal air is 20.95%. Because the pressure at sea level is 760 mmHg, the partial pressure of oxygen will be 0.2095 * 760 mmHg = 159 mmHg. The solubility of oxygen in a solution is dependant on the weak dipole-dipole interactions between water and oxygen molecules and is hence influenced by temperature and the concentration of oxygen in the gas above the solution. The solubility can thus be calculated according to Henry's law

$S_g = k_H \cdot p_g$

Where S_g is the solubility of oxygen in water (mg/L), k_H is Henry's law constant at the given temperature (K), solute and solvent, and the p_g is the partial pressure of oxygen over the solution (Torr). Our experiments were conducted at 37^{o}C which gives us a Henry's law constant of 0.04223 and hence a solubility of oxygen of **6.716 mg/L** at one atmospheric pressure (1 atm, sea level). Since our calibrations of the oxygen sensors were done in a Ringer's solution (to imitate the extracellular fluid *in vivo*) the oxygen saturation has to be corrected for salinity. Ions will not decrease the actual solubility of oxygen but they will decrease the portion of oxygen in the solution.

One liter of Lactated Ringer's Solution contains sodium (130 mmol/L; MW: 22.99; m: 2.9887g), chloride ions (109 mmol/L; MW: 35.45: m: 3.86405g), lactate (28 mmol/L; MW: 90; m: 2.52g), potasium ion (4 mmol/L; MW: 39.10; m: 0.1564g), and calcium ions (1.5 mmol/L. MW: 40.8; m: 0.0612g), which gives a total of 9.4496 g, hence a concentration of 9.5 ‰. The dissolved oxygen (DO) for certain salinity can thus be calculated with the formula:

$\ln(DO) = A_1 + A_2 \cdot (100/T) + A_3 \cdot \ln(T/100) + A_4 \cdot (T/100) + S \cdot [B1 + B2 \cdot (T/100) + B3 \cdot (T/100)2]$

Where A_1 is -173.429, A_2 is 249.6339, A_3 is143.3483, A_4 is-21.8492, B_1 is -0.0331, B_2 is 0.014259, B_3 is -0.0017, S is the salinity and the temperature is 310.15 Kelvin

(273.15 + 37°C). This equation gives a solubility of oxygen of 4.70 ml/L in a Ringer's solution which is **6.71** mg/L when converted (by multiplying with 1.4276).

The equation for calculating the partial pressure of oxygen (mmHg) from DO is

$$P_{O2} = V_m \cdot P_N \cdot 0.75 / (\alpha(T) \cdot 1000 \cdot M_{O2})$$

Where V_m is the molar volume of oxygen (22.414 L/mol), P_N is the standard pressure (1013 bar), $\alpha(T)$ is the Bunsen absorption coefficient for the given temperature ($24.00359 \cdot 10^{-3}$ for 37°C), and M_{O2} is the molecular mass of oxygen (32 g/mol). This equation gives us a partial pressure of oxygen of **149.8 mmHg** in a Ringer's solution equilibrated with room air (conversion factor of 22.173). In one of the experiments, 100% oxygen was administered to the animals. The partial pressure in a solution with 100% oxygen is hence interesting to calculate. The oxygen solubility in such a solution would be 32.0948 mg/L (0.04223·760), which may be converted into a partial pressure of 711.6 mmHg which is thus the maximum oxygen pressure in a solution.