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Locomotor Recovery after Spinal Cord Injury: a Multidisciplinary Investigation of the Role and Potential of Preserved Tissue.

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Mémoire présenté en vue de l'obtention du grade de
Docteur en Sciences Biomédicales et Pharmaceutiques (Bologne)

Année académique 2007/2008

Abstract

Background

Spinal cord injury is devastating for its victims because of the resulting motor, sensory and autonomic deficits, i.e., paraplegia and tetraplegia. The different deficits are mainly due to the interruption of the long white matter tracts which connect the supraspinal central nervous system to the spinal cord. In many cases, the interruption is partial and some recovery may be observed. The preserved spinal cord tissue plays a role in the recovery which, to date, has only been incompletely elucidated. The preserved spinal cord tissues have been investigated in the present work from two angles. The first part describes an attempt to recruit the locomotor generator of the sub-lesional cord, using several therapeutic strategies, in order to improve locomotor recovery. The second part describes a new approach for the investigation of the injury site and the preserved white matter; correlating magnetic resonance imaging and histology with locomotor recovery.

Methods

Subdural balloon compression was used in the rat to induce incomplete spinal cord lesions which allow partial locomotor recovery. Locomotion was assessed with a widely employed, detailed behavioural scale. In the first set of experiments, physiotherapy (body weight supported treadmill training), repetitive transcranial magnetic stimulation (rTMS), and monoaminergic pharmacotherapy (clonidine and fluoxetine) were used individually or in combination, five days a week, over several weeks. Spinal cords were analysed for histological and immunohistochemical correlates of recovery, including sub-lesional serotonin content. In the second set of experiments, experimental high field magnetic resonance imaging (MRI) was used *post-mortem* to investigate the spinal cord lesion and spared white matter. A comparison was then

made between the MRI and subsequent histological data. Morphometric parameters assessing the lesion extent, spinal cord atrophy, and white matter sparing were correlated with locomotor function. In a supplementary *post-mortem* MRI investigation, an explanted sample of human sub-lesional thoracic cord was analysed after a severe cervical spinal cord injury.

Results

Body weight supported treadmill training had a clear beneficial effect when initiated early after injury. Repetitive TMS also appears to increase locomotor recovery after low thoracic spinal cord injury, associated with an increase of serotoninergic innervation of the sub-lesional spinal cord segment. Combining rTMS with clonidine therapy appeared to have synergistic positive effects on locomotor recovery, but no statistically significant results could be obtained, due to the variability of the observed locomotor scores. Histology of the cords in the latter experiment did not allow sufficient anatomical or quantitative comparison of white matter sparing, a potential reason for the behavioural variability. In the subsequent series of untreated rats, *post-mortem* MRI of the spinal cord precisely showed the lesion size and topography, as well as white matter sparing, with high spatial precision. MRI could differentiate between lesion components. The evolution of the lesion was followed from the acute to the chronic stage. Different morphometric parameters were statistically significantly correlated with locomotor function. In the human spinal cord sample, the almost complete sub-lesional degeneration of the white matter tracts was precisely demonstrated with the same technique.

Conclusion

The present investigations confirm and extend the notion that partially preserved spinal cord tissue plays a key role in locomotor recovery after spinal cord injury. The intrinsic locomotor circuitry of the spinal cord is a promising therapeutic target for various strategies which can be combined and are potentially rapidly applicable in the clinical situation, as all the investigated treatments are already used in humans for different indications. The quantity and topography of white matter sparing is of major importance in locomotor recovery, and it can be precisely assessed with *post-mortem* MRI.

Résumé

Introduction

La tétra- et la paraplégie résultant d'un traumatisme médullaire sont principalement dues à l'interruption des voies longues qui connectent les centres supraspinaux aux circuits intramédullaires. La majorité des lésions médullaires sont anatomiquement incomplètes et une récupération fonctionnelle peut être observée dans bon nombre de cas. Le rôle du parenchyme médullaire épargné dans la récupération n'est que partiellement compris—que ce soit celui de la substance blanche périlésionnelle ou celui des circuits médullaires situés à distance de la lésion. Premièrement, nous avons tenté de recruter les circuits médullaires sous-lésionnels par différentes stratégies thérapeutiques, dans le but d'améliorer la récupération locomotrice. Ensuite, nous avons mis au point une technique d'imagerie par résonance magnétique (IRM) permettant l'étude de la substance blanche préservée et corrélé les images obtenues et l'histologie à la récupération fonctionnelle.

Méthodes

Après un traumatisme médullaire expérimental incomplet, nous avons suivi l'évolution locomotrice au moyen d'une échelle comportementale détaillée, largement utilisée. Dans la première partie du travail, différents groups de rats ont été traités pendant plusieurs semaines par physiothérapie (entraînement sur tapis roulant avec support du poids du corps), par stimulation transcrânienne magnétique répétitive (STMr), et par substances monoaminergiques (clonidine et fluoxétine), seuls ou combinés. Des analyses histologiques et immunohistochimiques des moelles épinières ont été réalisées, visant notamment à mettre en évidence la sérotonine dans le parenchyme sous-lésionnel. Dans la deuxième partie du travail, nous avons étudié la lésion médullaire et la substance blanche épargnée par IRM *post-mortem*, comparant

sa précision à celle de l'histologie standard. L'étendue de la lésion, le degré d'atrophie médullaire et la quantité de parenchyme préservé ont été mesurés et corrélés au comportement locomoteur. Dans une étude supplémentaire, un échantillon de moelle épinière humaine thoracique sous-lésionnelle a été analysé par la même technique d'IRM.

Résultats

L'entraînement par tapis roulant précoce potentialise la récupération fonctionnelle. La STMr semble également avoir un effet favorable sur la récupération dans les lésions médullaires thoraciques basses, augmentant l'innervation sérotoninergique de la moelle épinière sous-lésionnelle. Parmi les stratégies thérapeutiques combinées, nous avons également observé que les rats traités par STMr et clonidine montraient globalement une meilleure récupération locomotrice. Toutefois, cet effet n'était pas statistiquement significatif, en raison d'une variabilité trop importante des scores locomoteurs—possiblement attribuable à une hétérogénéité de préservation de substance blanche au sein des groupes expérimentaux. L'histologie n'a cependant pas permis de confirmer cette hypothèse. Chez les rats non traités, dans la deuxième partie du travail, l'IRM *post-mortem* de haute résolution nous a permis d'évaluer très précisément la lésion médullaire et la substance blanche préservée. Elle a également permis de distinguer différents composants histologiques de la lésion et son évolution du stade aigu au stade chronique. Les paramètres morphométriques ont pu être corrélés significativement au comportement locomoteur. Dans la moelle épinière humaine, une dégénérescence quasi complète des voies descendantes sous-lésionnelles a été démontrée.

Conclusion

La moelle épinière partiellement préservée joue un rôle clé dans la récupération locomotrice après un traumatisme médullaire. Les circuits locomoteurs intrinsèques sont une cible prometteuse pour différentes stratégies thérapeutiques qui peuvent être combinées et qui sont potentiellement rapidement applicables en clinique, puisque tous les traitements investigués sont déjà utilisés à d'autres fins chez l'homme. La quantité et la topographie de substance blanche épargnée sont d'une importance primordiale dans la récupération fonctionnelle, et celles-ci peuvent être précisément étudiées par l'IRM *post-mortem*.

Nomenclature List

5-HT	Serotonin (5-hydroxytryptamine: monoaminergic neurotransmitter)
BBB	Basso, Beattie, Bresnahan locomotor rating (a non-linear behavioural scale)
BDNF	Brain Derived Neurotrophic Factor
BWSTT	Body Weight Supported Treadmill Training
CNS	Central Nervous System
CoST	Coeruleospinal Tract
CPG	Central Pattern Generator (here, generally, the lumbar locomotor CPG)
CST	Corticospinal Tract
dti	diffusion tensor imaging
dpo X	X days post-operatively (i.e., X days after experimental SCI)
GABA	Gamma-Aminobutiric Acid (neurotransmitter)
GFAP	Glial Fibrillary Acidic Protein (marker for astrocytes)
ip	intraperitoneal(ly)
MBP	Myelin Basic Protein (marker for myelin)
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
NA	Number of Averages (MRI parameter, influencing image resolution)
NF	Neurofilament (marker for axons)
NT3	Neurotrophin 3
PBS	Phosphate Buffered Saline
PD	Proton Density (weighted): one MRI acquisition sequence.
PD8	Proton Density weighted images, NA = 8 (lower resolution)
PD32	Proton Density weighted images, NA = 32 (higher resolution)
PFA	Paraformaldehyde
RaST	Raphespinal Tract
RST	Rubrospinal Tract
RtST	Reticulospinal Tract
rTMS	repetitive Transcranial Magnetic Stimulation
SCI	Spinal Cord Injury
T	Tesla (SI Unit for magnetic field strength)

TE	Echo Time (MRI acquisition parameter, influencing tissue contrast)
TI	Inversion Time (MRI parameter: annihilates specific protons' signal)
TMS	Transcranial Magnetic Stimulation
TR	Repetition Time (MRI parameter, influencing tissue contrast)
trkB	tyrosine receptor kinase B (receptor for BDNF)
VST	Vestibulospinal Tract

Remerciements

Beaucoup de personnes ont contribué de près ou de loin à ce travail et à mon épanouissement dans l'apprentissage des sciences neurologiques. Qu'il me soit permis de leur exprimer à toutes ma reconnaissance.

Le Professeur Gustave Moonen m'a accueilli dans son laboratoire et m'y a initié à la recherche fondamentale. Je lui suis particulièrement reconnaissant de son soutien sans réserve pour mes recherches, en plus d'avoir assuré ma formation clinique en neurologie.

Le Professeur Jean Schoenen a donné au travail expérimental son orientation scientifique, dès sa conception, grâce à sa connaissance approfondie de la moelle épinière, à ses inspirations inépuisables et à son esprit critique incisif et constructif. Sa capacité d'interprétation des données scientifiques est exceptionnelle.

Les qualités de médecin, d'enseignant et de chercheur du Professeur Didier Martin m'ont profondément influencées, y compris son esprit structuré et didactique, sa capacité d'aller à l'essentiel, son bon sens et sa persévérance. Il a marqué de son empreinte mon évolution dans la neurochirurgie académique en encourageant ma double formation fondamentale et clinique.

Le Professeur Achille Stévenaert m'a accueilli dans le service de neurochirurgie à l'Université de Liège. Je lui dois le plus grand respect pour sa clairvoyance et sa rigueur, et je le remercie pour son enseignement neurochirurgical et général, pour ses précieux conseils et pour ses clins d'œil.

Les nombreuses discussions et heures passées sur divers travaux avec le Professeur Agrégé Gary Brook, lors de son séjour ici ou au Klinikum à Aix, ont toujours été un immense plaisir. Il incarne pour moi la compétence, la rigueur, l'honnêteté, et l'humilité scientifiques.

Je remercie également le Docteur Rachelle Franzen, sans laquelle ce travail aurait été impossible à réaliser. Je lui suis reconnaissant de l'aide et de la motivation qu'elle y a apportées.

Mes remerciements particuliers vont à mon fidèle compagnon de travail, Rémy

Phan-Ba. Merci pour ta disponibilité, ton investissement, ta bonne humeur, et pour tous les bons moments passés ensemble.

Merci aussi à tous les autres membres du laboratoire qui ont contribué à la réalisation de ces recherches, dont les Docteurs Sylvie Multon (tapis roulant), Delphine Bouhy et Anne-Lise Poirrier (rTMS).

Coralie, tu as travaillé avec courage et persévérance. Merci pour ta participation importante au travail pratique, *in vivo* et *post-mortem*, pour la préparation toujours impeccable de notre petite salle d'opération et pour ta mémoire infaillible qui gardait trace de tout et savait où chercher quand il fallait.

Jeanine, merci pour votre calme, votre aide, vos conseils dès mes premiers pas au laboratoire de neuroanatomie et d'avoir été présente au moment où la poursuite des expériences en dépendait. Murielle : merci pour toute ton aide ; je me souviendrai toujours de ta bonne humeur lors de nos interminables évaluations BBB du lundi. Arlette, merci d'avoir fait avec moi mes premiers pas expérimentaux—et chirurgicaux !

Ce travail repose en bonne partie sur une collaboration étroite avec l'équipe du Professeur Jan Gelan à Diepenbeek. Je remercie les Docteurs Peter Adriaensens, Lisbet Storme et Evi Theunissen pour la mise à disposition du précieux matériel de résonance magnétique, leur compétence et leur investissement au long cours.

J'exprime ici également ma gratitude au Professeur Charles H. Tator qui m'a accueilli dans son laboratoire et dans le service de neurochirurgie du Toronto Western Hospital, et à toute l'équipe, dont plus particulièrement les Docteurs Mojgan Hodaie, Eve Tsai, Christopher Wallace et Sarah Woodrow pour leur compagnonnage.

J'adresse mes remerciements chaleureux au Docteur Martin Scholsem, qui reprenait sur ses épaules le travail clinique des assistants quand j'étais au laboratoire. J'ai eu la chance de pouvoir partager trois ans de ma formation avec ce "grand frère chirurgical". On a traversé des étapes cruciales ensemble et passé de merveilleux moments.

Merci aussi aux Docteurs Frédéric Collignon, Annie Dubuisson, Colette Fransen, Bruno Kaschten, Jacques Lenelle, Pierre Robe et à l'équipe infirmière d'avoir participé à créer ce terrain fertile sur lequel j'ai pu commencer mon chemin vers la neurochirurgie.

Anne-Françoise Donneau a courageusement réalisé les analyses statistiques, sous la direction du Professeur Adelin Albert ; je les en remercie.

Last but not least, merci à Jean-Christophe Leyder et Rémi Lambert pour leur introduction à LaTeX, avec lequel j'ai écrit et mis en page ce travail.

Ce travail a bénéficié du soutien du Fonds National de la Recherche Scientifique, de la Fondation Léon Frédéricq (Bourse Lejeune-Lechien), et de la Fondation Internationale pour la Recherche en Paraplégie.

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Introduction

Chapter 1

Spinal Cord Injury—the Clinical and the Neurobiological Problem

RESTORING LOST FUNCTION AFTER INJURY TO THE NERVOUS SYSTEM is one of the great challenges of current neuroscientific research. This is particularly true for spinal cord injury (SCI): despite massive research efforts, traumatic paraplegia and tetraplegia still remain incurable.

1.1 Clinical Background

1.1.1 Epidemiology and Public Health

Although the general prognosis of SCI is not as dismal as it used to be in the not so distant past [149], life expectancy still remains lower than that of uninjured persons, and the socio-economical impact of SCI is considerable. Approximately 80% of the patients are young males, with an average age at injury of less than 40 years. About half of the patients suffer from tetraplegia, the other half from paraplegia. Almost two thirds of the patients report being employed before the injury; 10 years after, only one quarter of tetraplegics and one third of paraplegics are [4].

The yearly incidence is estimated at approximately 20 cases per million population in France [2], and approximately 40 per million in the United States of Amer-

ica. Estimated lifetime costs range, e.g., from approximately 500,000 US$ for a 50 year old incompletely injured patient to 3,000,000 US$ for a 25 year old completely tetraplegic patient. This does not include indirect costs of about 60,000 US$ per year, accounting for losses of wages, fringe benefits and productivity [4].

1.1.2 Clinical Presentation

It is far from trivial to reiterate how catastrophic traumatic SCI is for the individual, and the first and major complaint of any victim will be the inability to walk, i.e. *the loss of locomotion*. But para- and tetraplegia are more than that—spinal cord injury means to be, at once, deprived of private and professional perspectives, to lose working capacity, autonomy, and sexual function... many previously basic daily routines become challenging daily obstacles [179].

The clinical manifestations result from the interruption of the connections between the supra-spinal control centres and the spinal cord circuits caudal to the lesion site, i.e., the *deafferentation of the sub-lesional cord*. The so-called spinal cord syndromes may be "incomplete" or "complete".

In incomplete cord syndromes (approximately 50% of the cases), the loss of sensory and/or motor function below the level of injury is partial; the type of neurologic deficit is variable.

In complete cord syndromes (the remaining 50%), motor and sensory function below the level of the lesion is entirely lost.

So-called discomplete cord syndromes are *clinically complete*, but *neurophysiologically incomplete*: there is electrophysiological evidence of residual brain influence on spinal cord function below the lesion [91]. In one study, more than four out of five clinically complete syndromes could therefore neurophysiologically be classified as discomplete [277].

Although the deficits are for the most part irreversible, the clinical presentation may evolve over time. Spasticity develops, bladder function changes, and patients may progressively recover some of the lost sensory or motor function.

1.1.3 Magnetic Resonance Imaging of the Spinal Cord

Magnetic Resonance Imaging (MRI) is used to assess the injured spinal cord, in particular for the presence of haemorrhage and oedema [70, 68].

1.1.3.1 Technical Background

MRI is based on the application of a magnetic field to the protons that are present in biological tissues and which, being electrically charged, behave like small magnets and align along the axis of the field. In addition to the constant magnetic field, MRI scanners deliver electromagnetic pulses. After the application of a pulse, a certain proportion of the protons moves to a higher energetic state. Their spontaneous return to a lower energy level, called "relaxation", is associated with the emission of energy, called "echo signal", which can be captured and analysed. The time needed for relaxation of the protons depends in part on their physical environment. This environment and the proton content (or proton density) differ between the tissues of the body, explaining the high contrast that can be obtained by MRI, in particular in the central nervous system.

1.1.3.2 Image Contrast

Contrast in the MR images depends on the biological properties of the different tissues. In addition, it is influenced by the magnetic pulse sequences that are applied, as well as the time point in the proton relaxation process when the echo signal is captured. Parameters that influence contrast include "echo time" (TE, the time between the application of the pulse and the peak of the "echo" signal), the "repetition time" (TR, the amount of time that exists between successive pulse sequences), inversion time (TI, the time period between the first pulse and a second pulse which annihilates the echo signal of protons in a specific type of environment), etc. These parameters define the MRI acquisition sequences.

Among the standard acquisition sequences, the most basic ones are T1 weighted (based on the so-called "longitudinal relaxation" of the protons), T2 weighted (based on the so-called "transverse relaxation"), and proton density (PD) weighted (measuring mainly the concentration of protons in a tissue).

- In T1-weighted sequences, cerebrospinal fluid is black; they are useful to evaluate the size of the spinal cord.
- In T2-weighted sequences, cerebrospinal fluid is white; these are much more sensitive to the presence of tissue abnormalities, including spinal cord oedema and haemorrhage. Transverse T2 weighted images are also used evaluate the shape of the cord and the presence of spinal canal compromise because of high contrast of the tissues with the bright cerebrospinal fluid surrounding the cord.
- PD-weighted images do not show high tissue contrast in the normal human central nervous system, but may be used to detect loss of myelin.

- There are many other sequences, including so-called inversion recovery sequences (IR). The latter are based on the above T1, T2, or PD sequences, but the signal of one specific "tissue component" is "removed" from the image, e.g., fat, on the basis of the specific behaviour of the protons in that particular tissue.

- Diffusion based techniques, like diffusion tensor imaging (dti), are based on the Brownian motion [6] of water molecules. In fact, the signal of the protons contained in the water molecules varies depending on their Brownian motion, which may be restricted by highly structured tissues like myelin. Thus, highly myelinated regions may be detected with this kind of technique, and dti precisely depicts the white matter tracts of the human central nervous system (CNS) in the three dimensions. So called probabilistic tractography, based on dti, is presently used in neurosurgical preoperative planning to avoid injury to important white matter pathways.

1.1.3.3 Image Resolution

The resolution of the MR image depends on many factors (table 1.1), including the strength of the magnetic field (measured in tesla, T). In recent years, the field strengths of clinical MR scanners have increased to up to 3 T for standard MRI. However, the resolution of the images is influenced by factors as diverse as the homogeneity of the magnetic field, the size of the coil (influencing the signal to noise ratio), the acquisition time, especially in *in vivo* and functional imaging, etc.

Lack of motion
Size of the radio-frequency coil
Homogeneity of the magnetic field
Stability of the linearity of the gradient fields
Strength (tesla) of the magnetic field
Duration of the acquisition

Table 1.1: Factors contributing to MR image resolution.

In the research setting, small bore-high field spectrometers, which can address some of these aspects (table 1.1), have been used for years for basic science applications, and can be used on biological tissues.

1.1.3.4 Clinical Use of MRI for the Evaluation of Spinal Cord Injury

In the clinical setting, prognostic criteria have been defined on the basis of gross alterations of the cord parenchyma that can be detected by MRI in the acute state, concerning global functional outcome [190] as well as long term neurological deficits [116, 117]. In a recent study, prognostic criteria have been redefined and prospectively validated, including abnormal signal in the cord (oedema, haemorrhage) and various measures of spinal cord compression [220].

However, despite the high contrast that can be obtained, the densely packed functional subdivisions of the cord cannot be demonstrated with current clinical MRI resolution, which presently remains low. One of the reasons is the limited magnetic field strength used in general imaging practice, with a maximum of 3 T. Currently, field strengths up to 9.4 T are being evaluated for clinical application [5].

1.2 The Neurobiology of Spinal Cord Injury

1.2.1 The Spinal Cord Lesion

1.2.1.1 Physiopathology

The initial traumatic "blow" to the spinal cord, caused by vertebral fractures and displacement of bone and other structures, not only causes primary damage. It also *initiates a complex, destructive cascade of secondary events* leading to local extension of the damage [11, 148, 296, 297]. The necrotic lesion spreads, and, in the spinal white matter, more and more axons are damaged [8], resulting in increased deafferentation of the sub-lesional spinal cord and aggravated neurological deficits.

The secondary processes may be variable despite reproducible trauma to the spinal cord. This is not without importance for SCI research, since *"injury reproducibility is an important characteristic of experimental models of spinal cord injuries because it limits the variability in locomotor and anatomical outcome measures"* [22]. Therefore, if—and only if—a lesion and the degree of motor impairment and recovery following SCI are reproducible among experimental groups, differences in behavioural outcomes may be attributed to an investigated therapeutic intervention.

1.2.1.2 Histology

The spinal cord's volume initially increases at the lesion site, due to tissue oedema (figure 1.1B). Later on, the distal components of severed axons degenerate in so-called Wallerian degeneration, neurons undergo apoptosis, and glial scar tissue forms [144]. Glial scarring occurs at the lesion site itself [108] and has been demonstrated in the

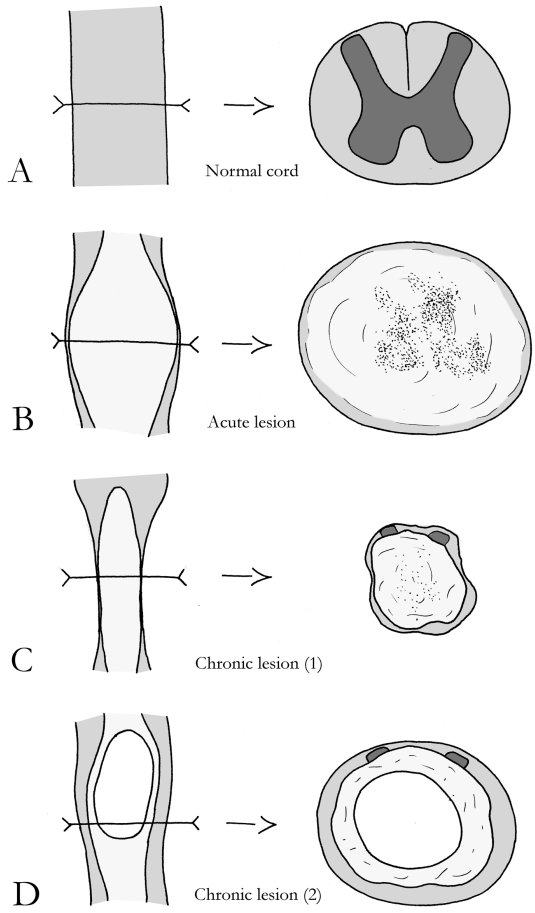

Figure 1.1: Schematic representations of gross cord changes after SCI. (A) Normal cord. The central, butterfly shaped grey matter is depicted in dark grey. (B) Acute lesion. Cord volume is increased at the lesion site. At the lesion centre, central haemorrhage can be seen (black dots), mainly in the grey matter areas. Oedema and beginning necrosis have invaded almost the entire transverse section (clearer shade of grey). (C) Chronic lesion. The scarring processes have resulted in retraction of the lesion and atrophy of the cord at the lesion site and haemosiderin deposits where the lesion was haemorrhagic (black dots). Spared white matter is seen around the lesion centre and the tips of the dorsal horns (dark grey) are preserved. (D) Chronic lesion with a central cyst (white). Total volume is increased.

human corticospinal tract [53]. Retraction of the tissue can be observed and the spinal cord undergoes atrophic changes, reducing its volume at the lesion site (figure 1.1C). In many cases, cyst formation can be observed (figure 1.1D).

1.2.2 Axonal Regeneration

The clinically important neurological deficits result from the loss of axonal connections of the supra-lesional centres to the sub-lesional cord. "Rewiring" the spinal cord by regenerating lost axons therefore appears as a promising therapeutic perspective (see below). However, after CNS injury, axonal regeneration was long thought to be impossible. In his classic treatise on nervous system degeneration and regeneration [55], Ramón y Cajal argued that:

> Once development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centres, the nerve paths are something fixed, ended and immutable. Everything must die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.

Nonetheless, as early as 1911, Cajal's student J. F. Tello had grafted peripheral nerve segments onto CNS tissue and shown that central nervous system neurons could regenerate into such a favourable environment [299]. At the beginning of the 1980s, the group of Aguayo published descriptions of the growth of CNS axons into peripheral nerve [78] and found that these CNS axons were basically functional, although with limited or altered synaptic contacts (probably due to the absence of their target) [225]. During the same period, fetal CNS neurons were shown to integrate into spinal cord tissue and even to "rescue" injured supraspinal neurons in the neonatal brainstem [44, 75, 250]. In the lesioned adult rat spinal cord, a substantial intrinsic axonal regenerative attempt was demonstrated, although it spontaneously regressed after a few weeks [46].

1.2.3 The Sub-Lesional Segment

The spinal cord, as part of the CNS, is more than a passive conduit and relay for neural messages between the supraspinal centres and the peripheral nerves. A complex intrinsic circuitry exists, mainly in the cervical and lumbar enlargements of the spinal cord. These circuits autonomously generate structured neural activity, including rhythmic output which results in basic locomotor patterns [139, 86]. After cervical or thoracic SCI, the lumbar circuitry remains anatomically intact, and plastic changes appear to be responsible for the change in neurological presentation that can be observed over time (see below).

1.3 Basic Therapeutic Approaches

Three types of strategies to treat the traumatic deafferentation of the sub-lesional segment result from the above [252]:

1. **Neuroprotective treatments** are designed to decrease the initial damage to the spinal white matter by interfering with the cascade of events that lead to secondary tissue injury [147, 148, 237, 305], in order to potentially decrease the final extent of the lesion, therefore to protect connections between the supraspinal centres and the sub-lesional cord, and thus to reduce the ensuing neurological deficits.

   In a series of clinical trials, the steroid antiinflammatory drug methylprednisolone appeared to have shown some promise as a neuroprotective agent at very high doses [40, 41, 42][1]. However, the initial enthusiasm has largely been tempered [164] and certain clinicians do not consider high dose methylprednisolone a standard of care anymore, but rather a treatment option [163]: according to a set of 2002 Guidelines for Management of Acute Cervical Spinal Cord Injuries, "treatment with methylprednisolone for either 24 or 48 hours is recommended as an option in the treatment of patients with acute spinal cord injuries that should be undertaken only with the knowledge that the evidence suggesting harmful side effects is more consistent than any suggestion of clinical benefit" [1]. However, in clinical practice, methylprednisolone is often used "despite the well-founded criticisms that have been directed against the [NASCIS] trials, given the devastating impact of SCI and the evidence of a modest, beneficial effect of MP" [109].

   Other substances have been investigated in the human. They include tirilazad, a (non glucocorticoid) steroid with more specific anti-oxidant effects and potentially fewer glucocorticoid side effects, but which appeared to be neither more efficient nor better tolerated than methylprednisolone. Another compound which was experimentally promising, the monosialoganglioside compound GM1, has not shown any significant clinical benefit so far [148].

   Thus, although neuroprotective strategies have been conceived for potentially rapid clinical application, the "magic bullet" is yet to be found.

2. Once the lesion size is established, and the sub-lesional cord disconnected, the ideal therapeutic approach would be the **repair of the lesion and reconnection** by regrowing axons. Since the experimental demonstration that CNS

[1] National Acute Spinal Cord Injury Study (NASCIS), USA

neurons have the potential to regenerate, at once, functionally useful repair of SCI appeared achievable. An expanding research movement has been investigating CNS axonal regeneration and the mechanisms inhibiting it [107], in particular the glial barrier [108], which had already been described by Cajal [55], and growth inhibitory molecules in the adult CNS which are mainly associated with CNS myelin [271, 273].

Axonal regeneration is, however, *only a first step* in the complex sequence which could potentially lead to useful spinal cord repair: after penetration into the lesion site through a fibroglial scar, an axon would have to grow in an orientated manner over a sufficient distance of lesion tissue to cross the lesion site, then exit the lesion site, continue growth in the sub-lesional central nervous parenchyma, reach an appropriate target, stop its growth there and form synaptic contacts with remaining, intact neuronal circuits, and myelinate, to finally re-establish a viable, functional circuitry. This sets an ambitious goal.

Currently, the measurable functional improvement after experimental repair attempts remains moderate at best [334]. Nonetheless—and despite a number of fundamental structural problems which hinder efficient translation into human investigations—an impressive number of repair strategies have already found their way into clinical trials [295].

3. The third therapeutic access is **the recruitment of remaining, preserved nervous tissue**, including the remaining intrinsic circuitry of the spinal cord, and especially the sub-lesional locomotor circuits. The goal is to modulate these circuits to enhance structured neuronal activity, and thus to increase neurological recovery, while avoiding undesirable modifications like spasticity.

1.4 Summary

In summary:

- The main clinical problem is the deafferentation of the sub-lesional part of the spinal cord, resulting in neurological deficits, in particular the loss of locomotion.
- Remote from the lesion, the spinal cord is anatomically intact.
- In most cases, spared tissue can be observed at the level of the lesion, including perilesional white matter.

- In most cases, even of clinically complete SCI, the brain retains some electrophysiologically detectable influence on the sub-lesional cord.
- The neurological functions of the deafferented sub-lesional cord circuitry are restructured after SCI, including a certain degree of functional recovery.

Thus, the preserved spinal parenchyma, including the intrinsic spinal circuitry as well as the perilesional white matter, appears to be the key player in the study of locomotor recovery after SCI.

Chapter 2

The CPG, White Matter Preservation, and Recovery after Spinal Cord Injury

2.1 The Central Pattern Generator

THE LOCOMOTOR ACTIVITY OF THE LOWER LIMBS in mammals is based on the finely tuned interaction of a tripartite system. The three components are the following:

- The basic, rhythmic neuronal activity of a cellular network located in the lumbar spinal cord called the locomotor "central pattern generator" (CPG) [86, 92, 99, 135, 182, 183, 206, 232, 239, 337],
- Supraspinal input, modulating the rhythmic activity of the CPG to adapt to complex external requirements (e.g. maintaining equilibrium of the body when mechanical constraints change and initiating locomotion when needed by voluntary commands, ...), and
- Sensory feedback from the lower limbs, allowing for example adaption to external conditions (e.g., uneven terrain, ...) or modulation of the supraspinal input to the CPG [337].

This neural network can be found in the spinal cord of all vertebrates, like the lamprey [138] and mammals [139] including humans [86, 92, 337]. In the human newborn (before the maturation and myelination of the corticospinal tract (CST), which controls voluntary movement), and even in anencephalic children, stepping movements can be observed, both in the absence of peripheral stimuli, or in response to the latter [119]. The lumbosacral circuitry controlling locomotion in the rat and in the human appears to be very similar [128].

2.1.1 Anatomy and Neurophysiology of the Locomotor CPG

The locomotor CPG corresponds to neuronal populations located in the ventral part of the caudal spinal cord (predominantly in lamina VII, but also in laminae VIII and IX, figure 2.1). The circuitry contains, among others, excitatory interneurons, which appear to play a role in rhythm generation, as well as commissural interneurons [290] connecting one spinal cord half to the other, in one segment or over several levels, playing a role in left-right coordination. In addition, there are intersegmental interneurons in the ventral cord, connecting the rostral part of the CPG to its caudal part [182, 337].

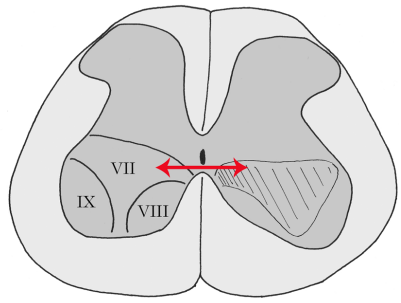

Figure 2.1: Anatomy of the CPG in the rat lumbar cord (hatched area). VII-IX: Rexed's laminae. The density of oscillating neurons is highest towards the central canal, and in the ventral grey commissure, there are inhibitory interneurons connecting the right and left laminae VII (double arrow) and mediating so-called reciprocal inhibition, a key process in the generation of locomotion with alternating limb movements [60].

The precise rostro-caudal distribution of the network in the low thoracic, lumbar and sacral cord differs between species; in rodents, it extends from the lowest

thoracic segments to the lower lumbar cord. In mammals, in general, the CPG appears to be composed of multiple rhythm-generating core networks ("modules") distributed over several cord segments [182], and the locomotor output capacity is greater in rostral than in caudal segments, in the rat [209] as well as all the other studied species [182].

In rats, the locomotor output appears to mainly originate at the thoracolumbar junction (T13-L2 [61, 209]). In a photolesion study of spinal cord injury in the low thoracic (T8) *versus* the upper lumbar cord (L2), the locomotor deficits were more severe in L2-lesioned rats [126]. In rats, cats, and humans, electrical stimulation of the lumbar cord can induce locomotor-like neuronal activity patters, and L2 is the most responsive level in rats and in humans [128]. The neurophysiological rostrocaudal gradient is influenced by the differential distribution of neurotransmitters like serotonin and dopamine. This particular neuronal organisation appears essential for the generation of a stable locomotor pattern [65].

The locomotor activity of the CPG is activated by supraspinal input, which originates in the brainstem and midbrain, resulting in rhythmic walking, ipsilateral coordination of flexors and extensors, and left-right coordination. In addition, the CPG activity is modulated by somato-sensory afferents reaching the spinal cord through the peripheral nerve. This creates dynamic sensorimotor interactions, either directly with the CPG, or, *via* ascending and descending long white matter tracts, after processing by supraspinal motor centres [258].

Monoamines (like serotonin) from the brain stem appear to play a key role in the modulation of motor neuron activities [294] and locomotor pattern generation [130, 169, 182, 254, 267], as well as other functions of the sub-lesional spinal cord which rely on central pattern generation, such as ejaculation [58, 59]. Serotonin is mainly of supraspinal origin in the rat spinal cord [267], and the loss of serotonin in the rat lumbar cord after SCI correlates with the loss of locomotor function [153].

2.1.2 Consequences of Spinal Cord Injury

Most human spinal cord injuries are located in the thoracic or cervical cord. Thus, the CPG is generally caudal to the lesion and not directly injured itself. Anatomically intact, it is—at least partially—disconnected from supraspinal control by the interruption of the white matter tracts.

In most species, CPG neuronal activity patterns sufficient to generate locomotion depend on supraspinal input [337], even though the locomotor CPG may produce coordinated motor patterns in isolation (in cats, locomotion recovered after low thoracic transections can be attributed to autonomous CPG activity and is abolished by a subsequent L3 lesion [213]).

SCI interrupts the long white matter tracts, leading to the neurological deficit, particularly the loss of walking ability [86]. Therefore, functional locomotor output may be recovered if the interplay between supraspinal control, persisting peripheral input, and the CPG could be reconstructed. This depends on CNS plasticity.

2.2 Spinal Cord Plasticity after Injury

The spinal cord's circuitry is capable of plasticity throughout life [330]. This has been shown for the relatively simple monosynaptic reflex arc [331]: training effects on the reflex arc observed in uninjured monkeys persisted after spinal cord transection, i.e., after complete isolation of the trained spinal cord segment from supraspinal influence [332]. Neuronal circuits within the human spinal cord also appear to be capable of a certain degree of "learning" [104, 222, 329].

After SCI—as mentioned before—spontaneous motor recovery can occur, as well as the development of spasticity, central neuropathic pain, autonomic dysreflexia, and emergence of bladder and sphincter dyssynergy after initial areflexia. As these functions rely on lumbar spinal cord circuitry, the deafferented human sub-lesional cord appears to spontaneously undergo plastic changes. Human and laboratory investigations confirm that:

- In SCI patients who are asked to execute volitional motor tasks, electromyographic recordings of the activity of the sub-lesional cord have shown the presence of a **"new" electrophysiological anatomy**, correponding to disconnected circuits, in addition to the "reduced" electrophysiological anatomy, which is still under cortical control [93].

- After CNS injury, neuronal circuits can undergo **synaptic reorganisation**, much like classical CNS learning processes [100, 154, 168, 226]. The recovery of locomotor function after experimental SCI in the cat is associated with changes in synaptic structure, including size, number and distribution [293, 302], as well as function [27, 160, 161]. A review of clinical, neurophysiologic, and neuropathologic data from SCI patients, compared with data from animals, concluded that the long term functional recovery occurring slowly and progressively (after the early post-traumatic phase when recovery is rapid, if it occurs) was likely to be the consequence of "presynaptic" plasticity, such as local synapse growth [195].

- Another important type of plasticity, which has been demonstrated extensively after SCI, is based on the so-called **sprouting** of nerve fibres. Sprouting

is the growth of a new branch from an axon. It can be "regenerative", by injured nerve fibres (see also 2.3.2.2), or "collateral", i.e., growth of a new axonal branch arising from a non-injured axon [12, 143, 334]. The new axonal branches may connect to deafferented circuits.

- After experimental SCI, the **neurochemical environment** below the lesion changes: not only are monoamines lost, like noradrenalin and serotonin [145, 153, 211], but others, such as GABA, increase [303].

  In addition to modifications of neurotransmitters and -modulators, the neurotrophic environment of the spinal cord changes. For example, BDNF expression is up-regulated—in a biphasic pattern. First, neurons and glia express BDNF for approximately three days. In the second, more sustained response, beginning seven days after inury, BDNF is mainly found in macrophages/microglia [166] and could thus influence the re-structurating of neuronal activity. This is because BDNF (like NT-3) not only enhances *sprouting* of nerve fibres [270, 339], including that of supraspinal descending tracts [85, 172, 197], but also plays a role in *synaptic plasticity*, with long term effects [157, 201, 217, 266, 278, 314, 316]. BDNF and markers of plasticity (like synapsin for synaptic terminals and growth associated protein 43 for sprouting) were also found to be increased in the *cervical* region after complete transection [133], resulting in a potentially favourable environment for plastic changes *rostral* to the lesion.

2.3 The Role of White Matter Sparing after Spinal Cord Injury in Locomotion and Recovery

2.3.1 Human Evidence

Anatomically, human SCI is usually incomplete; complete spinal transection occurs in only a minority of patients. Thus, even in the majority of clinically complete cord syndromes, a rim of white matter is usually preserved at the lesion level [50, 51, 175]. This has been confirmed by clinical imaging.

The spinal cord white matter contains the long axonal tracts which connect the supraspinal centres to the spinal cord circuits. This explains that, in most of the clinically complete lesions, which are in fact anatomically incomplete, some "subclinical" influence on the sub-lesional cord persists. This corresponds to the so-called discomplete spinal cord syndromes. Unlike the total loss of limb feedback after peripheral nerve sections (which results in the plastic reorganisation of cortical limb

representations), persisting sub-clinical spinal cord conduction may maintain the cortical representations of the limbs. It has been reported that sensory stimulation of the limbs could elicit topographically appropriate electrophysiological responses in the somatosensory cortex in a patient with a clinically almost complete syndrome (i.e., absence of overt movements or conscious sensations for several years after spinal cord injury) [69]. This confirms the persistence of functionally significant connections between the supraspinal centres and the sub-lesional cord, even after severe SCI.

Conversely, neurological recovery can be extensive after partial spinal cord injury, even with moderate white matter sparing [94]: some authors estimate that, even with a loss of as much as 80% of the axons, *post-SCI* neurological function can be almost normal [106].

Thus, white matter tracts, even when they are partially damaged, seem to play an important role in functional recovery after SCI. There is evidence in humans that white matter tracts influence spinal cord plasticity [89, 150], and some of the spontaneous recovery in patients with incomplete SCI may be explained by increased cortical drive to spared CST neurons, since electrophysiological measurements have shown spontaneous down-regulation of motor cortex inhibition after incomplete SCI [76, 77].

2.3.2 Experimental Evidence

2.3.2.1 The Role of the Amount of Spared White Matter in Recovery

In the rat, white matter tract preservation directly mediates recovery after SCI [23, 45, 151]. After complete transections of the cord, there is usually no significant locomotor recovery, but very limited tissue sparing at the lesion site is sufficient to influence the functional reorganisation of the sub-lesional cord [20, 22]. Very small increases in spared tissue at the lesion centre have profound effects on basic locomotor recovery (but more skilled, detailed locomotor functions were not enhanced by these small increases) [22].

In one representative experiment, rats were observed to recover a certain degree of locomotion after a severe but partial contusion injury. The spinal cord was then transected. Even after the secondary transection, this group of rats performed more hind limb movements than rats that had undergone a single, primary transection. The authors conclude that, after the initial partial lesion, spared tissue at the lesion level (sometimes as little as 1-2%) had facilitated functional reorganization in the caudal circuitry which persisted after complete transection [21]. Sparing of 5–10% of the fibres at the lesion centre is sufficient to help drive the segmental circuits

involved in the production of basic locomotion [22].

The importance of the spared perilesional white matter has been confirmed by a more recent study of attempted spinal cord transection at the T8 level, where significant recovery was observed with accidental, limited white matter preservation. Locomotor behaviour approached normal levels when approximately one third (28-35%) of the white matter was spared[1]. Unilateral sparing could participate in limited bilateral locomotor recovery [336].

2.3.2.2 The Potential for Plasticity of the Spinal White Matter Tracts

The fundamental potential for plasticity has been demonstrated for all known descending motor tracts of supraspinal origin, including attempted regrowth of the corticospinal, rubrospinal, coerulospinal, vestibulospinal, reticulospinal and raphespinal tracts after SCI [19, 18, 85, 159, 246, 248, 323][2]. These descending pathways have demonstrated sprouting and reorganisation in a targeted and functionally meaningful way [247], as well as the control of plastic events within the spinal cord [63]. Also, some of the lost function by damage to one part of the white matter tracts may be taken over by another part (see below).

In addition, the recruitment of *intraspinal* connections appears to play a role in functional recovery after incomplete SCI. In a recent investigation, transected descending CST axons, which initially targeted hindlimb motoneurons in the lumbar cord, sprouted into the cervical grey matter to establish contact with propriospinal neurons. Connections of these sprouts with long propriospinal neurons that bridged the lesion and arborised on lumbar motor neurons were durable and formed a new intraspinal circuit. The circuit relayed cortical input to its original targets in the sub-lesional cord and was functional, as established by behavioural and electrophysiological testing. Conversely, connections with short propriospinal neurons, which did not bridge the lesion site, were lost [19]. Therefore, the remodeling of intraspinal circuitry with preserved long propriospinal neurons occurs spontaneously after incomplete SCI and may serve as a relay for severed but sprouting long tract axons. Additionally, these data confirm that the stability of plastic "re-routing" events in the spinal cord depends on the presence of target structures.

[1] In comparison to the contusion injury study [22], for similar recovery, the percentages of white matter sparing are lower in the attempted transection study [336]; this could represent the fact that contusion injury spares dorsal tissue, unnecessary for basic locomotion.

[2] for a recent comprehensive review, refer to Deumens et al, 2005 [85]

2.3.2.3 Descending Tract Anatomy

Most of the supraspinal centres that control locomotion are located in the brain stem: the red nucleus, the vestibular nuclei, the reticular substance, as well as all the monoaminergic neurons projecting their axons to the spinal cord (in particular, noradrenergic areas A5 and A7 adjacent to the locus coeruleus ("A6"), and the serotoninergic medulla raphe nuclei in rats).

The descending tracts may run in well-circumscribed bundles, such as the CST and the rubrospinal tract (RST), or in ill-defined, scattered fibre systems, like the monoaminergic descending fibres. Figure 2.2 illustrates their anatomical distribution.

The CST is a well defined fibre tract, the main portion of which, in the rat, lies ventrally between the dorsal horns (unlike the human situation); fibres crossing at the bulbar level (pyramids); the ventral, uncrossed portion can be found (as in humans) in the ventral cord, close to the midline [47, 48, 307]. The RST runs controlaterally and is equally well defined in the dorsolateral funiculus [7]. The reticulospinal tract (RtST) and the vestibulospinal tract (VST) are ill-defined tracts mainly occupying the ventral, ventrolateral, and lateral funiculi; the RtST descends bilaterally [229, 230, 288, 319], the VST mainly ipsilaterally [85]. The raphespinal tract (RaST) fibres run ipsilaterally in two major areas: the ventral and ventrolateral fibres (mainly from the raphe obscurus and the raphe pallidus nuclei) project onto the ventral horn and the intermediolateral horn. The dorsolateral fibres (from the raphe magnus, not shown) project to the dorsal horn where pain perception can be modulated [174, 304]. The noradrenergic descending tract consists of scattered, mainly ipsilateral fibres in the ventral and dorsolateral funiculi from areas A5 and A7, as well as intragriseal fibres from A6 descending in the the dorsal horn itself. The latter appear to implicated in pain modulation [66, 67, 85, 124, 285]. For simplicity, all the noradrenergic descending projections from areas A5–A7 are described here as "the coeruleospinal tract" (CoST).

Furthermore, besides the long tracts of supraspinal origin, intraspinal neurons are present in the spinal cord white matter. The ventrolateral funiculus contains long ascending and descending axons, which have propriospinal inter-enlargement (cervical and lumbar), commissural and ipsilateral connections [249].

In addition to the scattered nature of some of the long tracts, there are functional subdivisions among these fibre systems. In the cat, the key locomotor component of the RtST appears to be located in the ventrolateral funiculus [288]. The functions of the dorsal and the ventral raphespinal fibres are fundamentally different [174, 304]. The noradrenergic fibres are involved in pain modulation (intragriseal fibres) as well as motor control (dorsolateral and ventral funiculi) [124, 294].

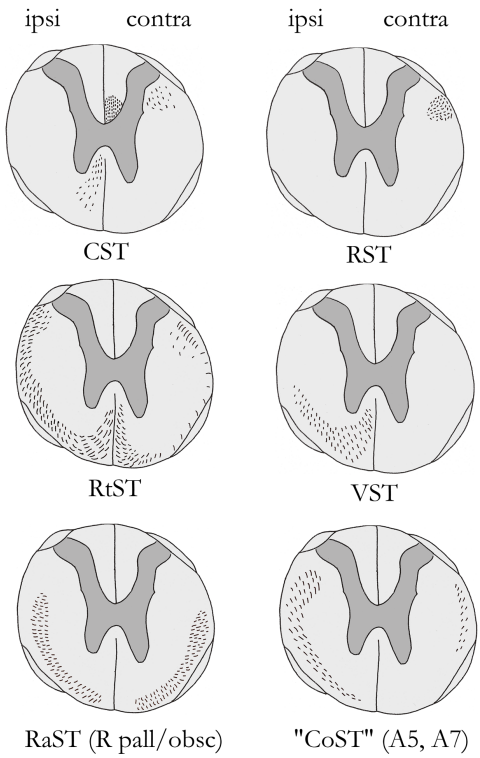

Figure 2.2: Transverse section drawings illustrating the approximate anatomical distribution of the most significant long descending motor fibre systems of right supraspinal origin [7, 47, 173, 264, 215, 231, 272, 304, 321]. CST: corticospinal, with the main dorsal (90–95% of the fibres from layer V in the motor cortex), the dorsolateral (approximately 2%), and the ipsilateral ventral (<5%) components; RST: rubrospinal; VST: vestibulospinal; RtST: reticulospinal; RaST: raphespinal, mainly from raphe pallidus and raphe obscurus; and CoST: "coeruleospinal", which originates in the A5 and A7 groups in the brainstem. It is essential to understand that these fibre distributions vary along the rostrocaudal axis and that scattered fibres of the ill-defined tracts can be found in various regions of the cord funiculi, which are not necessarily limited to those shown in the graph.
In the ventral and ventrolateral funiculi, fibres of various origins are found; including part of the reticulospinal tract (RtST) and the vestibulospinal tract (VST), as well as the mainly serotoninergic ventral fibres of the raphespinal tract (RaST) and the noradrenergic coeruleospinal tract (CoST).

2.3.2.4 Contribution of Different White Matter Tracts to Locomotion and Recovery

The basic stepping pattern produced by the interaction of the oscillating neurons and the inhibitory side-to-side interaction in the CPG, as well as the environmental input from the sensory afferents, depends on supraspinal input. The latter, including input from the the mesencephalic motor region [325], activates the CPG. Only under supraspinal influence can the complexity and precision needed for normal locomotion be developed [23]. However, the precise role of the different white matter tracts in locomotion and their respective contributions to locomotor *recovery* after SCI are complex and not yet completely elucidated [23].

2.3.2.4.1 The Role of the Descending Tracts According to Topographical Distribution in the Spinal Cord

The dorsal funiculi: the influence of the dorsal tracts on open field locomotion appears to be limited [288]; the CST and RST play a role in more complex locomotor tasks, like walking on a grid [272].

The ventral and lateral funiculi play a more important role in basic locomotion and central pattern generation in the rat, as described *in vivo* [151, 196, 200, 272] and *in vitro* [210]. In fact, as little as 20% of ventral or lateral white matter sparing after SCI can sustain locomotion with forelimb-hindlimb coordination in the rat, and limited white matter sparing either in the ventral, ventrolateral, or dorsolateral funiculi may result in significant recovery of basic locomotor patterns [272]. When the dorsolateral *and* ventrolateral funiculi or the complete ventral *and* ventrolateral funiculi are lesioned in the rat, significant locomotor deficits appear, whereas isolated dorsal hemisections, isolated ventrolateral or isolated central ventral funiculus lesions alone produce only insignificant locomotor deficits. Therefore, myelinated locomotor command pathways may be diffusely distributed within the ventral, the ventrolateral and the dorsolateral funiculi at low thoracic levels in the rat spinal cord [199, 200].

In addition to deficits in overground locomotion, ventral lesions may induce locomotor alterations not assessed by the standard BBB scale (see below), e.g., severe plantar flexion during locomotion, resulting in walking on toes in the absence of spasticity, or "noisy" stepping [199].

2.3.2.4.2 The Role of the Descending Fibres According to Suprapinal Origin

The CST appears to be involved in fine motor control [155, 323] and neither to play a major role in gross motor function [110], nor any significant role in normal overground locomotion in the rat [223]: the tract may be completely interrupted by a predominantly dorsal spinal cord lesion, and rats still recover normal open field locomotion [22, 23]. Even the so called "placing response" (rats lift the paw and place it on an obstacle after dorsal stimulation of the paw), which was thought to be under the strict influence of the CST [82], may in fact depend on an intrinsic spinal circuitry under the influence of more ventral tracts [218]. However, the tract may control more skilled locomotor behaviours in the rat, e.g. walking on a rope [155].

The RST: Multiple roles have been attributed to the rubrospinal system, including the initiation of fore limb reaching and tracking, muscular force production, coupling of different joint movements, and associative functions [23, 326]. Overground locomotion is affected to a moderate degree by lesions of the red nucleus [224, 320]; transection of the RST results in initially significant locomotor deficits, but which are compensated for after about six weeks by remaining dorsal and ventral tracts, although coordination deficits remain [155]. Therefore, the RST does appear to play an important role in the organization of locomotor behaviour, but its function can be substituted for by other tracts after injury.

The RtST plays a key role in locomotion. Its neurons synapse on the CPG, and the ipsilateral reticular formation monosynaptically activates lumbar commissural interneurons in the cat [170]. The RtST is thought to initiate the CPG's motor output [54] and locomotor activity such as stepping [95, 272], but also to influence posture and modulate somatic sensory and autonomic functions [319]. Most of the evidence for a major role in locomotion comes from electrophysiological studies in the cat, where the discharge patterns of reticulospinal neurons exhibit phasic modulations that correlate with hindlimb motor activity [95], and where the RtST influences the coordination of flexor and extensor activity of the hindlimbs during stance and during locomotion [96]. In addition to the initiation of locomotion, the RtST appears to influence *"the phase-specific control of individual limbs, postural adjustments to ensure sustained stepping, limb and postural responses to perturbations, and interlimb coordination."* [23].

In the cat, fibres responsible for controlled treadmill locomotion evoked by electrical stimulation of the mesencephalic locomotor region (which contains the reticular substance) run in the ipsilateral ventrolateral funiculus [288]. In

the rat, RtST fibres run in the ventral, ventrolateral, and dorsolateral funiculi [12], and the preservation of a small amount of RtST fibres in the ventral, ventrolateral, or dorsolateral funiculi may provide sufficient input to the lumbar sub-lesional spinal cord to initiate locomotion [272].

The VST not surprisingly appears to play a significant role in balance and posture control and has been implicated in the recovery of static as well as dynamic balance and locomotion, and different cell populations in the lateral vestibular nucleus are responsible for these different aspects [23].

The monoaminergic descending tracts play important roles in the facilitation of locomotion; this has been shown especially for the serotoninergic descending raphe neurons [129]. Their role in the recovery of locomotion after SCI appears to be major [23].

There is less data for the CoST in the rat; however, noradrenergic neuromodulation and neurotransmission may participate in the modulation of CPG activity (see below) and the CoST seems to exert an effect on spinal motoneuron activity and/or motor behaviour [294].

2.3.2.4.3 Mechanisms of Locomotor Recovery: Several potential mechanisms of motor recovery have been described, suggesting the possibility for intact white matter fibre tracts to substitute for the lost tracts' function.

- Intact portions of one tract may compensate for lost fibres of the same tract. For example, the ventral CST may take over motor functions of the severed main tract [323]; in fact, CST fibres, regardless of their spinal trajectories, innervate the same target areas and possibly even the same target cell populations [48]. In case of partial damage to the more diffuse tracts involved in basic locomotion, recovery might be comparable regardless of the portion of a specific tract which was injured. This is seen for example in the comparison of dorsal and ventral lesions of the low thoracic spinal cord [272].

- In the cat, different long descending tracts interact: for example, the RtST can transmit CST actions onto the lumbar commissural interneurons and motoneurons [170, 171, 287].

- The locomotor deficit induced by transection of the RST can partially be compensated if the dorsal columns containing the CST are intact, but not if the latter are also transected [155].

- After unilateral red nucleus lesions, unilateral striatal dopamine depletion, or unilateral pyramid (CST) transections, the observation of abnormal gait patterns and recovery suggests the existence of compensatory strategies for unilateral injuries in the CNS [224].

- After thoracic hemisections, the changes in projection patterns of the RtST below the injury (L2) can be correlated with improved locomotor function; the RtST's unpatterned input from the intact side, possibly *via* commissural interneurons, may be able to trigger the CPG as a whole and, thus, explain the temporal relationship between the changes in projection patterns and locomotor recovery [12].

- The transection of lumbar commissural interneurons abolishes recovery observed after a cord section sparing left lateral and ventral funiculus fibres [151].

- Unilateral ventrolateral lesions result in moderate locomotor impairment in certain behavioural tests (ladder climbing, estimation of ground forces) in the immediate post-operative period. In the long run, this impairment disappears, indicating the possibility that the functional contribution of the ventrolateral pathways can be subserved by remaining pathways [321].

- The RtST and VST, both projecting collaterals to cervical and lumbar enlargements, appear to play a role in the recovery of fore and hindlimb coordination [23]. The ventrolateral funiculus contains long ascending and descending axons with propriospinal inter-enlargement, commissural and ipsilateral connections. If spared after SCI, these are therefore anatomically well-suited to mediate interlimb coordination [249].

- As mentioned before, a severed descending tract may connect to instrinsic spinal neurons which serve as a stable functional relay to transmit the severed tract's input to the sub-lesional segment [19].

2.3.2.4.4 Summary: The relative contributions of the different descending motor systems to recovery after SCI still remain somewhat unclear, due to the multitude and complexity of the behaviours to be assessed, the limited means to quantify functional recovery, the anatomical dispersion of the different fibre tracts which can be found in different regions of the cord, the functional redundancy of different tracts and of different portions of the same tract, interspecies differences in behaviour, and interspecies differences in anatomical locations and functions of the tracts. It still appears clearly that, in the rat, the RtST, probably the RaST, and possibly the CoST,

which all run in the ventral and lateral funiculi, play an important role in locomotor recovery (figure 2.3).

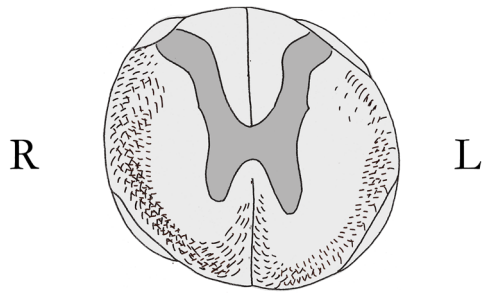

Figure 2.3: Fibres of the major tracts involved in basic open field locomotion (RtST, RaST, CoST (A5-A7)). Only the descending fibres of unilateral (right) supraspinal origin are shown.

2.3.2.5 Ascending White Matter Tracts

In addition to the descending motor tracts, *ascending* pathways transmit a rhythmic neuronal activity pattern to the magnocellular red nucleus in the cat [317]. This indicates that ascending long tracts provide direct feedback from the CPG to the supraspinal motor centres, in addition to the proprioceptive and other sensory feedback from the periphery.

2.3.2.6 SCI Models and White Matter Sparing

Therapeutic strategies after SCI are often assessed in incomplete SCI models with some degree of white matter sparing [22, 155, 180, 214, 256], not only because they resemble the most common human setting [214, 218], but also because a certain degree of recovery is seen and, therefore, neurological treatment benefits may be observed. However, the lesion extent in the cord, and thus white matter sparing, should ideally be reproducible from one animal to another for the correct interpretation of behavioural differences in homogeneous experimental groups.

Although operative techniques may be reproducible, it is impossible to precisely control or predict the definitive extent of the spinal cord lesion [180, 181]. This is not only due to the technical impossibility to induce identical traumata to the spinal cord, but also to the inherent unpredictability of the secondary extension of the spinal cord lesion. Thus, white matter sparing after experimental SCI may be different from one animal to another despite "the same" experimental trauma.

2.4 The Central Pattern Generator as a Therapeutic Target

In order to restructure the neuronal activity in the CPG circuitry with the goal to increase behavioural recovery, different therapeutic approaches can be proposed:

1. Modulation of activity dependent plasticity *via* structured input from the peripheral somatosensory afferents;

2. Recruitment of anatomically preserved pathways of supra-lesional origin in order to restore the influence of the supraspinal afferents;

3. Substitution of missing neurochemical factors, e.g. neurotransmitters/neuromodulators of supraspinal origin, to create a favourable neurochemical environment for CPG plasticity.

2.4.1 Recruiting the Peripheral Afferents—Locomotor Training

2.4.1.1 Experimental Evidence

The spinal cord can learn to perform a task that it practices [101] and exercise can modulate the plasticity of the spinal cord [333] and enhance the expression of neurotrophins in the CNS [228, 266]. An abundant literature clearly establishes that the afferent input generated by exercise modulates the expression of neurotrophins (BDNF, NT-3), including synaptic release, changes of their receptors (tyrosine kinase (trk) receptors trk-B and trk-C, for BDNF and NT-3, respectively), as well as the synaptic plasticity of the spinal cord. Neurotrophins themselves can increase trk receptor expression and thus positively reinforce their own effects, affording a certain stability of the exercise-induced changes [56, 125, 132, 133, 134, 137, 152, 204, 283, 284, 335]. Thus, the present evidence suggests a link between exercise, the expression of neurotrophins like BDNF and NT-3, and possibly functional recovery after SCI, especially as some of the descending long tract neurons which trigger CPG

activity express neurotrophin receptors like tyrosine receptor kinase B (trkB) in the RtST, the RaST and the RST [187]. Neurotrophins (e.g., BDNF) may enhance fibre regrowth of the CST [270, 314], RST [197], and possibly the RaST and the RtST [85].

Treadmill locomotor training has therefore been investigated in the experimental setting, hoping that patterend sensorimotor input to the CPG can improve locomotor recovery [222]. Most studies have been conducted in spinal cord-transected ("spinalised") cats [198]. The spinalised cat can recover weight-supported standing [79] as well as stepping by training [80] with a durable effect after the training period [81]; this is possibly enhanced by pharmacotherapy [62]; in addition, after complete spinal transection, the cat locomotor CPG recovers the ability to adapt the locomotor pattern to the speed of a treadmill, purely on the basis of afferent sensory information [16]. In the mouse, wheel running improves locomotor recovery from a moderate SCI [103].

In rats, locomotor recovery is enhanced by spontaneous exercise without any other therapeutic intervention (enriched housing) [189, 312]. Treadmill training has been investigated after partial SCI. The two studies that can be found in the literature report conflicting evidence. In the first one, no beneficial effect of treadmill training on locomotor activity could be seen after a rather moderate SCI [121]. In the second study, treadmill training improved functional recovery; the locomotor recovery was assessed on the treadmill (and not in an open field) [301].

2.4.1.2 Neurorehabilitation after Human Spinal Cord Injury

In humans, as in the laboratory animal, the CPG may be influenced by sensory input [86, 337]; e.g., locomotor circuits in complete paraplegics can adequately respond to different speeds when the patient steps on a moving treadmill [203]. The afferent input from hip joints and load receptors plays a crucial role in the generation of locomotor activity in the isolated spinal cord after complete injury [88]. In incomplete injuries, stepping on a treadmill creates proprioceptive input which appears to be processed in the spinal cord itself and to allow temporally appropriate activation of the motor neuron pools that generate stepping; this may be independent from the capacity to voluntary generate movements in the lower limbs [208]. Locomotor training after SCI may have an effect on plasticity *via* the peripheral afferents [150].

Training may have a facilitatory role on motor tasks after incomplete SCI *via* volitional supraspinal neuronal activity, by actively recruiting spared motor tracts. The progressive and long-lasting recovery observed after SCI may be explained by plasticity of spared descending pathways; this plasticity appears to be modulable and enhanced by active use of the concerned pathway [195].

In SCI patients, body weight supported treadmill training (BWSTT), other in-

tensive neurorehabilitative measures including functional electrical stimulation [14], and combined approaches [114] have been increasingly used since the first reports of the potential benefits of BWSTT more than 15 years ago [86, 94, 324]. Encouraging preliminary clinical and electrophysiological results have been obtained [15, 26, 158, 300, 327]. One single patient, after several years of stability in the absence of nearly all sensori-motor function below the shoulders, recovered some function (evolution from ASIA grades A to grade C[3]) following sophisticated and intense rehabilitative measures, including bicycle training and functional electrical stimulation [216].

The potential beneficial effects may however mainly be limited to incompletely injured patients [309]: in a comparative study of the effect of training on complete and incomplete cord syndromes, differences in neurological examination occurred only in incomplete SCI syndromes over the training period [89].

All in all, the clinical evidence remains tenuous, because of the heterogeneity of the clinical presentation, the limited range of beneficial effects, the possibility for spontaneous beneficial evolution, the limited number of patients, and the lack of randomised studies. Currently, the effectiveness of automated locomotor training in patients with chronic incomplete spinal cord injury is being evaluated in a multicentre trial [15, 328][4].

In conclusion, some beneficial training effects have been observed after incomplete SCI. They may reflect adaptive changes in spinal cord circuitry under the influence of the peripheral afferents. Partially preserved long white matter tracts may play a role in incomplete SCI syndromes (which appear to respond better than complete SCI syndromes). Many questions remain. The reproducibility of the observed beneficial effects must be shown and underlying physiological mechanisms studied, including the potential participation of voluntary recruitment of the supraspinal tracts in incomplete injuries.

2.4.2 Recruitment of Descending White Matter Tracts

2.4.2.1 Experimental Evidence

A considerable degree of locomotor recovery in mammals with a spinal cord injury can be attributed to the reorganization of spared neural pathways [86]. Therefore, the direct recruitment of the long white matter tracts appears to be an interesting therapeutic strategy, be it indirect as described for locomotor training, or direct,

[3] see appendix, page 167 for the ASIA scale

[4] for reviews concerning: locomotor activity in SCI patients, see Dietz and Harkema [87]; current evidence for beneficial effects of different aspects of clinical locomotor training, see Barbeau et al [15]; clinical trials investigating neurological recovery, especially locomotor, see Tator [295]; and general neurorehabilitation including sphincter problems, central pain, etc. see Ragnarsson et al [245].

e.g., using electromagnetic stimulation to induce neuronal activity in the supraspinal centres where the long tracts originate.

Electric fields applied to lesioned spinal cord [36, 37, 38, 39, 111, 112, 242, 276, 318], peripheral nerve [243, 244], or hippocampus [241] promote functional recovery, axonal regeneration and guidance, or both. There is one clinical trial assessing the efficacy of pulsed electric fields after complete SCI [275]. **Magnetic** fields can atraumatically induce electric fields in deep underlying structures, like the motor-evoked potentials obtained by transcranial magnetic stimulation of the motor cortex and commonly used in clinical neurophysiology. In rats, motor potentials measured in the hindlimbs are chiefly due to the activation of extrapyramidal subcortical motor pathways located in the ventral and ventrolateral white matter of the spinal cord [177, 202, 218, 282]. Long term stimulation by rTMS does not appear to have any deleterious effects on the rat brain [193].

Therefore, rTMS appears as a promising and safe therapeutic strategy in anatomically incomplete SCI, since magnetically induced electric fields have the potential to recruit preserved pathways after incomplete SCI, and more particularly the ventral descending tracts in the rat.

2.4.2.2 Repetitive TMS for Human Spinal Cord Injury

White matter sparing plays an important role in recovery after SCI, and, in incomplete lesions, long tracts may influence plasticity of the sub-lesional spinal cord (see above). The activity of supraspinal motor systems is influenced by rTMS: new generation devices, which can stimulate at very high frequency (50-100Hz), modulate cortex physiology and behavior in a long-lasting manner [162]. Repetitive TMS influences the plasticity of the motor systems in the long run [191, 281, 340].

This has not only spawned a series of therapeutic studies using rTMS outside its well established psychiatric indications[5], but confirms the potential usefulness of rTMS in SCI. There is one report in the literature describing potential benefits of rTMS after SCI. The protocol, which reduces inhibition of the corticospinal motor system, has been shown to improve neurological function, including dexterity, in patients with incomplete cervical SCI [28].

[5]For recent reviews of these clinical applications, refer to [115, 212]

2.4.3 Restoring the Neurochemical Environment

2.4.3.1 Experimental Evidence

The descending inputs and the sensory afferents act on the CPG in different ways: rapid **activation** (usually through glutamate *via* ionotropic receptors) or inhibition; slower **modulation** of the pattern generation (peptides/amines, *via* G-protein coupled receptors); and they interact with the local neurochemical environment in so-called **"meta-modulation"**[6] [239].

A number of pharmacological studies have investigated the possibility of increasing functional recovery by the pharmacological substitution of lost agonists or antagonists of recepters of the caudal cord circuitry. This includes monoaminergic drugs, which much of the research has focused on[7].

In fact, monoamine receptors offer a ready target: their role in the control of reflex activity and central pattern generation has been known for decades [120, 239, 267], including in the rat [113]. Following deafferentation after SCI, which results in reduced sub-lesional noradrenaline and serotonin content, post-synaptic 5-HT receptors remain functional on spinal cord neurons. A certain degree of super-sensitivity to monoamines appears and might increase their therapeutic potential in the acute phase (observed at 15 and 30 days post-injury in the feline spinal cord, before they return to control values) [131]. α_1-adrenergic receptors are up-regulated globally in the spinal cord, and α_2-adrenergic receptors in the lumbar region [260, 261].

The serotoninergic system: A seminal investigation in rats with transected spinal cords—where there is usually no significant recovery—describes the recovery of locomotion by transplantation of monoaminergic cells [253]. The recovery was linked to the serotoninergic re-innervation of lumbar levels L1 and L2[8] [254]. Several experimental serotoninergic drugs have since been tested after experimental SCI [9, 10, 43, 49, 186], some inducing sustained behavioural recovery.

The noradrenergic system: Noradrenergic substances have also been investigated, especially in the spinal cat where the noradrenergic system seems the most

[6] The concept of metamodulation describes the additive or substractive effects as well as the novel modulatory patterns that can arise from the immediate or prolonged interplay of several neurotransmitters and neuromodulators in a neuronal network [239].

[7] For a thorough review, including other neurotransmitters than the monoamines, refer to Parker [239]

[8] It is interesting in this context to note that serotonin exerts most of its effects *via* nonsynaptic, paracrine, or volume transmission [52, 156, 255]. This has potential implications for the study of the plasticity of serotoninergic systems: axonal regeneration of serotoninergic fibres simply approaching the CPG might be sufficient to restore serotoninergic influence.

efficient in triggering locomotion. In particular, α_2-adrenergic agonists like clonidine[9] initiate locomotion when applied to restricted lumbar segments [17, 257], as does noradrenaline itself. The latter has also been shown to modulate locomotor rhythm-generating networks *in vitro* (i.e., in the isolated neonatal rat spinal cord) [184, 185], *via* various receptors [286]. Clonidine enhances recovery of locomotion in spinal cats when used in combination with locomotor training [62]. To date, there is no published study addressing the effect of clonidine on functional recovery after SCI in the rat.

Furthermore, neurotransmitters can modulate spinal cord plasticity in the lamprey [238]. Thus, in addition to their possible immediate effects on locomotion pattern generation, the stimulation of the CPG by monoaminergic drugs could potentially *modulate plasticity in the long term*. This was confirmed by a serotoninergic stimulation study where the observed behavioural benefits of the treatment did not decrease after the discontinuation of the treatment [9].

In addition, serotoninergic activity can *protect* spinal cord plasticity in the rat in the experimental setting of so-called "uncontrolable stimulation" (through peripheral afferents) [74], which normally inhibits learning and undermines recovery after spinal cord injury [136]. Serotonin and serotoninergic agonists, as well as clonidine, can counteract this inhibition.

Thus, in the experimental setting, monoaminergic substances appear to have the potential to durably influence the activity of the mammalian lumbar locomotor CPG after SCI.

2.4.3.2 Human Pharmacotherapy

In human experimentation, clonidine and cyproheptadine (a serotonin antagonist) have been tested [234, 251]. Some locomotor improvement was noted, but, currently, the results are not conclusive[10]. The authors observed a clear reduction in spasticity with both drugs, which may have participated in the observed locomotor improvement. The reduction of spasticity might in some cases be undesirable, though: some patients depend on the spastic lower limb muscle tone for stance and locomotion.

[9] In addition to its effects on adrenergic receptors, clonidine cross-reacts with serotonine receptors [239].

[10] In one of the studies, the authors mention that none of the patients treated desired the implantation of a clonidine pump [251]. Tolerance of clonidine has regularly been an issue for discussion, but its clinical interest should not be neglected because of potential side effects (mainly sedation): clonidine has been used for decades in the long term treatment of arterial hypertension; the observed side effects frequently regress after a few weeks.

Currently, human volunteers are being recruited to test the role of selective serotonin reuptake inhibitors on volitional and spastic motor behaviors following motor incomplete SCI and their effects in combination with specific physical interventions (i.e., BWSTT) [3].

2.5 Summary

The currently available evidence indicates that:

- The spinal cord is more than a hardwired pathway and simple synaptic relay between the brain and the periphery—it possesses an active intrinsic circuitry.
- Locomotion, including human walking, relies to a certain extent on patterned neuronal activity in the lumbar spinal cord. The activity of the responsible intrinsic circuit, named locomotor CPG, is modulated by peripheral and supraspinal afferents:
  - The peripheral afferents provide the locomotor CPG with feedback from the limbs in order to adapt locomotion to the external conditions.
  - The supraspinal afferents are responsible for various aspects of the control of locomotion, including voluntary adaptations. The supraspinal centres also receive feedback from the sensory system and the CPG itself.
- After injury to the more rostral spinal cord, the intrinsic circuits are, at least partially, disconnected from supraspinal control and malfunction. This results in a loss of neurological function—in the case of the locomotor CPG in humans, walking capacity.
- The neuronal circuitry of the spinal cord is capable of plasticity, and remains so after SCI. The plasticity may be largely activity-dependent and potentially modulated *via* the activation of motor systems by:
  - locomotor training, including BWSTT, which recruits proprioceptive afferents and, in partial injuries, may also recruit ascending and descending spinal white matter long tracts,
  - direct recruitment of the long tracts, as may be expected with repetitive transcranial magnetic stimulation, and finally
  - monoaminergic pharmacotherapy.

This is different from stimulating the CPG's immediate motor output (as often studied *in vitro*).

- The modulation of plasticity in the CPG by neuronal activity or neurochemical factors is common to all these approaches.

However,

- The evidence for a beneficial effect of human BWSTT remains, at present, incomplete. Experimental studies in the rat are rare and yield unequivocal results. Therefore, in the most common SCI animal model, little is known about BWSTT. An experimental BWSTT model in the rat is needed which (1) could confirm the potential beneficial effect of BWSTT and (2) could subsequently serve for the study of the contribution of activity dependent CPG plasticity to recovery, as well as the role of the supraspinal centres and perilesional tissue preservation (for ascending feedback and descending CPG modulation).
- Repetitive TMS has not been studied after experimental SCI, although a small clinical study has shown a certain degree of therapeutic potential.
- Monoaminergic pharmacotherapy has gained in interest over recent years and shown promise, but the antihypertensive noradrenergic drug clonidine and the serotoninergic antidepressant fluoxetine, frequently administered in the clinical situation for other indications, have not been studied in rat SCI.
- Knowledge about the precise roles of the spared white matter tracts after partial SCI remains somewhat sparse, and, in therapeutic investigations comparing treated to untreated SCI animals, the assessment of white matter sparing is not always performed.

2.6 General Objectives

The objectives of the present work were:

1. to recruit the spared spinal cord tissue's potential for enhancing locomotor recovery, using experimental therapeutic strategies which are potentially rapidly transferable to the human, and
2. to better identify spared tissue and to correlate tissue preservation with spontaneous locomotor recovery.

Part II

Personal Contribution

Chapter 3

Recruiting Preserved Tissue

3.1 Strategy

IN KEEPING WITH THE CLASSICAL APPROACH to found clinical neurorehabilitation treatments on evidence from basic research [13, 298], therapeutic strategies aimed at the locomotor CPG were investigated in the laboratory setting. In order to reproduce the most common clinical situation, a partial SCI model was used in the rat, since bipedal and quadrupedal locomotion appear to share common spinal neuronal control mechanisms [86, 71].

3.1.1 Hypotheses and Choice of the Treatments

The goal was to modulate the CNS environment in the long term in order to enhance structured, durable reorganisation of the locomotor CPG, as well as its supraspinal and peripheral connections.

Therefore, the strategies in the present investigations were chosen:

1. for their potential to **enhance activity dependent plasticity** in the CPG and
2. for their potential to **restructure CNS input** to the CPG in the long term, as the *isolated* CPG cannot produce functionally significant locomotor output.

   CNS input to the CPG may be generated by:

   - the **physiological recruitment of nervous pathways** which were spared by the injury, but are silent;

- sprouting and the **reconnection of existing, preserved pathways**, either rostral to the lesion (creating a new intraspinal circuit) or caudal to the lesion (connecting these pathways directly to disconnected targets); or, lastly,
- regeneration of nervous fibres across the lesion site, ultimately **restoring lost pathways** between supraspinal centres and the sub-lesional cord.

The different strategies described in the present work concentrated on the first two points.

With this approach, expectations for functional recovery are moderate, and the therapeutic goal is not necessarily the complete restoration of locomotion. Nonetheless, moderate functional improvements, which may in the experimental setting even correspond to statistically non-significant effects, may have a profound impact on a patient's quality of life. As an example, a patient will clearly benefit from a gain in the capacity to stand and support the body's weight; if a patient was already able to stand, even very limited walking could increase autonomy.

Therefore, conservative approaches recruiting preserved nervous tissue may provide clinical benefit. In addition, ethically sound and rapid translation into the clinic is possible when the investigated treatments are already commonly used in humans and known to be relatively innocuous. In the present work, treatment strategies were investigated that could be immediately applicable in the human.

3.1.1.1 Locomotor Rehabilitation: BWSTT

BWSTT, as a technical sophistication of the more standard, current clinical rehabilitation strategies, is already being used in humans.

Experimental hypothesis: Hind limb stepping activity creates structured proprioceptive input both *directly* into the CPG, and *indirectly*, *via* partially preserved ascending and descending long white matter tracts and the supraspinal control centres. The descending output of these centres is modulated by the ascending input, which corresponds to either

- direct proprioceptive feedback to supraspinal centres (altered after SCI by abnormal limb motion), or
- CPG feedback (altered after SCI by deafferentation and by altered proprioceptive feedback to the CPG).

In any case, the restructured proprioceptive and supraspinal inputs modulate the neuronal activity in the deafferented locomotor CPG, and thus restructure activity-dependent plasticity and functional output (i.e., locomotion) in the long term.

3.1.1.2 Repetitive TMS alone

Repetitive TMS is an clinical routine treatment for other indications than SCI, at present mainly in the psychiatric domain.

Experimental hypothesis: Activation of supraspinal motor centres recruits descending tracts and modulates spinal cord plasticity, including the locomotor CPG.

3.1.1.3 Combined Strategy

The combination of rTMS, pharmacological treatment, and BWSTT is clinically feasible in rehabilitation centres, and the drugs that are used can be rapidly introduced.

Experimental hypothesis: Combining different strategies with beneficial effects on CPG plasticity creates synergy. In particular, monoaminergic drug treatment restores a favourable neurochemical environment to an extent that increases recovery by itself and/or enhances the effect of other treatment strategies by facilitating plasticity.

- Clonidine was used as an α_2-adrenergic and, in part, serotoninergic agonist and potential neuromodulator.

- Fluoxetine was used a selective serotonin reuptake inhibitor, to enhance the neuromodulatory activity of preserved serotoninergic tracts.

3.1.2 Objectives

The main objectives therefore were:

1. To investigate the potential benefits of the treatments in experimental rat SCI, using a lesion technique that induces a partial cord lesion at a the thoracic level, resulting in significant deafferentation of the CPG. The motor deficits are sufficiently severe to observe potential beneficial effects of the treatment, but not too severe, in order to allow behavioural assessment and training on the treadmill.

2. To investigate a potential syngergy between different strategies, designed to recruit the CPG motoneuron pools from three different therapeutic angles.

3.2 Methods

All experiments were performed on adult female Wistar rats (190-240g) in accordance with the rules and regulations of the Belgian National Fund for Scientific Research concerning the use and care of laboratory animals.

3.2.1 Preparation and Handling of the Animals

The animals were handeled for several days before surgery, in order to be accustomed to the investigators and to the experimental settings used for physical therapy and behavioural evaluation. In the treadmill study, three days of daily handling in the open field (behavioural evaluation) and exposure to the treadmill in harnesses (figure 3.3) eliminated any observable sign of anxiety. Additionally, in the rTMS series, the rats were exposed to the treatment environment and to the noise of the rTMS stimulator for at least a week before surgery.

3.2.2 The Lesion Model and the Surgery

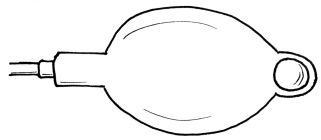

Figure 3.1: Schematic drawing of the normal shape of an inflated "Goldvalve" balloon, with a tip containing a small metallic sphere.

The balloon model developed in our laboratory is biomechanically similar to the most common clinical situation, i.e., prolonged compression of the cord in a closed spinal canal (figures 3.1 and 3.2). It has been used as the main SCI model in our laboratory because it addresses some of the recognised limitations of the most common partial lesion model, i.e., contusion injury by calibrated weight drop: "*...a single blow at one angle does not reenact all of the torsional forces and prolonged compression that occur with non-penetrating traumatic SCI from accidents*" [94]—even if the latter technique has shown a certain degree of relevance to human SCI [218]. In addition, extensive laminectomy can potentially damage the posterior vertebral joints, which may result in vertebral body dislocations [122] and spine deformities, which

may influence locomotor biomechanics [personal observation] and possibly induce supplementary spinal cord trauma.

In the subdural balloon compression model, 10 μl compressions induce mild lesions and rats recover complete overground locomotion, whereas 30–40 μl lesions produce very severe lesions; in the latter, functional recovery remains limited to isolated, non-periodic movements of the hind limbs. Five minute compression at a volume of 20 μl results in a partial spinal cord lesion with sufficient tissue sparing to allow residual locomotion, sufficient to be observable, but sufficiently impaired to leave space for potential behavioural amelioration by the treatment [214].

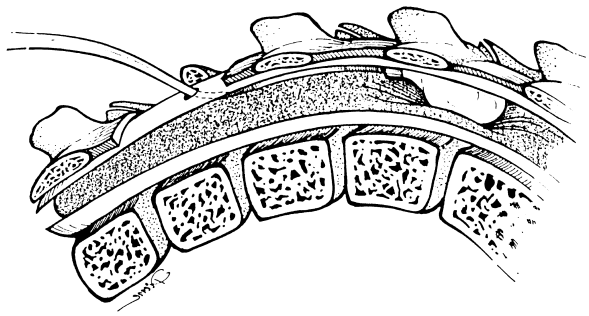

Figure 3.2: Schematic drawing of the surgery (from [214]).

After dorsal balloon compression, the dorsal corticospinal tract is always completely interrupted [unpublished tracing data]. The descending tracts that are crucial for locomotion are partially spared in the ventral and lateral funiculi.

In the different experimental groups (see below), the lesion levels were

- midthoracic (T7) in the treadmill group
- high thoracic (T4–5) and low thoracic (T10–11) in the rTMS group, and
- mid-low thoracic (T8–9) in the combined treatment group.

Surgery was performed in the vast majority of rats by the same operator, the only exception being the rTMS-only study, where another operator participated for the high thoracic lesions.

Before the procedure, rats were anaesthetised by intraperitoneal (ip) injection of xylazine (10mg/kg) and ketamine (75mg/kg). Depth of anaesthesia was adjusted to

corneal and plantar areflexia. The dorsal skin was shaved, the eyes protected with artificial tears, and the operative field disinfected. After incision of the skin, subcutaneous tissue and muscle aponeuroses, paraspinal muscle was retracted, the bony spine exposed, and a minimal central laminectomy performed. An inflatable microballoon (model GV15; CathNet Science, Paris, France), mounted on a catheter, was introduced through a small dural incision and moved approximately 6 mm rostrally, inflated with 20 μl of distilled water, left in place for 5 minutes, then deflated and carefully removed. The surgical wound was closed in two layers (muscle and skin).

After surgery, dehydration was prevented by ip physiological saline injections, and infection by immediate post-operative ip injection of amoxicilline-clavulanic acid. The bladder was manually expressed daily until the rats recovered spontaneous micturition. Urinary infections were treated as needed.

The rats were housed separately. Food and water were provided *ad libitum* during the entire study duration.

3.2.3 Behavioural Assessment

Motor function of the hind limbs was evaluated weekly using the Basso, Beattie, Bresnahan (BBB [21]) open field locomotor rating scale (page 170) over eight to twelve weeks. The BBB method evaluates locomotor behaviour on a non-linear scale from 0 to 21, where "0" corresponds to the absence of active movements of the hind limbs, and "21" is normal walking, according to precise criteria like the placing angle of the foot, the tail position, and trunk stability. Three ranges of scores can be distinguished:

1. Scores 1–7 evaluate single joint movements; for example, "1" corresponds to slight movement of a single or two joints, and "7" corresponds to extensive movement of all three joints (hip, knee, ankle).

2. Scores 8–13 evaluate the evolution of coordinated hind limb activity, from the absence of weight support. The score of 8 corresponds to so-called sweeping, which is a cycle of repetitive, rhythmic flexion-extension movements of the hind limbs through the whole range of movement, but in the absence of weight support; this gives the impression that the rat's hind limb "sweeps" the floor. Alternatively, the score of 8 is given to rats capable of placing the paw on its palm (plantar placement). The following score, "9", is given for plantar placement with weight support at stance, indicated by the fact that the rear is lifted from the ground, but, as the rat moves forward, this weight support cannot be maintained, and the rear collapses. Scores of 10 and more evaluate

the progressive recovery of basic stepping. Stepping is defined as a weight-supported cyclic mouvement: plantar contact with the ground followed by hind limb advancement and re-establishment of plantar contact. This occurs first in the absence of coordination with the fore limbs, then coordinated.

This range of scores probably scores the clinically most significant recovery of locomotor behaviour, in particular the progression from the absence of weight support (8) to weight support (9), stepping (>9), and consistent fore and hind limb coordination (>13).

3. Scores 14–21 evaluate fine locomotor behaviour during coordinated hind limb and fore limb stepping, including the position of the paws at the initiation of steps and when the foot is placed on the ground, the capacity to completely lift the paw from the ground (audible "toe clearance"), etc.

Characteristically, rats recovering from SCI show these behaviours sequentially and thus progressively increase their BBB score. The rapidity and degree of locomotor recovery have been histologically correlated with lesion severity [22].

Some authors have criticised the non-linearity of the scale. For example, the scores of 8 and 14 appear to represent "threshold" values where BBB recovery values might cluster [272], despite clear differences in lesion intensities (or, possibly, different treatment effects!). However, among the presently available behavioural tools [219], the BBB-scale represents the most widely used and accepted behavioural assessment methods for rat SCI. It has shown inter-laboratory reproducibility. It is reliable and the most independent from the animal's motivation. BBB scores assess overground locomotion, a CPG-dependent behaviour [219] which is known to be influenced by the monoamines and may be ameliorated by regeneration of the RaST and the CosT [85]. The BBB scale was therefore chosen for the present investigations.

Before behavioural observation, bladders were emptied to avoid the hind limb activity associated with voiding. Each rat was evaluated by two examiners (blinded to the rat's identification number and to the treatment group) over four minutes in a standardised open field. Motricity was scored from 0 to 21 and noted for the left ("L") and right ("R") hind limbs. Movements occurring immediately after contact with the examiners were disregarded. The mean BBB score was calculated: (L+R) x 0.5[1].

In the combined treatment group, locomotion was evaluated on Mondays (after >48h of treatment interruption) to avoid any interference of possible immediate effects of the drugs on the CPG and locomotion.

[1]In the first combined treatment series, the evolution of the mean maximal BBB score (L or R) was also compared between groups; there was no difference between the evolution of the curves.

3.2.4 Tissue Processing, Histology and Morphometric Analysis

At the end of each *in vivo* study period, the rats were deeply anesthetised and perfusion-fixed with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). Immediately after the perfusion, the spinal cord was carefully excised and post-fixed in the same fixative for 24 h at 4°C.

3.2.4.1 Treadmill study

In the treadmill study, histology was used to estimate white matter preservation at the lesion level. Tissue blocks from the centre of the lesion were osmified and embedded in resin (Epon 812). Semi-thin (1 mm) transverse sections were stained with toluidine blue and the area of microscopically normal appearing white matter was outlined and colored using a *camera lucida*. Digitalised black and white versions of the drawings were subjected to image analysis, and the spared white matter was expressed as a percentage of a T7 white matter section of a non-lesioned rat.

3.2.4.2 Repetitive TMS study

A 12 mm tissue block centred on the lesion centre and two 5 mm rostral and caudal adjacent blocks were cryoprotected in 30% sucrose for 48 hours at 4°C, then frozen for cryostat sectioning at a thickness of 20 μm.

Several mounted sections were stained with haematoxylin-eosin or thionin to assess the lesion extent, others processed for serotonin (5-HT) immunohistochemistry: after blocking endogenous peroxidase and nonspecific antibody binding, sections were incubated overnight with a polyclonal anti-5-HT antibody (dilution 1/100,000; Incstar Co., Stillwater, MN). The next day, sections were washed and incubated for 1 hour in biotinylated goat anti-rabbit antiserum, (dilution 1/500, Vectastain Elite ABC kit; Vector, Burlingame, CA) followed by a 1-hour avidin-biotinperoxidase complex incubation (dilution 1/1,000; Vectastain Elite ABC kit) and a 5-minute incubation in 3,3'-diaminobenzidine (DAB; concentration 0.02%). Sections were counterstained with 0.02% thionin.

Tissue blocks with optimal immunostaining for 5-HT were were viewed with a 40X objective for blinded morphometry of the surface occupied by serotonergic fibres in sections from (1) normal non-lesioned thoracic spinal cord (high-T3 and low-T11), and (2) in sections from blocks located immediately rostral and caudal to the lesion from 6 "low thoracic" rats (3 control and 3 stimulated) and in 4 "high thoracic" rats (2 control and 2 stimulated). Microscopic fields were captured with a CCD camera (XC-77CE; Sony) equipped with an amplifier and transferred to a Macintosh IIci computer running NIH Image 1.52 software (public domain program written

by W. Rasband, NIH, Bethesda, MD). The surface of 5-HT-immunoreactive profiles (5-HT immunoreactive area) was measured for each rat on evenly spaced fields, covering the most densely stained portions in superficial dorsal horns (Laminae I and II) and ventral horns (Laminae VIII and IX), and in the intermediolateral columns. Immunoreactive fibres were separated from the background by an interactive method of thresholding and converted into black and white digital images. All objects in the preselected areas were measured automatically. The surface occupied by black pixels (i.e., 5-HT-immunoreactive profiles) was calculated as a percentage of the total surface of all white pixels within the selected field. The mean percentage area (±SEM) occupied by 5-HT fibres was computed from all values obtained in each section and the ratio between rostral and caudal segments calculated.

3.2.5 Statistical Analysis

3.2.5.1 Treadmill training study and rTMS study

Repeated measures analysis of variance (ANOVA) were used to assess the effect of the treatment on motor recovery and to compare BBB scores between treated and untreated animals. Week by week comparisons between the two groups were performed with a "planned comparisons" test. The Mann-Whitney *U* test was used to compare spared white matter between the two groups of animals in the treadmill study, and Pearson's test correlate the final BBB score and the distance from the centre of the lesion to the obex and the density of serotonergic fibres. The level of significance was set to $p<0.05$.

3.2.5.2 Combined treatment group

In order to account for the higher number of treated groups (see below), a different approach was chosen. Zerbe's non parametric method for response curves (obtained by simple linear interpolation between consecutive time points) was used to compare groups two by two. This method allows the comparison of means values not only at each time point but also over any specified time interval.

3.2.6 Treatments

3.2.6.1 Experimental Body Weight Supported Treadmill Locomotor Training

Up to three rats were trained simultaneously on a treadmill. The rats were attached by hanging harnesses which allowed for body weight support and free movement of

all four limbs (figure 3.3). The height of the attachment could be adapted depending on the rat's anatomy and capacity for body-weight support. During the training sessions, the investigator could stimulate motor behaviour in order to simulate human physiotherapy (with intervention from the therapist depending on the achieved activity level), or to mobilise spastic limbs.

Rats were trained on the treadmill during 30 minutes, once daily, five days a week. In the combined treatment series, training was completed each day before rTMS. For each rat, three 10-minute training sessions were separated by 5 minute breaks.

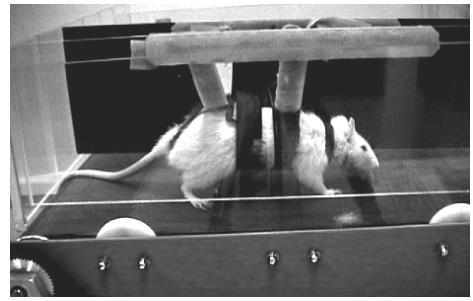

Figure 3.3: Rat on the treadmill.

In the **BWSTT group**, treatment was begun when the rat had reached a BBB score of 1, i.e., after 2–4 days. The speed of the treadmill was 58 mm/sec. Total duration of the treatment was 12 weeks.

In the **combined treatment series**, all rats were treated from the 4th post-operative day on. The treadmill speed was adapted to the rats' locomotor performance: initially, the treadmill surface speed was 33 mm/sec; progressively, during the second week, as rats recovered minimal hind limb motor activity, the speed was increased to the final value of 66 mm/sec. Total duration of the treatment and behavioural follow-up was 11 weeks.

During the first weeks, when paralysis was flaccid, manual paw placing by the investigator or, depending on motor recovery, gentle tail rolling between two fingers were used to initiate hind limb activity until a sufficient motor performance for spontaneous stepping on the moving treadmill was obtained. If necessary, and depending on the resulting performance, the rats' trunks were detached to stimulate locomotion.

3.2.6.2 Repetitive Transcranial Magnetic Stimulation

Rats were treated by rTMS once daily, five days a week, for 11 weeks. A Magstim Rapid, 8-shaped coil was used (Magstim Ltd., Whitland, Dyfed, United Kingdom). For transcranial stimulation, the rat was gently held in the opened cage: two fingers were placed laterally to the neck in order to fix the skull in an immobile, flat position. The coil rested on the head, centred on the skull between the ears and eyes.

Ten 5 second bursts at 10 Hz and 50% of maximal stimulator output were delivered in two series of five trains, at an intertrain interval of 15 seconds. The two series of trains were separated by a 10 minute break.

The maximal magnetic field strength of the stimulator was 2 T at the midpoint of the intersection of the two loops, and the applied magnetic field was thus 1 T.

3.2.6.3 Monoaminergic pharmacotherapy

Fluoxetine and clonidine were dissolved in sterile water according to the manufacturers' instructions and administered ip or subcutaneously once daily, 5 days a week, before rTMS or handling. Doses were 10mg/kg/day for fluoxetine and 0,25 mg/kg/day for clonidine. The medications were administered before rTMS in order to increase chances of possible synergistic interaction between the two treatments while monoaminergic substances were present in the rat's organism. Rats receiving fluoxetine were supplemented with normal saline subcutaneously once daily (1 ml) to avoid dehydration.

3.2.6.4 Controls

Controls were handled daily in their opened cages and underwent "sham rTMS", which corresponded to fixing the rat's head and positioning a sham coil on the head. The fear reactions in controls were comparable to those occurring during rTMS.

3.2.7 Experimental Groups

In the three studies, the rats were distributed in the following experimental groups:

1. Treadmill training **(N=13)**: trained n=7, control n=6.
2. rTMS only **(N=23)**:
   - High thoracic (T4-6): rTMS n=5, control n=6
   - Low thoracic (T10-11): rTMS n=6, control n=6
3. Combined treatment series:

   In a **first series**, a total of 90 rats was operated in several steps; after each series of operations, rats were attributed to *all* experimental groups.

   - In the group that was statistically analysed **(N=54)**: Controls n=10, rTMS n=9, clonidine n=6, clonidine-rTMS n=9, treadmill-rTMS n=8, fluoxetine n=5, fluoxetine + rTMS n=7 (table 3.1)

- Fluoxetine **ip** (N=6): fluoxetine n=3, fluoxetine + rTMS: n=3
- Other controls: n=9 in smaller cages [2].
- Total number of deaths N=21. Peroperative deaths (before first BBB) n=6, later deaths[3] n=15.

Group	Treatment	Number of rats
1	Controls	n=10
2	rTMS	n=9
3	rTMS-BWSTT (treadmill)	n=8
4	Clonidine	n=6
5	rTMS-clonidine	n=9
6	Fluoxetine	n=5
7	rTMS-fluoxetine	n=7

Table 3.1: Combined treatment groups (I).

In a **second series**, a total of 40 rats were operated after methodological adaptations (see 3.3.3.4, page 56).

- In the group that was finally analysed **(N=11)**: Controls n=3, rTMS n=2, clonidine n=4, rTMS-clonidine n=2.
- Excluded rats **N=8**: 7 for asymmetric recovery (scores 2 and 3 on a scale from 0 to 3), and 2 for inconsistent BBB evolution.
- Deaths and exclusions for veterinary reasons **N=20**. 11 rats died in the perioperative period; 7 between BBBs 2 and 7. One rat was excluded for autophagia and another one for severe hydronephrosis[4].

[2]The high total number of control rats (19) is explained by the group equilibration

[3]Early deaths in the acute phase (first two weeks) due to urinary tract infections n=9, 1 anorexia in the treadmill group, 1 hydronephrosis, 1 unexplained death in the treadmill group, 3 late deaths in the fluoxetine groups.

[4]The high mortality, which had afterwards also been observed by other investigators in our laboratory, was, for its major part, attributed by a veterinary analysis to the change in drinking water associated with a move to new housing facilities. High calcium content and high urinary pH, in the setting of low urodynamic flow, lead to an excessive number of cases of urinary lithiasis, which increases the risk for urinary tract infections. This has since been corrected.

3.3 Results

3.3.1 BWSTT

In the BWSTT series, training had a clear and statistically significant beneficial effect on the recovery of spontaneous locomotion in the open field.

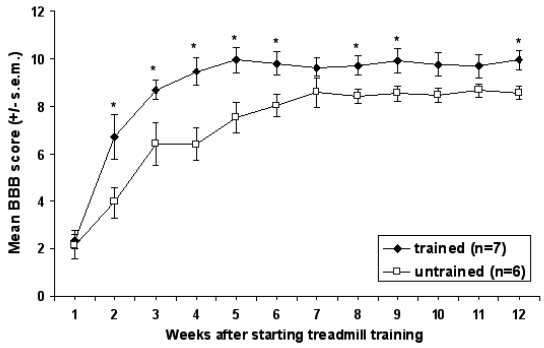

Figure 3.4: Evolution of the BBB scores over time in the trained and the control groups; *$p<0.05$. From publication 1, page 185.

In both the treated group and the controls, starting at the same mean BBB score, the recovery curve on the BBB scale expectedly showed rapid amelioration followed by a locomotor performance plateau after 5–6 weeks (figure 3.4).

The BWSTT group reached a statistically significant higher motor score than the untrained rats. This statistical significance was observed from the second week on. The mean plateau BBB values of the treatment group indicate *at least stationary weight support* (the pivotal BBB score of 9) or even some degree of "stepping" (scores >9), whereas the mean value of the untrained group peaks at approximately 8, which is a score translating either plantar placement of the paw, or so-called "sweeping" (full flexion-extension-flexion cycles of the hind limbs), both *without* weight support.

The histological analysis confirmed the presence of the usual chronic traumatic spinal cord lesion after balloon compression, including massive tissue loss in the dorsal two thirds of the cord at the lesion centre, and preservation of a part of the ventral and ventrolateral white matter tracts. The percentage of spared white matter was equivalent in the two groups (BWSTT 12.17 ± 2.20; untrained 11.42 ± 3.08).

3.3.2 Repetitive TMS in low and high thoracic lesions

The results of the initial rTMS investigation can be summarised as follows (figure 3.5):

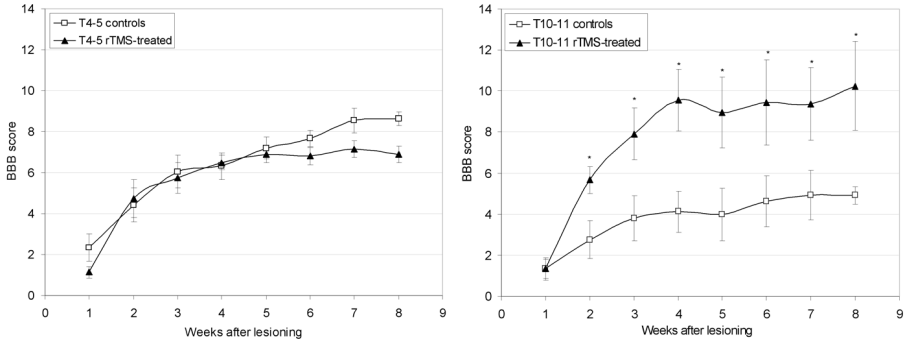

Figure 3.5: Evolution of the BBB scores in the four experimental groups: low thoracic, with or without rTMS (left); high thoracic, with or without rTMS (right). From publication 2, page 185.

- In the *high* thoracic cord lesions (approximately T4–5), recovery was moderate, with scores below 9 (indicating extensive movements of the limbs but no weight support), in the rTMS group as well as the control group.
- In the *low* thoracic cord lesions (approximately T10–11),
  - *in the absence* of rTMS, functional recovery was very limited (mean BBB plateau scores of approximately "5", i.e. moderate hind limb mobilisation on the ground), but
  - *with* daily rTMS treatment, recovery was significantly enhanced, with scores of >9 in three out of the six rats (which corresponds to weight support and stepping).

  The difference was found to be statistically significant ($P = 0.004$).

It is important to note that the motor recovery could vary within the treatment groups, especially in the rTMS treated *low*-thoracic group (final BBB score range: 4.5–17.5). However, even when the best score was excluded from statistical calculations, the detected difference was still found to be significant ($P = 0.05$).

In this series, spared white matter had not been not quantified because of the laborious nature of this type of analysis, and because there was no detectable difference between groups on simple microscopic inspection. Also, 5-HT positive fibers could be detected in superficial lateral funiculi in all animals at the lesion level. The sub-lesional serotoninergic fibre content was clearly reduced in all animals, compared to the supra-lesional area, as assessed by morphometry.

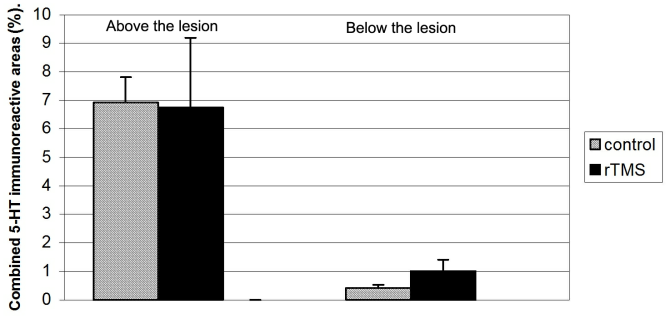

Figure 3.6: Percentage of the 5-HT positive areas in the rostral and caudal spinal cord sections (as compared to a normal cord) in rTMS (n=5) and control (n=5) rats. From publication 2, page 185.

In all rTMS treated rats, but more so after low thoracic lesions, the average area covered by 5-HT positive fibres in the caudal cord was *increased*, as compared to untreated rats (figure 3.6). This failed to reach statistical significance, but the extent of 5-HT immunoreactivity could be statistically correlated with locomotor recovery in the rTMS-treated rats. The morphology of the sub-lesional 5-HT terminals was similar in all animals.

3.3.3 The Combined Treatment Strategy

After these encouraging results, it was decided to confirm the potential beneficial effect of rTMS in a sufficiently large group of rats with low thoracic lesions, and to combine rTMS with BWSTT and pharmacotherapy.

The underlying principle was to investigate the recruitment of the partially preserved white matter tracts in order to influence plasticity of the partially *deafferented*

CPG, because this is the type of treatment that would be needed in the majority of spinal cord injured *patients* who suffer from the sub-lesional deafferentation deficit, the "spinal cord syndrome", as described above. The spinal cord compression lesions were thus induced slightly *above* the CPG (level T8-T9), in order to (1) remain in the lower thoracic region, at distance from the stimulation site, (2) avoiding damage to the CPG itself[5].

3.3.3.1 First Combined Treatment Series

The mean BBB scores of the seven different groups (n° 1-7, table 3.1) showed the following evolution (figure 3.7):

- Mean scores increased progressively, as expected, from an initial level that was the same in all groups, until reaching a plateau after 4–5 weeks.
- With the exception of fluoxetine-treated rats, the control rats (group n°1) had the lowest final mean BBB score, and the increase in mean BBB scores to the plateau level was slow.
- The rTMS (n°2) treated rats' mean BBB score initially increased more rapidly and to a higher level than the controls', but over the last weeks of the observation period, mean scores of the rTMS and control groups were almost the same (the final mean BBB was slightly higher in the rTMS group).
- The Clonidine (n°4) rats' mean scores reached the plateau more slowly than the other groups but clearly remained in the upper range until the end.
- The rTMS-Clonidine (n°5) group's mean BBB scores reached the plateau in the same time interval as the other groups, but are the plateau score was much higher and remained so until the end of the observation period.
- The rTMS-BWSTT (n°3) rats' mean scores recovered rapidly, and the ultimate mean BBB score remained higher than the controls'.
- The Fluoxetine rats' mean BBB scores initially increased as expected, with or without rTMS (n°6 and n°7), then progressively decreased.

[5]This is entirely different from lesions at the thoraco-lumbar junction (T10 and lower), which can result in some degree of destruction of the CPG, due to the rostro-caudal extension that is usually observed with the balloon-compression injury. In the latter case, local changes may influence recovery in a different manner than the long tract recruitment that was aimed for in the present work.

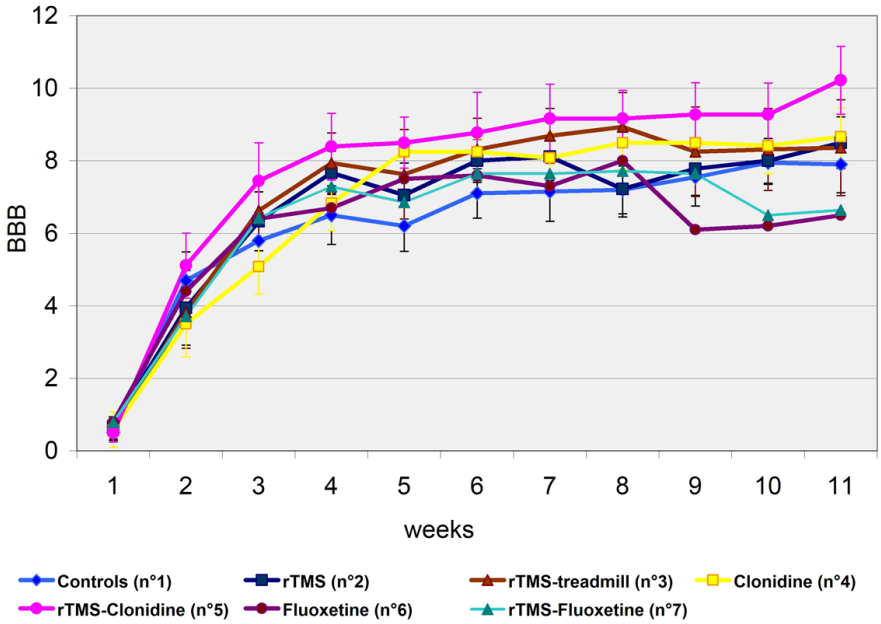

Figure 3.7: Evolution of the BBB scores in the combined treatment rat series over 3 months.

The statistical comparison of the different groups to the control group (Zerbe's analysis, table 3.2) showed *one single significant value* ($p=0.03$). At 32 days post-operatively (dpo32, the fifth BBB score), i.e., at the beginning of the plateau phase of recovery, there was a significant difference between the control group (n°1) and the rTMS-clonidine group (n°5). No other statistically significant difference could be seen, despite the relatively large experimental groups (e.g., controls $n=10$, rTMS-clonidine $n=9$). The global evolution of the rTMS-clonidine group tended towards a significant difference from the controls' ($p=0.09$).

dpo	1vs2	1vs3	1vs4	1vs5	1vs6	1vs7
4	0.63	0.83	0.90	1.00	0.59	0.56
11	0.55	0.48	0.34	0.73	0.81	0.44
18	0.64	0.49	0.54	0.22	0.63	0.62
25	0.27	0.24	0.79	0.14	0.88	0.54
32	0.45	0.31	0.05	0.03	0.27	0.64
46	0.38	0.20	0.44	0.12	0.90	0.69
53	0.98	0.14	0.22	0.07	0.42	0.63
60	0.86	0.65	0.48	0.16	0.25	0.96
67	0.95	0.76	0.64	0.22	0.12	0.25
74	0.59	0.72	0.54	0.07	0.28	0.39
Global	0.62	0.38	0.36	0.09	0.52	0.66

Table 3.2: Zerbe's analysis comparing each treated group's BBB scores to the control group ("1"), each week ("dpo"=days post-operation) and over the entire observation time ("global"). A single statistically significant value can be observed at dpo 32 for group 5 (rTMS-clonidine).

When the evolution of the single membres of the control group was analysed, the variability of the scores and the chaotic evolution of some of the BBB scores over time were striking (refer to the appendix for all the graphs, page 171). Four control rats reached scores of 9 or higher, up to 12; four rats reached 7–8; and two rats achieved very low scores and thus decreased the mean BBB of the control group. In the rTMS-Clonidine group, there were two rats with particularly high scores, i.e., who recovered much better locomotion than the remainder, and who clearly increased the mean BBB of that group. All in all, good mean recovery scores were seen in the most homogeneous groups, with the exception of the BWSTT group. Generally, lower scoring rats showed more fluctuating BBB-score evolutions and tended to show asymmetrical motor behaviour.

The first BBB score (dpo4) was not related to final recovery. For example, in the control and in the clonidine-treated group, the rat with the best final BBB scores

had a score of 0 at dpo4. In the control group, the rat with the second to worst final BBB score had the highest dpo4 score in this group, and, within the clonidine-treated group, the rat with the worst final BBB had the best initial score (i.e., BBB = 3).

3.3.3.2 Miscellaneous Observations

- For logistical constraints, 9 control rats had to be housed in lower cages. Spinal cord injured rats tend to "climb" on the metal grid of the lid and to lift the entire body from the ground; during "climbing", active hind limb movements can be observed. In the low cages, however, the lower trunk rested on the ground. Therefore, these rats had less spontaneous physical activity, and their BBB scores consistently tended to be lower (see appendix, page 175). These rats were excluded from the statistical analysis.

- During rTMS, fear reactions occurred despite the preparations and included hind limb activity.

  In addition, in all the treated groups (rTMS, BWSTT, monoamines), the rats showed spontaneous uncontrolled lower limb activity between the treatment sessions (rapid extensions of the lower limbs, sometimes repeatedly), and more so in the treated than in the control groups.

  Fluoxetine-treated rats showed frequent motor behaviour that was not observed in any other group: spontaneous lordosis for several seconds, repeatedly.

- Fluoxetine was administered intraperitoneally (ip) in the first treated rats. These rats, aside from being less hygienic than the rest of the groups, had malodorant urine, progressively appeared to be dehydrated and, over several weeks, developed an induration of the abdominal region. Autopsy revealed a fibrotic reaction which had invaded the entire abdomen.

  Initially, a local effect of the medication was suspected to be responsible for the abdominal fibrosis, and thus the next fluoxetine treated rats received the same dose subcutaneously. The effect was the same.

3.3.3.3 Intermediate Analysis

The obtained results did not exclude a favourable, synergistic effect of the strategy combining rTMS with clonidine treatment. It appeared biologically plausible that the rTMS group's and the clonidine group's recovery was somewhat increased, as

compared to the controls', and that recovery was even better in the combined treatment group "rTMS-Clonidine"—suggesting synergy.

However, the absence of a clear, statistically significant difference, and the high behavioural variability, prompted a review of the methodology for possible interfering factors. Aberrant locomotor recovery and behaviour could potentially have been related to specific factors, including but not limited to:

- the day of the operation: possibility of material failure or other technical or surgical factors;
- the localisation of the lesion, as estimated by distance to obex and vertebral level;
- variability of the lesion size—but the major cord atrophy in all cords with very little tissue sparing (which is in keeping with the possibility for significant recovery with very little white matter sparing) made morphometry of spared white matter or even topographical localisation of spared white matter impossible (figure 3.8);
- the rat's anatomy (possible differences in the size of the spinal canal resulting in a more or less compressive effect of the balloon).

No correlation of behavioural discrepancies with any of these factors was found (appendix, page 176).

Thus, in order to increase chances to obtain significant results, methods were adapted. The goal was to reproduce the favourable effect of rTMS and noradrenergic treatment in more homogeneous groups.

3.3.3.4 Second Combined Treatment Series

The following adaptations were made:

- First of all, the surgical technique and material were rigorously reviewed: the surgical anatomy of the lesion level was reviewed for highest possible precision of the lesion level, the insertion length was precisely marked at 6mm; a new balloon was used at each operative series, and changed in case of even the slightest doubt.
- In the second series, all rats were housed in cages of the same size to exclude possible influence from differences in spontaneous exercise [189, 312].

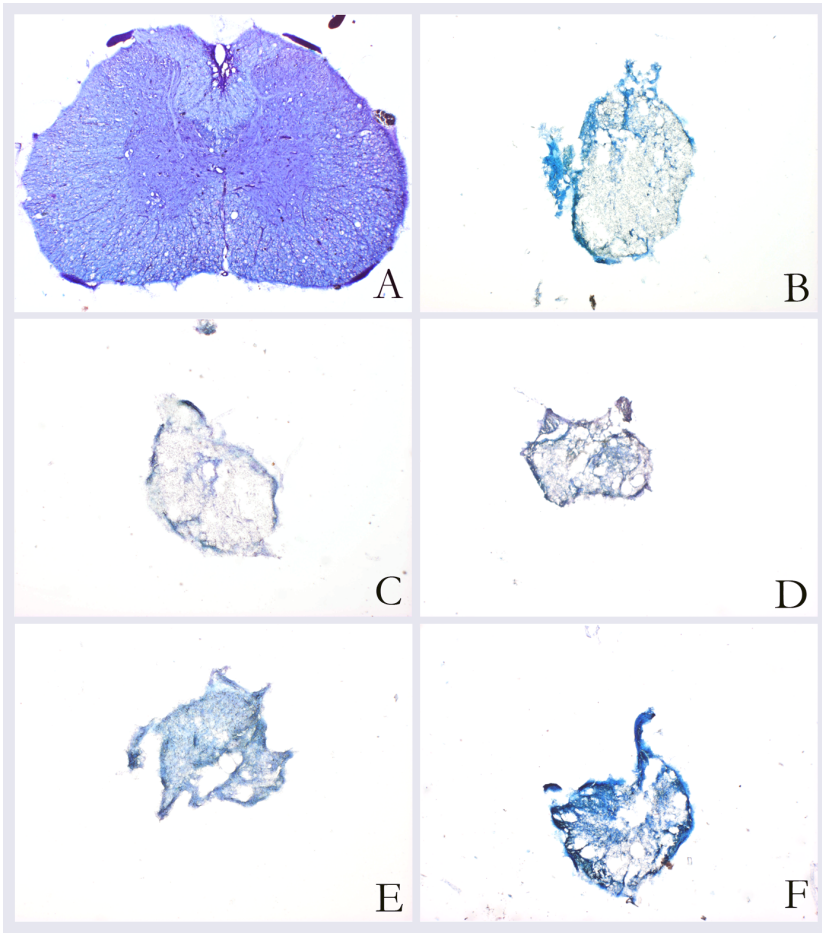

Figure 3.8: Atrophy in the first combined treatment study. (A) shows a section at a normal junction of thoracic and lumbar cord; (B–F) are samples from spinal cords from the control group (Luxol Fast Blue and haematoxylin stains). The sections do not necessarily represent the lesion centre, as the latter could, in most cases, not be located. Final BBB scores of the rats whose cords are depicted are 7 (B), 12 (C), 7.5 (D), 8 (E), and 9 (F).

- The nervous system "activation" by fear was even more taken into account, as well as possible interferences of fear with the interpretation of spontaneous motor behaviour: fear reactions were reduced to the inevitable minimum by longer pre-surgical handling period (two weeks), and rTMS sounds were played during sham-rTMS.

- To increase statistical power, the number of groups was limited to four and higher numbers of rats per group were aimed for (12/group).

- Asymmetry can potentially significantly alter behavioural recovery and its interpretation in the BBB scoring system: in the case of an asymmetrical lesion, the recovery of one hind limb can be "blocked" by another, less well performing hind limb. As an example, if one leg is not capable of weight support and hardly moves, the other leg will never be able to step; the mean BBB score of both hind limbs may be lower than that of a rat with a symmetrical lesion of a comparable size.

  Therefore, after 3 weeks, asymmetrically recovering rats or those showing abnormally fluctuating scores were excluded (scores of 2 or 3 on a four-step scale: 0=no asymmetry, 1=mild asymmetry, 2=moderate asymmetry, 3=severe asymmetry).

- In order to complete behavioural analysis with supplementary tests, as has been suggested [219], a narrow beam was constructed in the perspective to increase sensitivity of the behavioural analysis.

- Tracing of the descending tracts was planned and the technique had been developed, not only to investigate plasticity (using double labelling immunofluorescence techniques), but also to estimate their relative integrity in the different rats at the lesion centre.

At the same time, an investigation was undertaken to describe a technique that could detect potential anatomical causes for variable behavioural recovery, i.e., high resolution magnetic resonance imaging of the rat spinal cord (pages 72 *et seq*).

From the first 40 rats operated, 11 could finally be analysed.

The BBB recovery curves were shaped as expected and more homogeneous than in the first series, with stable plateaus, despite the low number of rats. This was confirmed by the rat-by-rat analysis (graphics: appendix, page 179).

In this series, the rTMS-clonidine rats performed worst (figure 3.9), but there were only two evaluable rats in this group.

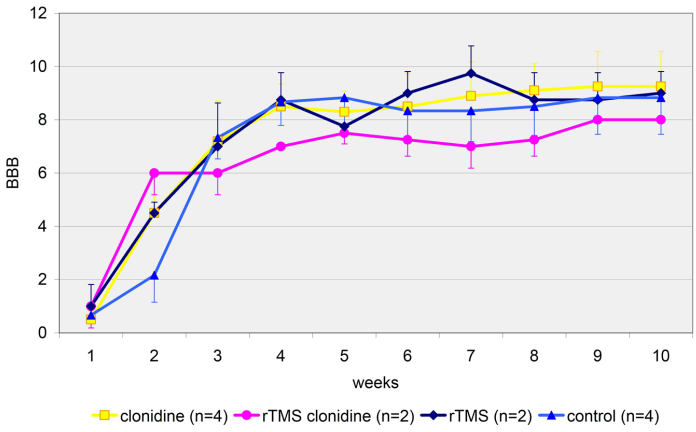

Figure 3.9: Mean BBB score evolution in the second combined treatment group.

This intermediate analysis showed an unacceptably high number of operated rats for a low number of evaluable rats. The investigation was halted until a new approach could provide more reproducible experimental conditions.

3.4 Discussion

The present work investigated treatment strategies which can potentially create a favourable environment for functionally useful and durable neuronal reorganisation in the preserved CNS tissue after SCI, i.e., the locomotor CPG and its connections. As mentioned earlier, this approach is not identical to stimulating immediate motor output by activating the CPG circuitry, although the two may be inter-dependent.

The different strategies appear promising in the rat.

3.4.1 Insights Gained from the BWSTT Study

Early BWSTT increased locomotor recovery, as assessed in the open field. In summary, with comparable white matter sparing at the lesion site after balloon compression injury, trained rats were significantly more likely than untrained rats to achieve a mean BBB score of 9. The score of 9 indicates the capacity for spontaneous hind limb weight support—a critical stage in locomotor recovery after SCI. This motor

activity is *different* from the one that was *trained* (unlike the evaluation on the treadmill by Thota and colleagues [301], which showed that treadmill training increased locomotor function on the treadmill itself). In addition, the score of 9 is just above the "cluster" score of 8, confirming its significance. Recovery was more rapid than in controls and the functional benefit persisted (but did not increase) in the plateau phase.

The usefulness of *early* locomotor training in partial lesions has been confirmed in the rat from another viewpoint: muscle function and size, as well as BBB-evaluated locomotor function, were increased after a short-course treadmill training program (one week) [289]. Conversely, delaying motor training after SCI has been shown to reduce recovery of motor function, i.e., accurate stepping on a horizontal ladder [235].

These findings have to be distinguished from another previously published experimental treadmill study in the rat which had *not* shown any beneficial effect of treadmill training on open field locomotor recovery [121]. In the cited study, the lesion was less severe (40% of preserved white matter). Rats rapidly achieved a BBB score of 9 (first week) and *independent stepping* without artificial body weight support on the treadmill (second week). Thus, as compared to the more severely injured rats in the present BWSTT investigation, the observed *benefit* from treadmill training was likely inferior because rats could rapidly "self-train" the hind limbs spontaneously, and, as mentioned earlier, spontaneous exercise increases recovery [189, 312].

Thus, on the one hand, treadmill training may be particularly useful to enhance recovery of locomotor deficits that are *sufficiently severe to require body weight support* during locomotor exercise. On the other hand, the beneficial effect was observed after a *partial* lesion, where a significant degree of recovery occurs spontaneously. This is very different from complete SCI (e.g., cord transection in the rat), where most authors describe no motor function of the hind limbs, or only slight recovery of movement of single joints [22, 336]. However, a recent publication reports the recovery of locomotor function after spinal cord transection with locomotor training over approximately six weeks [338]. This suggests a key role of direct proprioceptive feedback to the CPG *via* the peripheral afferents. Still, in the present investigation, white matter preservation may have played a role in the BWSTT-enhanced recovery: recovery after partial SCI may be influenced by the transmission of proprioceptive feedback to the supraspinal centres by preserved long tracts—either directly, *via* preserved ascending sensory tracts, or indirectly, *via* the peripheral afferents' modulation of the CPG's rhythmic feedback to suprapinal motor centres. This hypothesis is confirmed by the fact that, in the present investigation, the preserved white matter was mainly found in the ventral and ventrolateral columns. The latter appear to play a key role in the recovery of locomotion and the preserved matter may not only

have included long tracts of supraspinal origin, but also long propriospinal axons.

In summary, during BWSTT,

- the long tracts of **supraspinal** origin could have influenced the activity of the CPG directly: e.g., a rat on the treadmill is likely to recruit supraspinal (voluntary) locomotor programs because it is "forced to advance", as can be seen when observing the rats, in particular the intact forelimbs, on the treadmill; in addition,

- the ventrolateral thoracic white matter contains ascending and descending axons with **propriospinal**, commissural and ipsilateral connections. These propriospinal axons appear to connect the lumbar and cervical enlargements [249]. They can re-establish a functional circuitry with the severed long tracts after SCI, reconnecting the supraspinal centres to the sub-lesional cord and participating in locomotor recovery [19]. This type of circuitry may also have been recruited by BWSTT.

Preliminary clinical studies in human paraplegics indicate that the efficiency of treadmill training may depend on the severity of the lesion. The inability of patients with complete transections to achieve unassisted walking, unlike, e.g., the fully spinalised cat, suggests that the greater improvement observed in subjects with incomplete lesions may not solely be attributable to spinal mechanisms, since generation of stepping is probably more dependent on supraspinal and/or proprioceptive inputs in humans than in cat [100, 309].

Clearly, the mechanisms underlying the enhancement of locomotor recovery by structured stepping activity on a treadmill merits further study, especially the respective roles of the proprioceptive afferents and the intraspinal long tracts and connections, as well as their acitivity dependent plasticity, possibly mediated by BDNF and other neurotrophins. Partial SCI in the rat appears as a useful model for this type of investigation.

3.4.2 Insights Gained from the rTMS Study

In the second series of rats, treated by rTMS alone, the repetitive stimulation of the supraspinal centres appears to have recruited long descending fibres after low thoracic SCI, and increased their plasticity with functional repercussions.

In humans, among the long descending tracts, the CST would be a major target for this kind of stimulation; it is accessible to the clinically used rTMS and its excitability can be modulated (inhibited or stimulated), according to the stimulation frequency [207, 308]. However, since the superficial cortical areas (the origin of the

CST) do not play a prominent role in the recovery of open field locomotion in the rat, the descending monoaminergic tracts are a better target in the rat model for the study of open field locomotor recovery. The present investigation concentrated on the descending serotoninergic tracts, because the latter have shown a potential for regenerative plasticity [85, 123] and appear to play a major role in recovery after experimental SCI [23], as does serotonin [113, 254]. Therefore, the stimulation was aimed at the deeply situated brainstem neurons (reticular formation, raphe, noradrenergic groups A5 and A7, etc.); these are within reach of the magnetic field generated by rTMS [259, 263].

The first major finding of this study was that, despite the same lesion intensity, the low thoracic lesions induced much more severe locomotor deficits than high thoracic lesions. This can be explained by the known extension of the balloon-induced lesion over several metameric levels [214]; low thoracic lesions can thus partially disrupt the *rostral* part of the CPG, which reaches the low thoracic cord (T13-L2) and is crucial for the locomotor pattern generation [126, 182, 209].

In those partial low thoracic lesions, unlike the higher ones, daily rTMS appeared to be beneficial. In the rat, spinal cord serotonin is of almost exclusively supraspinal origin [57], and 5-HT immunoreactivity was preserved in the periphery of the lesion site. Thus, descending serotoninergic axons were partially preserved. After the injury, 5-HT immunoreactivity was markedly decreased but not completely lost caudally to the lesion site, and better recovery in the rTMS treated rats was found to be correlated with a caudal increase in 5-HT reactivity (including the ventral laminae where locomotor-related neurons are concentrated [182]). Knowing that serotoninergic neurotransmission appears to be predominantly nonsynaptic (so-called volume transmission) [255], the increased locomotor recovery could be attributable to a favourable paracrine *neuromodulatory* effect on the CPG of rTMS-induced serotonin expression by the stimulated long tracts, much like the improvement of locomotor recovery observed after transplantation of monoaminergic neurones in the same thoracic level (T11) [254].

These results thus indicate a facilitatory role (and not necessarily immediate locomotor pattern stimulation) of rTMS-induced serotonin expression on the neuronal reorganisation of the locomotor systems after SCI. The mechanism underlying the beneficial effects of rTMS are not certain. Chronic rTMS has been reported to have various region-specific effects on release and uptake of serotonin, as well as on its receptors [29, 140, 178, 192, 194], including in humans [280], and this may explain a serotoninergic neuromodulatory effect. In addition, pulsed magnetic fields were found to enhance and guide neuritogenesis both *in vivo* [167] and *in vitro* [97, 205]; therefore, sprouting of serotoninergic neurites is another plausible potential mechanism for the increase in 5-HT content and possible serotoninergic neuromodulation.

This particular aspect certainly merits further investigation.

It also remains unclear whether the best observed recovery, in the rat with the most caudal lesion, was attributable to a lesion "too caudal" to injure the locomotor generation region of the rostral CPG, or if a "lowest distance" to the injured CPG was beneficial, because re-expressed serotonin could more easily have reached the pattern generating network.

Last but not least, in this initial investigation of rTMS after experimental SCI, the lesion levels were quite variable, as were the behavioural scores, and this must be considered in the interpretation of the results.

Concerning the high thoracic lesions, the absence of an observable beneficial effect may be attributed to different causes. Treatment by rTMS could simply be inefficient. Or, on the other hand, the high thoracic region could have been reached by the magnetic stimulation. Direct stimulation of thoracic lesions has shown a possible detrimental effect on recovery in preliminary, unpublished studies [311]. This might explain the absence of a beneficial effect. Other possible reasons include the size of the experimental groups, which may not have been sufficient to detect an effect of the treatment. Or there may have been differences in white matter sparing. This is one limitation of the study: there was no quantitative assessment of white matter sparing; so there was definitely a need for further study, in order to ascertain the beneficial effect of rTMS.

Still, in summary, these initial results indicated that:

- For the same lesion intensity, spontaneous locomotor recovery after SCI may be better after high thoracic injuries than after low thoracic injuries, probably due to partial disruption of the central pattern generator by low thoracic lesions.
- Daily use of high-frequency rTMS may improve the extent of functional recovery after low thoracic spinal cord lesions; this effect was associated with enhanced serotonergic nerve fibre density in ventral horn grey matter caudal to the lesion and may therefore be due to plastic changes that influence serotoninergic neuromodulation of the CPG.

A series of questions are raised:

1. In low thoracic lesions: what is the precise role of potential serotoninergic reinnervation and neuromodulation of the injured CPG? Are other long tracts involved, like the RtST and the CoST, and what are the precise mechanisms of recovery? The regenerative and plastic potential of raphespinal axons has already been shown [85, 123, 248], but the regional effect of potentially regenerating RaST axons on the CPG circuitry must be investigated in more detail.

In this setting, it is interesting to note that some authors suggest a different, more diffuse, and supralumbar distribution of the 5-HT stimulated rhythm generating circuitry in the neonatal rat spinal cord, where supralumbar application of serotonin induces rhythm generation, whereas the application of serotonin to the lumbar region does not induce this kind of neuronal activity. The authors suggest, on the basis of their findings, that 5-HT does seem to play a *"role in organizing specific patterns of behavior, such as locomotion, on a network level"* [72]. This confirms the neuromodulatory potential of serotonin, and suggests that even a rostral 5-HT increase at distance from the CPG may be able to influence neuronal locomotor output.

Thus, from a basic scientific point of view, in order to better understand the mechanisms underlying enhanced recovery induced by rTMS in low thoracic lesions, as detected here in rather small and rather variable experimental groups, it would be useful to confirm the effect using precise lesions induced at different low thoracic and lumbar levels, and to observe the effects of rTMS (locomotor observation and investigation of the descending tracts and caudal circuitry, e.g. using immunohistochemical, immunoblotting, and tracing studies, and investigating plasticity).

2. In a more clinical perspective, where most lesions are remotely situated from the CPG region, it was important to know if the observed beneficial effect of rTMS was limited to lesions *injuring* the locomotor CPG in the low thoracic and upper lumbar cord, or if rTMS could recruit long tracts sufficiently to modulate sub-lesional plasticity in (or above) the partially *deafferented* but anatomically intact CPG.

3.4.3 Insights Gained from the Combined Treatment Study

Based on the principle of multi-modal approaches, as in human rehabilitation [114], and on the previous results, as well as the hypothesis that several treatments aiming at the same target may be synergistic, the "combined treatment strategy" was designed, in order (1) to confirm the beneficial effect of rTMS on the *deafferented* CPG and (2) to further enhance the recruitment of spared CNS parenchyma.

Since the first two treatment series indicated the possibility of a facilitatory role of BWSTT and rTMS on the neuronal reorganisation of the locomotor CPG after SCI *via* monoaminergic neuromodulation, rTMS was combined with: (1) the selective serotonin reuptake inhibitor **fluoxetine**, in order to potentiate the effect of existing sub-lesional serotonin; (2) the noradrenergic agonist **clonidine** as a neuromodulator which also exhibits some degree of intrinsic serotoninergic activity [239],

and which is as yet unstudied in the spinal cord injured rat; and (3) **BWSTT**, creating patterned peripheral sensory (proprioceptive) input in order to re-structure the neuronal activity in the CPG.

With the group design explained in 3.2.7, page 47, rTMS could on the one hand be assessed as a *primary* treatment modality—either as a stand-alone strategy, or in a combination setting where BWSTT or pharmacotherapy could create a neuromodulatory and plastic environment that could potentially enhance the effects of rTMS. On the other hand, in the absence of an effect as a stand-alone treatment, it could also be assessed as a *complementary* strategy to pharmacological treatment or to BWSTT, because, in this perspective, it could potentially itself induce a favourable activation state of the CPG *via* the long tracts, thus possibly enhancing beneficial effects of BWSTT or pharmacological treatments.

To increase statistical power and reproducibility, these strategies were tested in larger experimental groups than in the first rTMS study, after a partial lesion allowing for moderate locomotor recovery, and induced by a single surgeon at a reproducible lesion level in the low thoracic cord. The level (T8–T9) was chosen at sufficient distance from the CPG to avoid direct injury of the locomotor circuitry, but also sufficiently caudal to be at distance from the magnetic field in order to avoid direct magnetic stimulation of the lesion site.

3.4.3.1 Clonidine and rTMS Treatment

The mean BBB score evolutions initially showed a very promising trend: all groups starting at almost the same mean BBB score, the groups treated by rTMS alone and by clonidine alone performed better than the controls; when both treatments were combined, locomotor performance was even better—indicating synergy, or, at least, additive effects. However, these effects failed to reach statistical significance, except for a single mean BBB score comparison of the rTMS-clonidine treated group to the controls. A detailed analysis (page 55) revealed extremely variable behavioural scores within the groups (page 171), and a review of the single BBB scores showed that asymmetrically recovering rats played a major role in this variability.

Therefore, after the exclusion of different other potential factors that could have disturbed the experiment (page 56), a histological analysis of the spinal cord lesions was attempted in order to detect asymmetrical lesions, variable lesions sizes and white matter sparing. In the four treated groups in question, very little white matter preservation was observed, although many of the rats had recovered weight bearing and stepping capacities. This is in keeping with results of other studies [20, 22, 336]. Nevertheless, cord atrophy, deformation by handling and sectioning of the fragile material, and the severity of the lesion made the study of anatomical white mat-

ter preservation impossible, despite the efforts of a very experienced technician in the preparation of the material. It was in fact impossible to determine the cord's orientation (ventral or dorsal white matter?) and, despite myelin specific staining (Luxol Fast Blue), the preserved white matter's quantity, or even the rostro-caudal localisation of the lesion centre. Thus, there was no histological proof of variable white matter sparing; nevertheless, differences in the lesion severity and symmetry appeared as the most plausible explanation for the variable behavioural scores.

Because of the compelling rationale that monoaminergic treatment and rTMS may be *beneficial* and *synergistic*, another series of rats was operated in the methodologically revised setting (refer to page 56), with the goal of confirming the observed effect in more homogeneous groups. However, combining an excessive mortality rate and the necessity to exclude a number of rats, mainly for asymmetric recovery, the proportion of behaviourally "reliable" rats out of the total number operated was unacceptably low (12/40=30%), and in the 12 rats that could be analysed, the lowest scoring group on the BBB scale, unexpectedly, was the one treated with rTMS and clonidine. The investigation was halted.

Series:	Combined I	Combined II
rTMS	+	0
BWSTT	+	
Clonidine	+	0
Fluoxetine	−	
rTMS + BWSTT	+	
rTMS + clonidine	++*	−
rTMS + fluoxetine	−	

Table 3.3: Summary of the effects of the different treatment strategies on BBB locomotor recovery in the combined treatment series; *denotes a trend towards statistical significance (<0.1).

Thus, in the current experimental setting, the initially encouraging results could not be confirmed. The above experiments should be repeated, ideally in the following experimental conditions:

- High number of animals per experimental group.
- *Few different treatment groups* per investigated series, e.g. noradrenergic *or* serotoninergic, agonists *or* reuptake inhibitors, *or* rTMS with or without BWSTT.
- A partial lesion model that has high morphological and behavioural reproducibility (see below). Epidural balloon compression has been reported to result in rather reproducible behavioural deficits [313], and behavioural variability does seem to occur less in weight drop contusion injury, where scores

for this injury severity show a variability of one or two points on the BBB scale [R. Deumens, G. Koopmans, personal communication].

- The comparison of these partial lesions to a transection group of rats, and, in the partial lesion group, the use of secondary transection, to investigate the importance of long tract preservation and the long term modulation of the sub-lesional segment.
- Groups with longer survival than the treatment period, in order to observe locomotor recovery after the treatment has been halted. This could indicate if the potentially observed beneficial effects are durable.
- The additional use of pharmacological antagonists to reverse potentially beneficial effects of the investigated substances (e.g., Yohimbine for adrenergic substances, ...).
- A compensation for the limitations of the BBB scale, which not only evaluates a limited range of behaviours, but is also motivation-dependent. Additional behavioural analyses should be well chosen on the basis of the rats' motor capacity [219], but CatWalk Analysis, despite being costly, appears as a particularly useful method [188].
- A correlation of supraspinal plasticity and neuronal remodelling, using functional imaging techniques like functional MRI or positron emission tomography, as well as biological analysis (histology, molecular biology).
- In a convenient experimental setting, the effects of the investigated treatment strategies should be studied above and below the lesion, mainly plasticity, including synaptic plasticity as well as sprouting; e.g. double labelling 5-HT and growth associated protein 43; immunohistological and -blotting analysis of BDNF, trkB and synapsin; tracing experiments to investigate sprouting of particular tracts, with immunofluorescent double-labeling (tracer-GAP43) and analysis of specific neuronal populations of the CPG (ephrins).
- Last but not least, white matter sparing at the lesion centre must be assessed as precisely as possible, including quantification of the sparing of long tracts by tracing studies, in particular of the RtST and the RaST [146, 315].

3.4.3.2 The Role of White Matter Sparing

In the present investigation, the influence of the long white matter tracts on the CPG and on locomotion is clearly seen. A certain degree of locomotor recovery is

observed after the 20 μl balloon compressions which spare some white matter, as opposed to the more severe 40 μl lesion which results in almost complete destruction of the cord at the lesion level [214]. In addition, fear reactions and attempts to escape from the rTMS position resulted in vivid movements of the hind limbs during rTMS and sham stimulation, i.e., movements *a priori* initiated in supraspinal centres. This confirms that supraspinal connections of the CPG must be taken into account in the study of the motor function of the hind limbs after partial SCI. Since variable recovery may be due to variable white matter tract sparing, reproducible white matter tract injury appears essential in these models.

In this perspective, let us consider the potential usefulness of the interruption of single white matter fibre tracts. The deafferentation of the CPG from the input of a single tract could potentially induce perfectly reproducible behavioural deficits. This would, theoretically, be ideal for investigations of sub-lesional plasticity and recovery: behavioural assessment of the specific functional deficit and the following recovery could determine the differential contribution of the white matter tracts to spontaneous recovery, as well as in different treatment settings. However, the main locomotor white matter tracts run in ill defined, overlapping fibre systems (figure 2.2, page 21). In addition, they may be functionally redundant [12, 151, 170, 272, 321]: preserved axons of a partially interrupted tract may subserve the functions of the lost fibres; the corresponding contralateral tract may take over functions of the lost tract *via* intraspinal circuits, like those based on commissural interneurons; and other tracts may substitute for the lost function of one specific tract. The latter potential mechanism, however, does not appear crucial in spinal cord injury, because—topographically speaking—the key tracts for locomotion run in the same funiculi (figure 2.3, page 26), and, therefore, lesions will always damage fibres from several origins. Furthermore, the respective roles of different fibre tracts remain partially unknown—not only for basic locomotion as assessed by the BBB scale, but also for more precise motor functions. In addition, these different motor functions cannot necessarily be behaviourally quantified because of limited existing behavioural assessment tools. Thus, all in all, the lesion of isolated fibre tracts is neither practicable, nor desirable with the current state of knowledge.

Still, the ideal spinal cord lesion for the study of locomotor recovery would interrupt reproducible sets of descending tract fibres, resulting in reproducible locomotor deficits and reproducible recovery, assessed and followed by specific behavioural tests. Theoretically, this kind of precisely targeted white matter lesioning could be achieved, e.g., by partial spinal cord sections. But the lesions that would be the most interesting for the study of locomotor recovery—i.e. in the ventral and lateral funiculi [272]—are surgically challenging or even impossible to create. Another technique that could potentially produce deafferentation of the CPG with high spatial

precision is localised white matter tract demyelination using stereotactic injections of ethium bromide and irradiation [199, 200]; but the induced demyelination is temporary.

Thus, it still appears necessary to rely on "classic" partial lesion models, like compression injury (with more or less frequent lesion asymmetry, and the observed variable behavioural recovery), or contusion injury (as used in many laboratories, including behavioural follow-up), even for the study of biological phenomena spatially remote from the lesion itself, like CPG plasticity and locomotor recovery, as opposed to studies of the lesion's physiopathology.

However, neither compression nor contusion injury can perfectly control the quantity and the topography of white matter sparing, and both may therefore not appear ideal for the comparative assessment of locomotor recovery. Nonetheless, in both types of lesions, ventral and lateral white matter is usually partially spared. And there are scenarios where the comparison of locomotor recovery in different treatment groups with these lesion models could yield clear results:

1. if major tissue sparing or neuronal regeneration were to occur due to particularly successful neuroprotective or repair strategies. In that case, more or less reproducible lesions that imitate the biomechanics and physiopathology of human SCI are clearly of interest. However, behavioural ameliorations due to the treatments must be considerable to achieve biological and statistical significance. Even in this hypothetical setting, the lesion topography and resulting descending tract interruption must be taken into account in the interpretation of the results.

2. if white matter sparing could be anatomically and quantitatively assessed to confirm lesion homogeneity among experimental groups. Serial sectioning and histological analysis of the excised *post-mortem* spinal cord can be used. However, this is laborious, and, in the combined treatment group, despite a partial cord lesion with significant locomotor recovery, major spinal cord atrophy and fragility of the chronic lesion site have interfered with the histological quantification of spared white matter. Therefore, a different technique would be useful, either a less laborious *post-mortem* technique without the necessity to excise the cord from the spine, or an early *in vivo* assessment of the lesion (in order to assign the rats to homogeneous treatment groups with comparable lesions).

In the present work, we have therefore investigated *post-mortem*, high resolution MRI of the injured spinal cord, and assessed the usefulness of the technique in the investigation of spared white matter.

Chapter 4

Correlation of Locomotor Recovery with Lesion Severity and White Matter Sparing Using High Resolution MRI and Histology

4.1 Strategy

A DETAILED STUDY OF THE INJURED SPINAL CORD was undertaken using *post-mortem*, high resolution magnetic resonance imaging (MRI) in an experimental spectrometer with a magnetic field of 9.4 T.

4.1.1 Experimental Spinal Cord Injury MRI: Objectives

MRI was tested after experimental SCI for its capacity to demonstrate:

- anatomical changes of the spinal cord over time, like cord swelling and cord atrophy
- the spinal cord lesion and its histological components like oedema, necrosis, scarring, and haemorrhage

- the severity of the lesion (i.e., its volume)
- the microanatomy of the lesion (i.e., its precise transverse extent)
- the spared tissue (in particular the quantity and the anatomy of perilesional white matter sparing).

Its precision was evaluated by histology, and both were correlated with locomotion.

4.1.2 Sub-Lesional Cord Imaging in the Human: Objectives

Correlative investigations of high field MRI in traumatic spinal cord injury are relatively rare [24, 50, 73]. In the perspective the evolving technology introducing high field strength magnets into clinical MRI, up to 9.4 T [5], we investigated the potential of standard MRI sequences (PD) to visualise damage to the long white matter tracts in a sample of high thoracic human spinal cord, caudal to the injury site.

4.2 Methods

4.2.1 Material: the Spinal Cords

4.2.1.1 Rat Spinal Cord Compression Injury: Experimental Groups

A total of 36 rat spinal cords were analysed *post-mortem* in a 9.4 T magnet, following balloon compression injury (described above, page 40), and, when useful, weekly behavioural follow-up (pages 42 *et seq* and 170).

The following groups were analysed:

1. 2 dissected spinal cords from the combined treatment series were investigated, followed by 8 dissected cords with different lesion severities (10, 20, 30, and 40 μl) from a series of 12 untreated rats, sacrificed after 9 weekly BBB evaluations. There were striking behavioural differences between the groups: in the 10 μl group, locomotion approached normal (as evaluated by BBB) after two weeks; in the 20 μl group, recovery was partial; and in the 30 and 40 μl groups, only limited movements of the hind limbs could be observed (table 4.1).

μl	n°	BBB1	BBB2	BBB3	BBB4	BBB5	BBB6	BBB7	BBB8	BBB9
10	9	11	20	20	21	21	21	21	21	21
10	10	19	20	21	21	21	21	21	21	21
20	1	2	5,5	6	6,5	6	6,5	6	6	7,5
20	3	0,5	8	11	14	16	15	16	16	16
30	7	0	1	5,5	5,5	3	4	3,5	4	3,5
30	12	0	0	0,5	0,5	0,5	1	0,5	0,5	0,5
40	5	0	0,5	0,5	1	2	1	2,5	3	2,5
40	8	0	1	1	4,5	1	2	0,5	0,5	0,5

Table 4.1: BBB scores in the pilot study, weeks 1–9 (BBB1–BBB9).

2. In second series, 16 rats were operated (20 μl lesions) for survival delays ranging from 1 day to 5 months (table 4.2), and their spines analysed by MRI.

Survival	number of rats
1 day	3
4 days	3
7 days	3
14 days	1
21 days	1
28 days	3
56 days	1
150 days	1

Table 4.2: Spine MRI group with different survival delays.

3. The third study was conducted in a group of 12 rats which had initially been operated for the combined treatment series (20 μl balloon compression). However, the balloon malfunctioned and inflated abnormally (hourglass shape and variable inflation volume, figure 4.1).

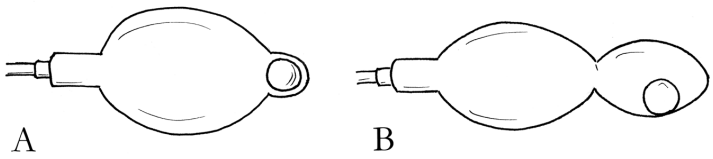

Figure 4.1: A: normal inflated balloon. B: abnormal inflation of the metallic sphere's chamber, resulting in a hourglass-shaped deformity.

Therefore, the induced lesions' topography and severity were variable. At the first behavioural evaluation (4 dpo), before the first planned treatment, the

behavioural deficits were very different. They corresponded to the interval of scores that could be expected after 10–20 μl balloon compression lesions. Therefore, the rats were not treated. But the variable lesions (that could be expected because of the variable locomotor deficit) offered the opportunity to investigate MRI for its potential to detect differences in spinal cord lesions. Thus, 11 rats were followed behaviourally for two months (8 BBB scores), and five were finally completely analysed by MRI and histology, including morphometry.

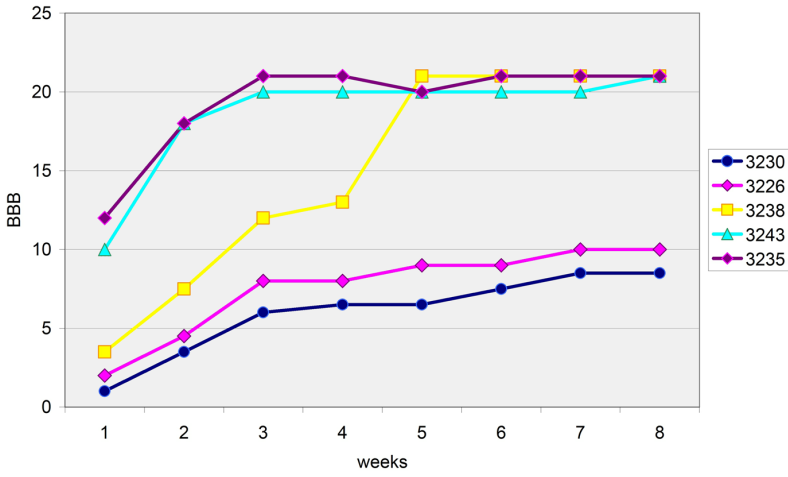

Figure 4.2: Behavioural follow-up of the variable lesion rat series over 8 weeks (BBB). There are three clearly distinct recovery patterns (see text).

Three locomotor recovery patterns could be observed (figure 4.2): in the first pattern, rats showed the expected locomotor evolution after 20 μl cord compression, achieving mean BBB scores of 8.5 (n° 3230), corresponding to weight support on one hind limb at stance, and 10 (n° 3226), indicating limited stepping. In a second pattern of intermediate recovery, rat n° 3238 attained the maximal score of 21 after a gradual recovery over about a month. In a third pattern, two rats (n° 3235 and 3243) showed recovery of almost normal loco-

motor capacity after approximately two weeks.

Several of the rats showed initially asymmetrical locomotor recovery, including n° 3226 and 3238 (see below). After the initial asymmetry, over the first weeks, both these rats increased locomotor performance of the weaker limb, and locomotor behaviour became symmetrical (i.e., inconsistent stepping for n° 3226, and progressive recovery up to the maximum score for n° 3238).

4.2.1.2 Human Sub-Lesional Spinal Cord

Human spinal cord injury material was studied in collaboration with Dr. Byron Kakulas, University of Western Australia, Perth, Australia, who managed one of the largest tissue banks of injured human spinal cords that currently exist (more than 1000 *post-mortem* spinal cords, without previous surgical intervention). Some of these cords have been preserved over decades, and several have already been analysed for pathological changes [269, 268, 53] in collaboration with Dr. Gary Brook, Rheinisch Westfälische Technische Hochschule, Aachen, Germany.

The presently used *post-mortem* spinal cord tissue was obtained in 1972 from a 29 year old male who, as a result of a diving accident, had sustained a severe compression fracture of the fifth cervical vertebra, resulting in tetraplegia with complete motor and sensory loss below the lesion level. Seven months after the initial injury, the patient died, and the spinal cord was removed and fixed in 10% formalin until further use. A high thoracic cord sample (approximately Th2-Th4) was investigated after 30 years of storage.

4.2.2 Magnetic Resonance Imaging

4.2.2.1 MRI of Rat Spinal Cord Injury

All animals were deeply anaesthetised and perfused with PFA. The spinal cords from the combined treatment series and the pilot study, and whole spines in the remainder, were removed and post-fixed in paraformaldehyde for 24–48 hours, then stored in PBS-azide. MRI was performed on specimens of 23 mm length. Based on a rapid localising MR scan, the acquisitions were centred on the level of the injury. Consecutive 1 mm sections were obtained in axial slice directions, and 0.5 mm sections in longitudinal horizontal directions, using the multi-slice spin-warp technique in a 25 mm birdcage coil on a 9.4 T vertical bore magnet (Varian Inova 400 spectrometer, Varian, Nuclear Magnetic Resonance Instruments, Palo Alto, California, U.S.A.).

In all series, the cords / spines underwent PD weighted imaging, and, on some, standard T1 and T2, echo gradient, as well as inversion recovery imaging suppressing

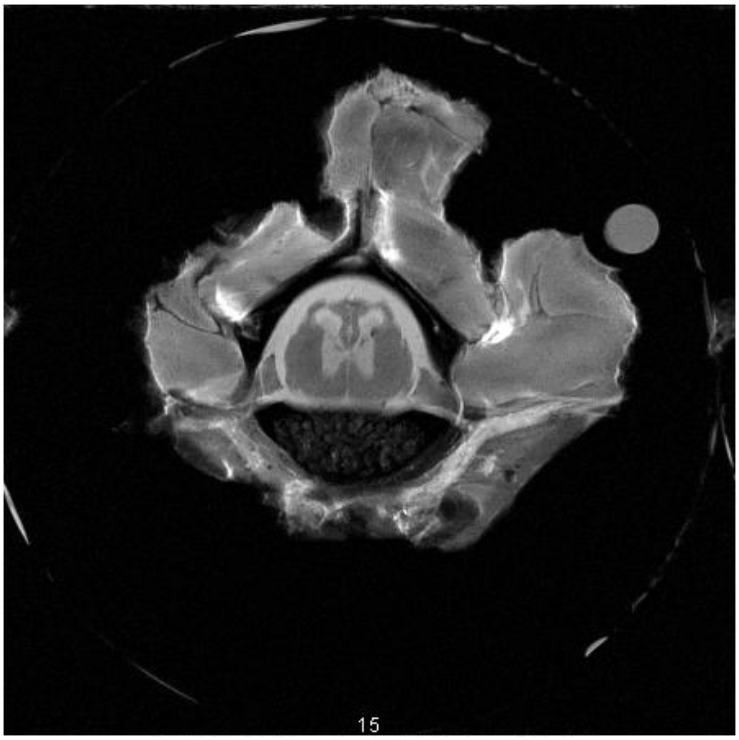

Figure 4.3: Sample image of thoracic cord from a MRI overview, high resolution proton density (PD): number of averages (NA): 32. Rat 3235.

free water or intact white matter were tested. In the behaviourally followed series, all sequences were used on the five selected spines, except for echo gradient imaging.

All PD images were acquired over a length of 23 mm with a repetition time (TR) and an echo time (TE) of 2500 and 18 ms, respectively. PD-images with an in-plane resolution of 37.8 x 37.8 μm^2 (and 33 x 33 for the horizontal images) were acquired with a number of averages (NA) being 32, which resulted in a total accumulation time of about 9-10 hours ("PD32"). PD-images were also acquired with NA=8 or NA=4, both with a resolution of 68 x 68 μm^2 (total accumulation time of about 50 minutes for "PD8" and 25 minutes for "PD4"). The parameters for T1 and T2 weighted imaging were: TR=400 ms/TE=18 ms and TR=2500 ms/TE=55 ms (TE=18 ms for the longitudinal images), respectively, and for gradient echo imaging TR=200 ms, TE=6 ms; 200 scans/slice, pulse angle=22.5°. Inversion recovery (IR) images were acquired at TR=2500 ms, TE=18 ms, and an inversion time (TI) of 525 ms or 1750 ms, suppressing the signal of normal white matter or free water, respectively. The IR-images were acquired over a distance of 11 mm centred on the lesion and had a resolution of 68 x 68 μm^2. IR-imaging was accomplished with 16 NA in about 7 hours for white matter suppression, and almost 21 hours 44 minutes for water suppression.

The raw acquisition data was used to create single slice images with high resolution, as well as overviews (see below, figure 4.4). As in clinical imaging, the tomographic right side corresponds to the anatomic left side, and *vice versa*.

4.2.2.2 Dissected Human Spinal Cord MRI

The high thoracic cord sample was investigated by proton density (PD) weighted nuclear magnetic resonance in three orthogonal planes in the same magnet as the rat spines. The in-plane resolution was 50 x 50 μm^2 and total acquisition time 20 hours.

4.2.3 Histological Methods

4.2.3.1 Histology of the Rat Spinal Cord

After careful removal from the spinal canal and cryoprotection, the spinal cords were cut transversely at a thickness of 15 μm, mounted onto gelatine coated slides. All rats cords were stained with haematoxylin and Luxol Fast Blue (for myelin, i.e., white matter).

4.2.3.2 Histology of the Human Spinal Cord

Following MRI, the spinal cord sample was cut transversely into small blocks and embedded in paraffin wax. Serial transverse sections (5 μm thick) were cut on a microtome and collected onto poly-L-lysine coated slides. Sections were then de-waxed and re-hydrated to undergo routine histological staining and immunohistochemistry.

Routine histology: Sections obtained from the lesion site as well as from the thoracic cord sample were processed using a number of histological stains.

Peroxidase immunohistochemistry: Sections were subjected to antigen retrieval by multiple cycles of microwave heating, inhibition of endogenous peroxidase activity, incubation in blocking solution, washing in 0.1 M PBS, and incubation in primary antibody overnight at room temperature. The next day, sections were incubated in biotinylated horse anti-mouse antibody or biotinylated goat anti-rabbit for 1 hour, followed by the avidin-biotin-HRP complex. Peroxidase activity was revealed by incubation in 3,3'-diaminobenzidine.

The primary antibodies used for peroxidase immunohistochemistry were anti-200 kDa neurofilament (NF, monoclonal), anti-vimentin (monoclonal), and glial fibrillary acidic protein (GFAP, polyclonal rabbit anti-bovine). Monoclonal antibodies for the detection of MHCII (anti-HLA-DR) and for monocytes and macrophages (anti-CD68) were also used but proved to be unsuccessful in this material. For controls, primary antibodies were omitted.

Fluorescence immunohistochemistry: Prior to microwave treatment, a mixture of 95% absolute ethanol/5% acetic acid at 4°C was used to incubate the sections for 20 minutes, followed by a 5 minute incubation in 70% ethanol. Sections were then blocked by a 20 minute incubation in 10% defatted milk powder followed by an overnight incubation at 4°C in polyclonal rabbit anti-myelin basic protein primary antibody (MBP). The next day, sections were incubated in Texas Red conjugated goat anti-rabbit antibody for 3 hours. Finally, sections were cover-slipped using Immuno Fluore mounting medium and epifluorescence microscopy performed.

For controls, primary antibodies were omitted.

4.2.4 Image Analysis and Morphometry in the Rat Spinal Cord

Morphometric data were obtained in the behavioural MRI series using an image analysis program. Two observers, blinded to the rat's behavioural scores by using a

different set of identification numbers, measured the lesion surface in all MR sections and in those histological sections of sufficient quality.

4.2.4.1 MRI Morphometry

For the MR measures, jpeg-formatted PD overviews, showing all transverse sections (figure 4.4), were used. Image analysis, using Olympus analySIS FIVE (Olympus, Tokyo, Japan), proceeded caudo-rostrally, from the sub-lesional lumbar area (L) to the supra-lesional thoracic area (T, see figure 4.5, from left to right). This order of analysis (from sub-lesional to supra-lesional) was used in all settings, including the histological analysis.

The lesion areas and preserved cord matter, as well as total cord surface and dural sac surface were measured as square pixels. When there was doubt in the interpretation of the PD image concerning the detection of preserved white matter (especially minimal preserved white matter), IR images were used to decide if a slight signal alteration was to be considered pathological or normal. The distinction of the IR induced hypo-signal from "paramagnetic" hypo-signal of bleeding (iron) was based on the presence or absence of this hypo-signal on the PD image.

4.2.4.2 Histological Morphometry

The histological surface measurements were obtained on transverse sections (15 mm slice thickness) using the same image analysis software. For practical reasons, one slice out of 33 was kept (two 6mm blocks) in the periphery, and one out of 22 in the central block (11mm). Thus, 10 sections were analysed for the rostral and caudal segments, and 30 for the lesion centre (i.e., a total of 50 sections over the length of the 23mm block).

4.2.4.3 Measurements and Calculations

Volumetry: The lesion volume was calculated as the area under the curve of histological and MRI surface measurements. For the MRI sections, the lesion volume was calculated in μm^3 by multiplying the lesion area by the pixel size in μm^2; the sum of all surface measurements in μm^2 was multiplied by the slice thickness (1000 μm). From the histological surface measurements, a graph was established (figure 4.8, page 84). The X-axis is the length of the cord specimen; the Y-axis is the surface (μm^2). When a histological slice was missing, or not sufficiently well preserved, the mean value of both adjacent data points was calculated. The area under the curve was divided in

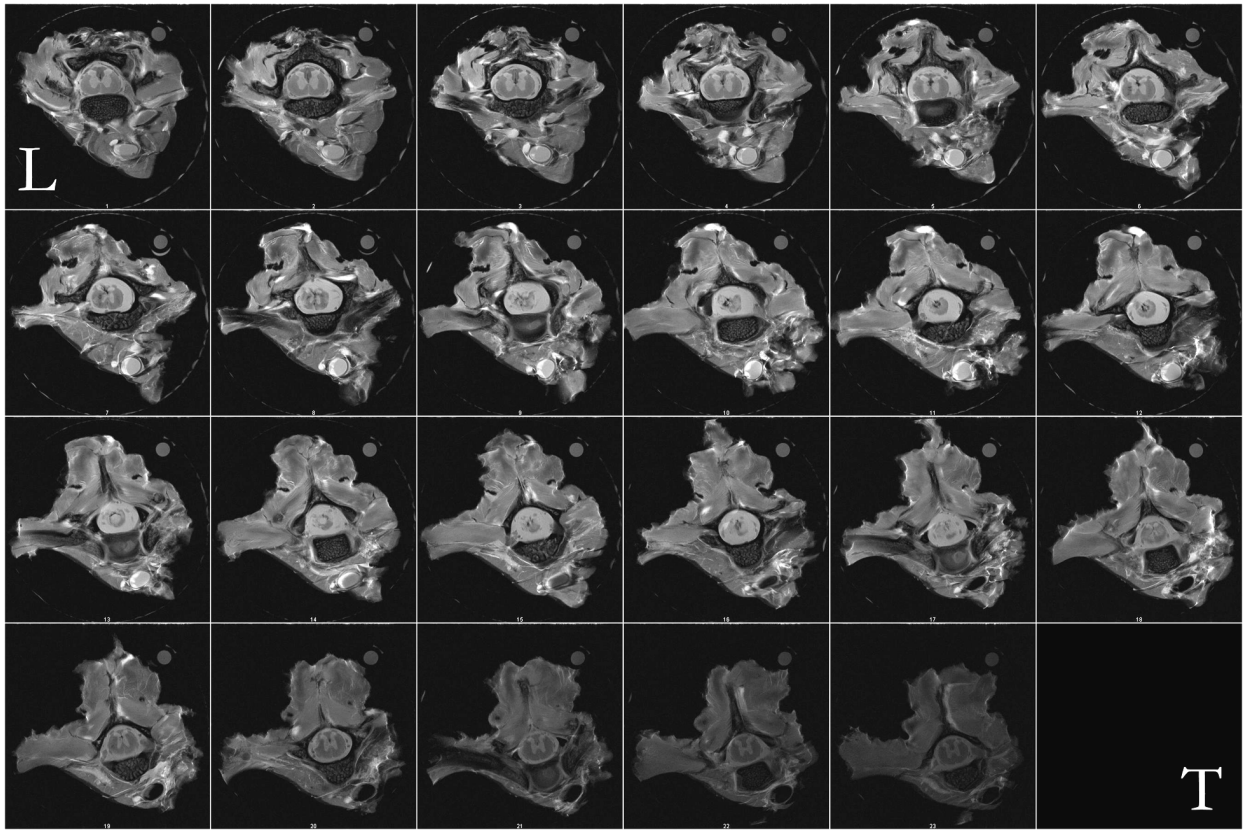

Figure 4.4: Illustration of the material used for MR image analysis: the MRI "overview" spans the entire length of the spine sample. Analysis was conducted from lumbar (L) to thoracic (T). The fading image contrast at the extremities is due to diminishing magnetic field strength.

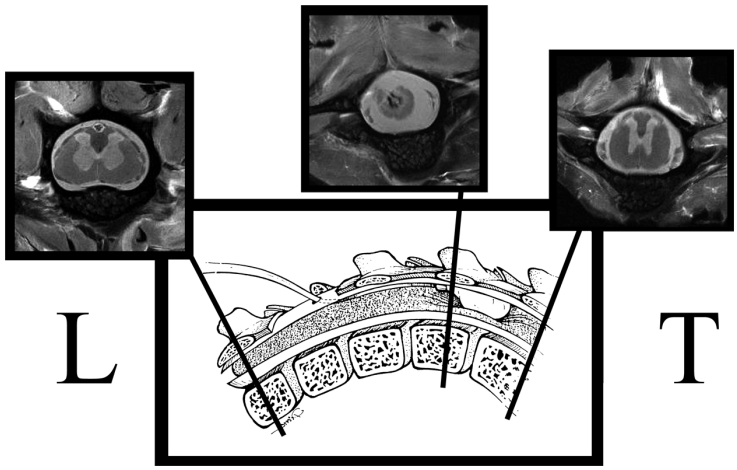

Figure 4.5: Caudo-rostral MRI acquisition, i.e., from the sub-lesional lumbar cord (L) to the supra-lesional thoracic cord (T). In the frames: typical normal lumbar and thoracic cord PD-MR images, as well as a sample image of a spinal cord lesion between both. The histological analysis followed the same direction. In the graphic illustrations of morphometric data (see below), left–right also corresponds to lumbar–thoracic.

small rectangular bars ($x * y$: x =distance between 2 consecutive histological slides; y =measured lesion surface). The area of each bar was calculated ($[(x_2 - x_1) * y_1] + [(x_2 - x_1) * (y_2 - y_1)/2]$) and the results added. The maximal lesion extension in the transverse cord sections was represented by a ratio dividing the lesion surface by the total cord surface on the transverse histological section.

Normalisations of cord atrophy: Atrophy was estimated by (1) measuring the minimal surface of the cord, and (2) calculating two types of ratio to normalize the atrophy, in order to avoid potential interference of anatomical differences between individual rats.

The "atrophy ratio" corresponds to the minimal cord surface (absolute atrophy) divided by the maximal cord surface of the same rat (i.e., the surface of its normal lumbar cord).

The "normalised atrophy ratio" corresponds to a supplementary normalisation of the atrophy ratio: the latter is divided by the highest cord/canal surface ratio in the same rat (i.e., the relative dimensions of the cord and the canal at a level where the cord is not injured); potentially taking into account anatomical differences in the cord and canal diameters between rats.

Spared Matter: Both total spared parenchyma and spared white matter were measured on MR images and histological sections. In severe lesions, the total spared parenchyma was identical with the spared white matter. In less severe lesions, where some grey matter was preserved, the spared white matter was measured separately. Furthermore, the ventral spared white matter was measured ventrally to a horizontal line centred on the ependymal canal on a transverse section.

For statistical analysis, the different parameters were labelled "X1"–"X11" (for detailed statistical data, refer to the appendix, page 182).

4.2.4.4 Graphic Representations of Morphometric Parameters

In addition, to illustrate the measurements, graphs were constructed, showing the evolution of the parameter over the spinal cord length, i.e. figure 4.6–4.10:

- the cord and lesion surfaces, (MRI and histology);
- the spared matter (cord surface − lesion surface, MRI and histology);
- the surface ratio lesion/cord (MRI, see appendix).

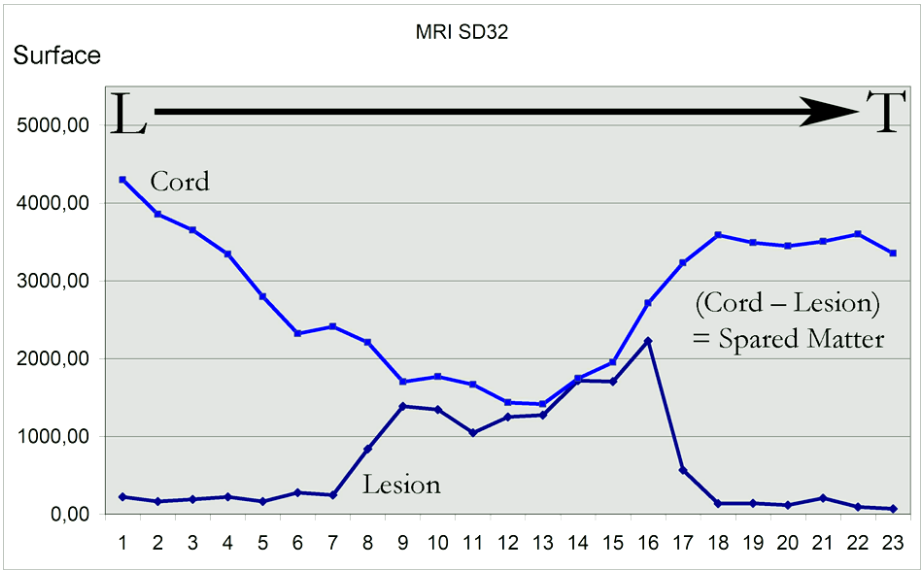

Figure 4.6: Illustration of the cord and lesion surfaces measured by SD32 MRI. L: lumbar (sub-lesional), T: thoracic (supra-lesional).

In figures 4.7 and 4.8, the MRI and histological graphs represent the lesion volume (lesion surface section by section along the cord, lower curve/brighter surface) and cord atrophy (cord surface, upper curve), as well as the spared matter (dark coloured surface between the two graphs).

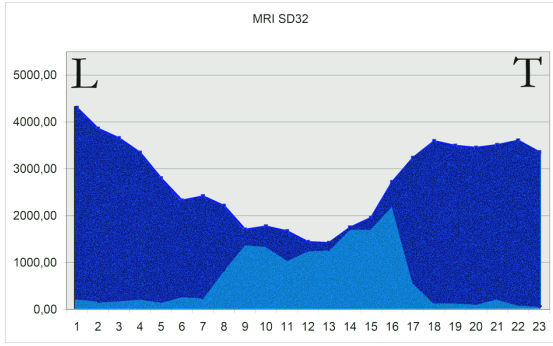

Figure 4.7: MRI (PD32). Upper curve: cord surface; lower curve: lesion surface. X-axis: MRI section number. Y-axis: surface (square pixels).

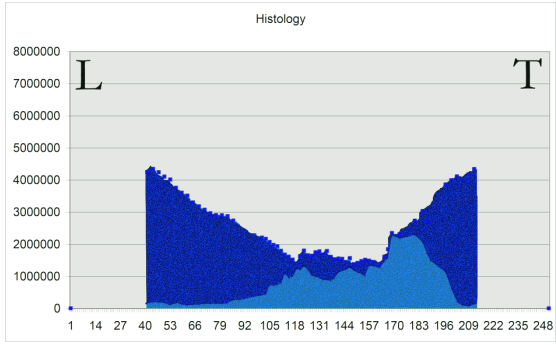

Figure 4.8: Histology. Upper curve: cord surface; lower curve: lesion surface. X-axis: section number. Y-axis: surface (μm^2)

Figures 4.9 and 4.10 illustrate the spared tissue in another manner, i.e., the spared parenchyma's surface from the lumbar to the thoracic cord.

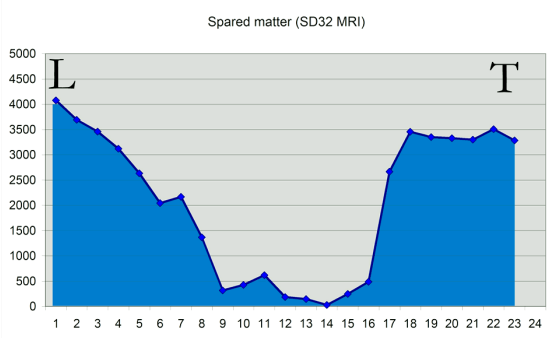

Figure 4.9: MRI PD32. Spared matter (total surface−lesion surface). X-axis: section number. Y-axis: surface (square pixels).

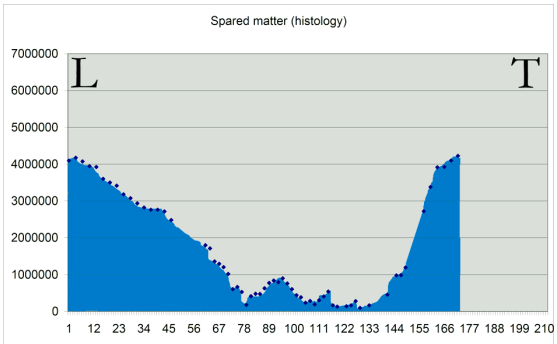

Figure 4.10: Histology. Spared matter (total surface−lesion surface). X-axis: section number. Y-axis: surface (μm^2).

4.2.5 Statistical analyses

Statistical analysis was used to correlate the different morphometric parameters both with the initial BBB score and with the slope of the recovery curve over time. Results were expressed as mean and standard deviation.

The effect of time, the different variables, as well as their interactions on the evolution of the BBB score, was assessed by means of the general linear mixed model (GLMM) which accounts for repeated observations on the sample units and a random intercept. All results were considered to be significant at the 5% critical level ($p<0.05$). Statistical analyses were carried out using SAS (version 9.1 for Windows) and S-Plus (version 6.2 for Windows).

4.3 Results

4.3.1 MRI of Spinal Cord Injury in the Rat

4.3.1.1 MRI of the Dissected Cord

In a first attempt, the excised spinal cords of rats from the combined treatment group were subjected to MRI, but cord atrophy was severe and very little information could be obtained from these images. The first interpretable images came from the analysis of the excised spinal cords from the pilot series of 12 BBB-followed rats, using different acquisition sequences (T1, T2, PD), in different planes (axial, sagittal, and horizontal), and at different resolutions (i.e., different numbers of averages/acquisition times). The most useful acquisition sequence appeared to be PD, as shown in human material [31] and rats [34], offering the best compromise for resolution and contrast. The level of the lesion could be located in the longitudinal plane (for images, refer to the CD) and the severity of injury appeared different between the mild (10 μl), moderate (20 μl), and severe (30-40 μl) compression groups (figure 4.11).

Although these results were promising, as they showed the potential of the technique to distinguish lesion severities, they were not entirely satisfactory, because the image quality remained low, and some of the limitations of the histological analysis could not be addressed:

- The cord's shape was significantly altered in the more severe lesions, possibly due to the lesion itself, but also by handling.
- The orientation of the cord and the localisation of ventral and dorsal parts were not clear at the lesion level. The deformities and rotation hindered the interpretation of both the coronal and the longitudinal sections.
- Pathological and preserved tissue could not be distinguished sufficiently clearly for measurements of the lesion or spared tissue.

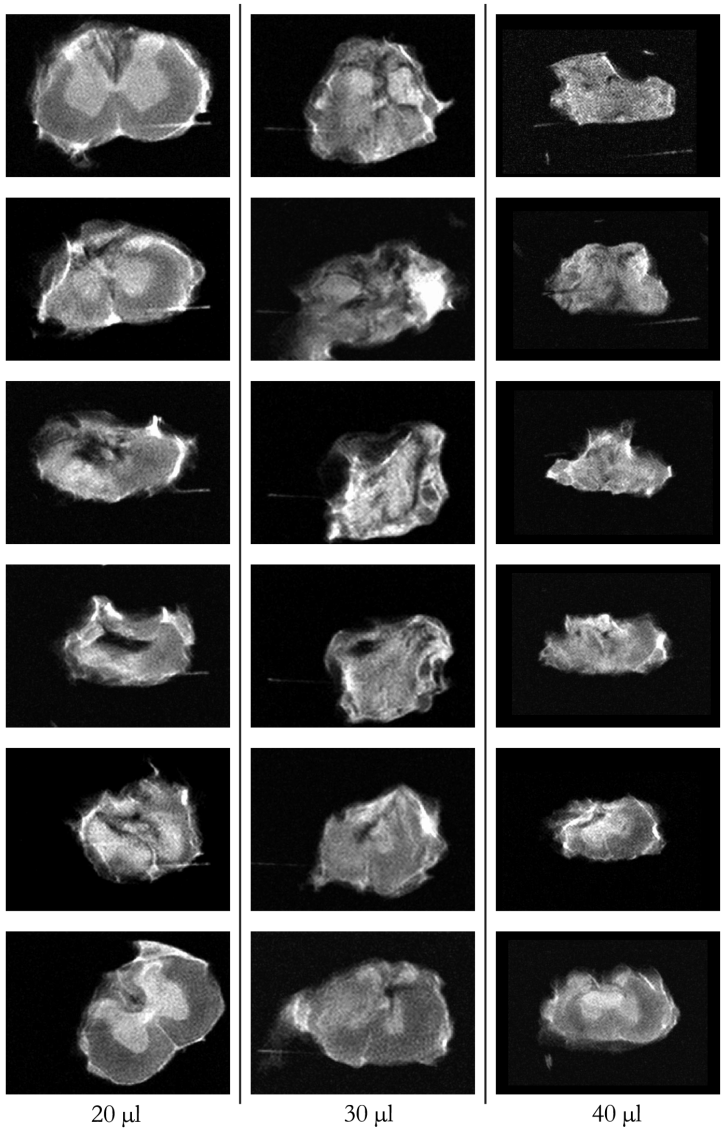

Figure 4.11: Sample sections from three pilot-studied isolated spinal cords (1 cord per column; from left to right: rats n°3, 12, and 5.)

4.3.1.2 MRI of the Spine Block with the Spinal Cord *in situ*

Therefore, after the above pilot study, all spines of the following experiments were dissected *as a whole* in an attempt to reduce image distortion. With this modification, the quality of the obtained PD MR images was very high in most of the cases (figure 4.12); in some of the spines, however, gas (probably air) had entered the spinal canal and reduced image quality. PD MRI offered the best compromise for resolution and contrast; acquisition time is reasonably short (see above). T1 and T2 weighted image quality was inferior to PD weighted imaging and did not add any supplementary information.

The images not only depicted the spinal cord parenchyma in detail, but also showed its relation to surrounding tissues including the subarachnoid space, the segmental level of the lesion (longitudinal planes, figure 4.13) and the integrity of the surrounding skeletal and muscular structures, including surgical traces of the balloon insertion.

4.3.1.2.1 Transverse Imaging of the Cord-Spine Block. Figure 4.12 shows the bony spine surrounding the spinal canal as low MR signal, i.e. almost black, except for the inter-trabecular spaces of the cancellous bone in the vertebral body, ventral to the cord. The spine itself was surrounded by paravertebral muscle. The spinal canal, filled with paraformaldehyde (replacing the cerebrospinal fluid) appeared as high signal, almost white. The dural sac could be seen around the spinal cord (figure 4.12A), and the two anterior and two posterior nerve roots appeared as lower signal (figure 4.12B).

In the centre of the canal, the spinal cord—at distance from the lesion—appeared normal; the anatomical delimitation of grey and white matter was precise; the signal of the central grey was more intense than the white matter, where the presence of myelin reduces the PD signal. Pronounced hypo-signal indicated the presence of the myelinated corticospinal tract in the ventral part of the dorsal columns, between the bases of the dorsal horns (figure 4.12A). Topping the dorsal horns, the substantia gelatinosa was hyper-intense (figure 4.12B).

4.3.1.2.2 Horizontal Longitudinal Imaging of the Spinal Cord-Spine Block. In figure 4.13, the spinal cord is shown in longitudinal horizontal images, from the ventral part to the dorsal part. The bony vertebral bodies were hypo-intense (figure 4.13A) and separated by intervertebral disks. The ribs can be seen (figure 4.13C) in the paravertebral muscle.

The the spinal cord itself is seen in the canal. Intact parenchyma surrounded the injury rostrally and caudally and the grey matter could clearly be distinguished

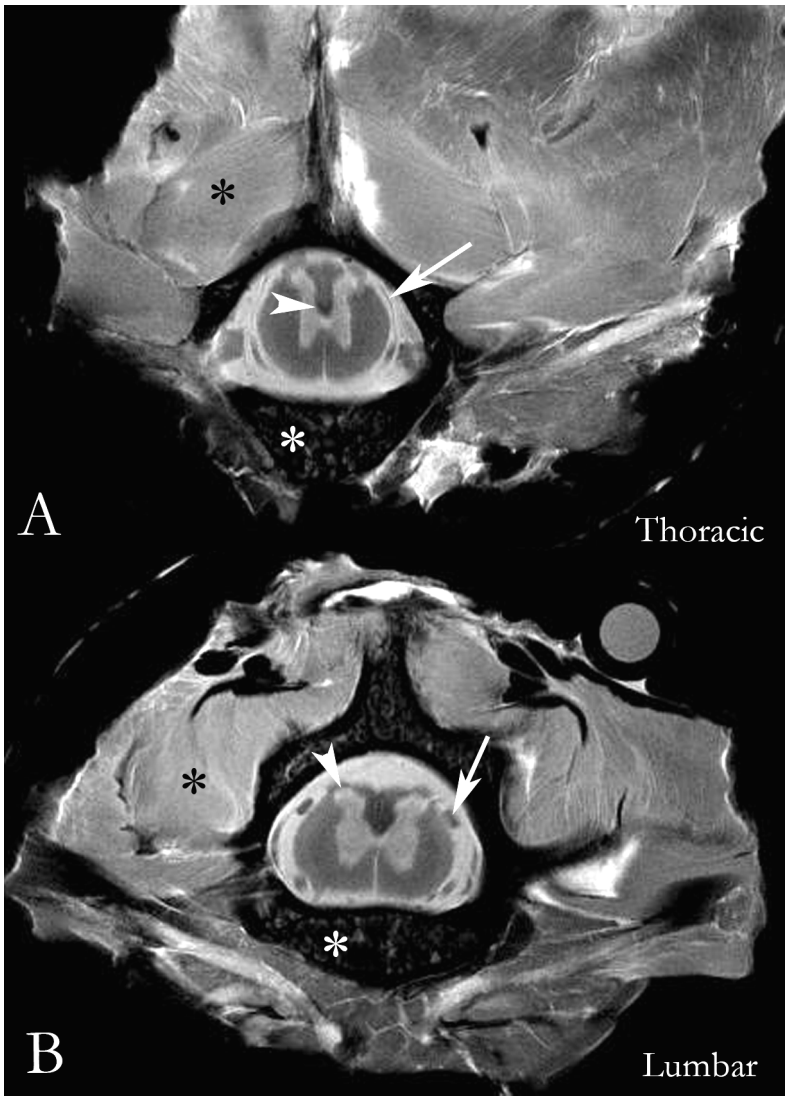

Figure 4.12: High Resolution PD weighted MRI of the spinal cord-spine block (NA=32); A: thoracic, B: lumbar. White asterisk: bony spine (vertebra). Black asterisk: paravertebral muscle. Arrowhead in A: corticospinal tract. Arrow in A: dural sac. Arrowhead in B: substantia gelatinosa. Arrow in B: nerve root.

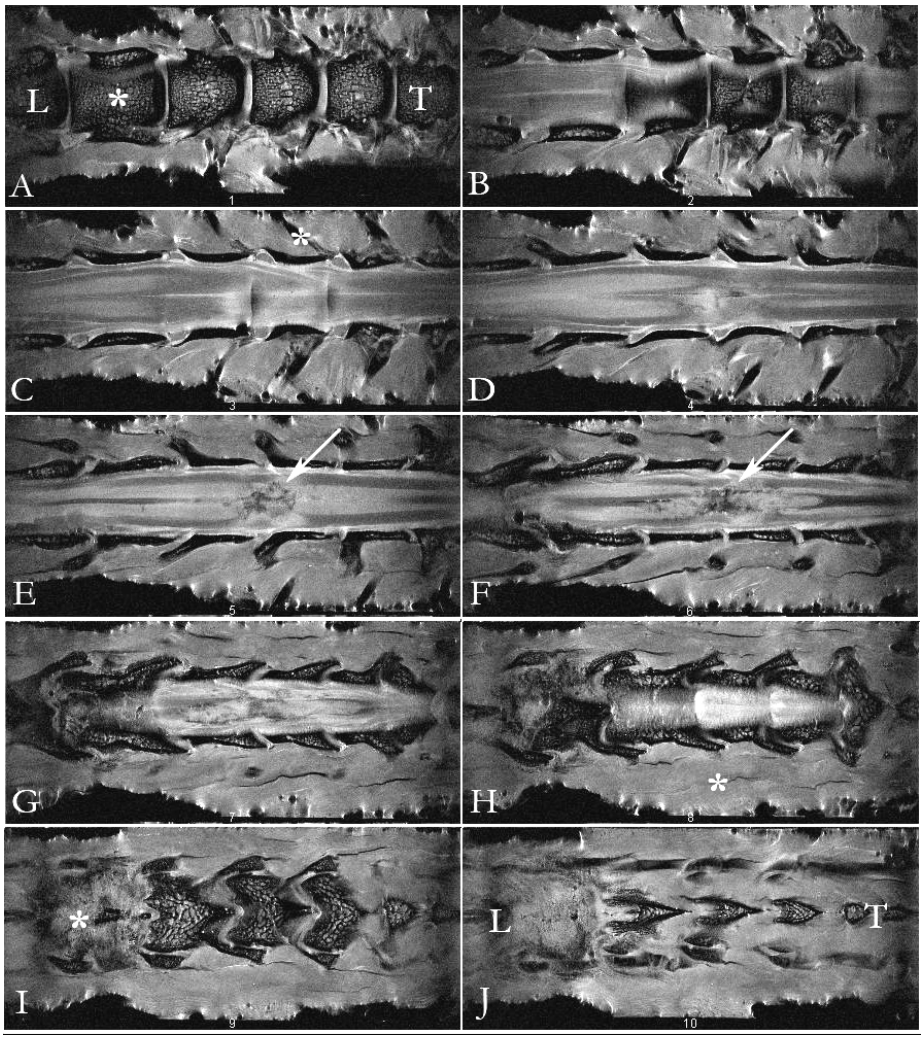

Figure 4.13: Longitudinal horizontal PD weighted images, from ventral to dorsal (A–J; L=lumbar, T=thoracic): Asterisk in A: vertebral bodies. Asterisk in C: ribs. Asterisk in H: paravertebral muscle. Arrows in E and F: the lesion with a hypo-intense centre. Asterisk in I: post-surgical scar tissue. 7dpo, NA=32.

from the white matter. The level of the lesion centre was precisely located in relation to the vertebrae and the nerve roots (figure 4.13E–F), two metamers rostral to the insertion site of the balloon (figure 4.13F). The spindle shaped lesion was hyper-intense for its major part, and mainly situated in the posterior half of the cord (figure 4.13D–G): the hyper-intense spindle extended rostro-caudally in the dorsal columns, between hyper-intense dorsal horns. The lesion centre showed some hypo-signal (figure 4.13E–F).

Dorsally, the laminae and the spinous processes closed the spinal canal (figure 4.13H–J). Traces of the balloon insertion surgery are seen caudally (figure 4.13I). Kyphosis of the rat spine at this level explains that each section shows different tissue planes.

4.3.1.2.3 Topography and Nature of the Lesion. Luxol Fast Blue staining for myelin (i.e., white matter) and haematoxylin as a counterstain (in order to facilitate orientation) demonstrated a precise correlation between abnormal PD MR signal and histopathological changes in the transverse sections.

1. MRI precisely located the spinal cord lesion, in all three planes (e.g., figure 4.13);

2. MRI distinguished between lesion *components*: increased MR signal (light) corresponded to oedema in the acute stage and necrosis in more chronic lesions; decreased MR signal (dark) corresponded to haemorrhage, i.e., haemoglobin in the acute stage, and haemosiderin in the chronic stage, both of these paramagnetic;

3. MRI accurately reproduced the lesion's *topography*, i.e. precisely delimited the lesion's extent, in particular in the transverse plane;

4. MRI depicted preserved tissue anatomy and showed a potential to detect *spared white matter* in the lesion periphery.

Figure 4.14 illustrates the topographical precision and the differences in signal due to necrosis and haemorrhage. In this sample section, necrosis appeared as a wing shaped area in the lateral funiculi, as well as in a smaller central dorsal area. The winged shape of the lesion in the lateral funiculi was regularly seen in the periphery of the lesion at the chronic stage (e.g., also refer to figure 4.24, page 102).

The MR signal of the lesion was heterogeneous: the periphery was hyper-intense and the centre hypo-intense. Haemosiderin laden macrophages were responsible for the hypo-signal. They appeared dark and brownish on the histology images

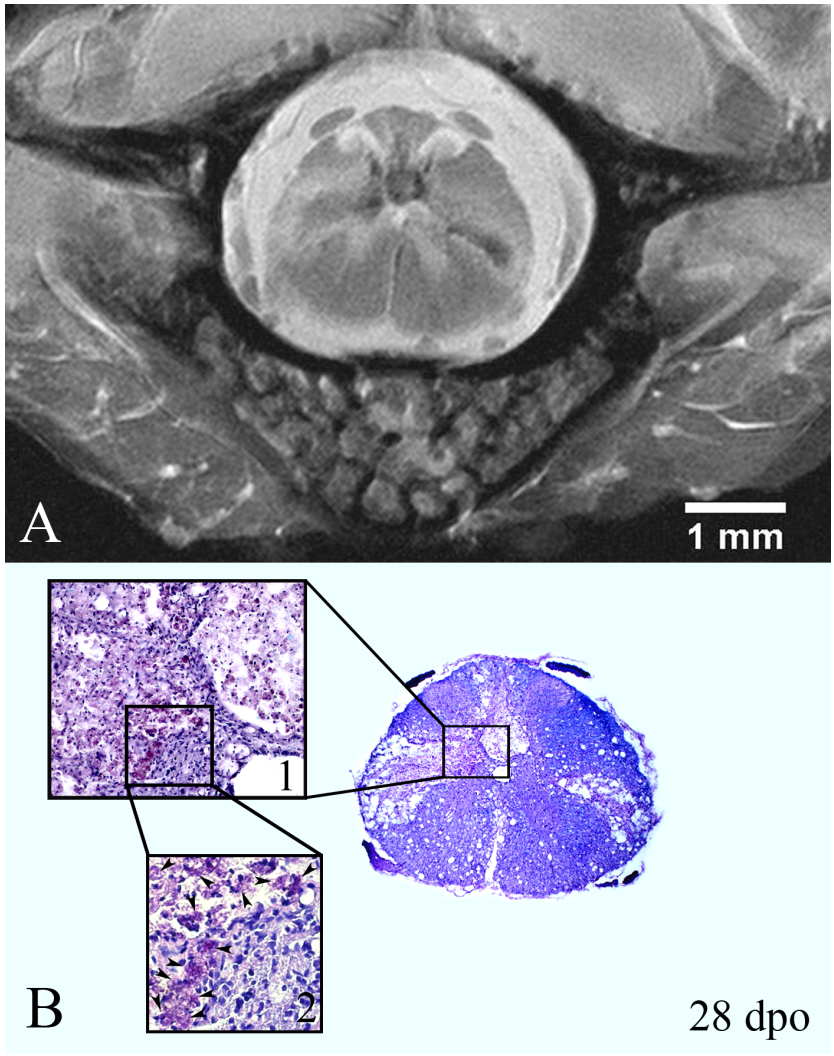

Figure 4.14: Correlation of histology (haematoxylin and Luxol fast blue) and PD MRI in the peripheral part of the lesion. Arrowheads in frame 2: hemosiderin laden macrophages. 28 dpo.

(frame 2), precisely reproducing the topography and intensity of the hypo-signal, e.g., adjacent to and in the central dorsal necrotic lesion (frame 1), as well as in the left lateral funiculus. Scarring and atrophy in the central grey matter resulted in the juxtapositioning of the two dorsal and the two ventral horns towards the centre of the cord, seen in both MRI and histology. The hyper-intense dorsal horns thus showed a characteristic "bunny ear" appearance, which was frequently observed in chronic lesions (e.g., figure 4.20; refer to the CD for more images).

Histology confirmed the topography of the necrotic lesion, as well as the presence of haemosiderin-laden macrophages, seen at higher magnification, in precisely those areas where the MR image is darker within the hyper-signal. This signal specificity was observed in all injured spinal cords that were imaged after survival delays of one month or more (figure 4.15).

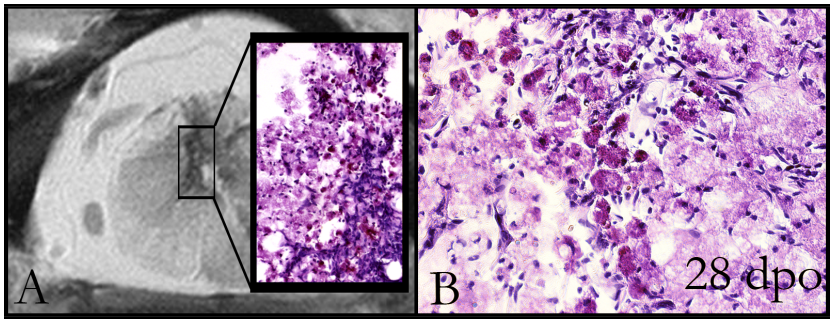

Figure 4.15: (A) Histology of a region conspicuous in PD MRI for its hypo-signal, showing abundant haemosiderin-laden macrophages. (B) High power photography of haemosiderin-laden macrophages at 28 days post-trauma.

4.3.1.2.4 Acute Lesions. As mentioned above, at survival times of one, four and seven days, the acute processes known to occur after traumatic SCI were detected as two types of abnormal MR signal.

Hyper-signal indicated the beginning of necrosis. Necrosis progressively replaces acute oedema in the first 24-48 hours [296]. In PD MRI, during the acute phase (survival delay of 4 or 7 days, figures 4.16C and D and 4.18), the cords appear "very white" and swollen—corresponding to massive necrosis and oedema—before entering the sub-acute and chronic phases of SCI, where scarring processes replace the injured tissue and cord atrophy appears.

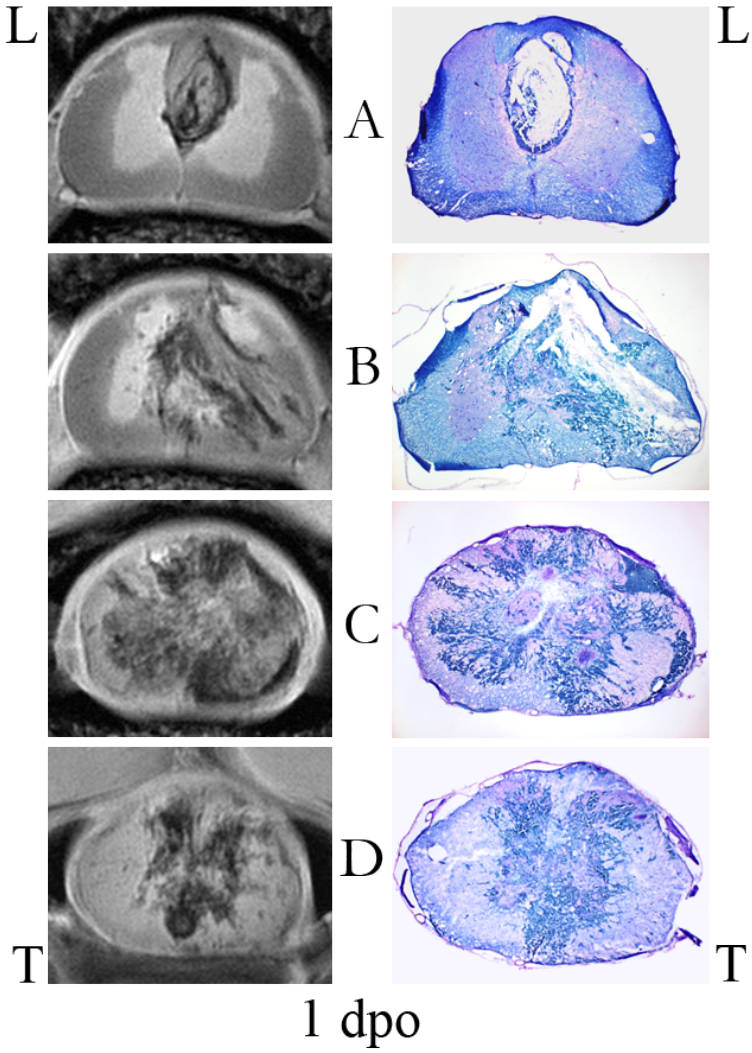

Figure 4.16: One day survival, from lumbar (L) to thoracic (T). The topographic correlation of histology and PD MRI is precise. Grey and white matter contrast is preserved in the periphery of the lesion (A, B). Row C shows the lesion centre with massive haemorrhage in the grey and white matter. In the more rostral part (row D), the haemorrhage concerns mainly the grey matter, in a characteristic topographic pattern.

Hypo-signal corresponded to haemorrhage (figures 4.16 and 4.18). The *quantity* and *spatial extent* of haemorrhage, which could be precisely detected at all survival times, was variable from one rat to another, independent of the survival time. The *topography* was however reproducible: bleeding occurred predominantly in the central grey matter and, if massive, ruptured first into the ventral funiculus, and eventually into the rest of the white matter (figures 4.16D and 4.17).

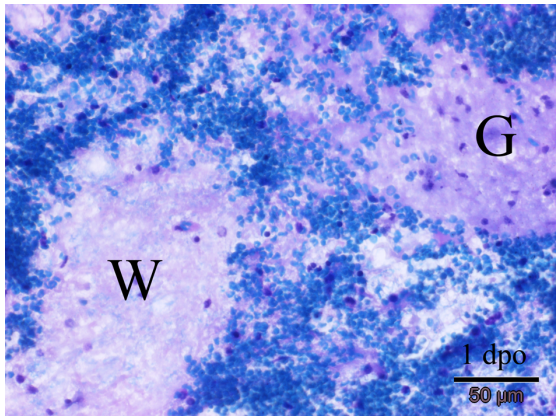

Figure 4.17: Acute haemorrhage infiltrating the white (W) and the grey (G) matter. One day *post-SCI* , haematoxylin and Luxol fast blue.

In the early stage after injury, i.e., at one day survival, differences in lesion severity could be seen, be it in the transverse sections or the longitudinal extension (for detail, refer to overviews on the CD). Even at the lesion centre where necrosis and haemorrhage were maximal, it was still possible to distinguish preserved grey and white matter from the lesion (figure 4.16).

Note that, after the short survival times (one, four, and seven days), the spinal cord was seen to fill the spinal canal, particularly strikingly after 7 days (figures 4.16 and 4.18). After a week, it was difficult to distinguish spared matter on MRI, as well as on histological sections, due to the massive tissue oedema invading even presumably spared tissue.

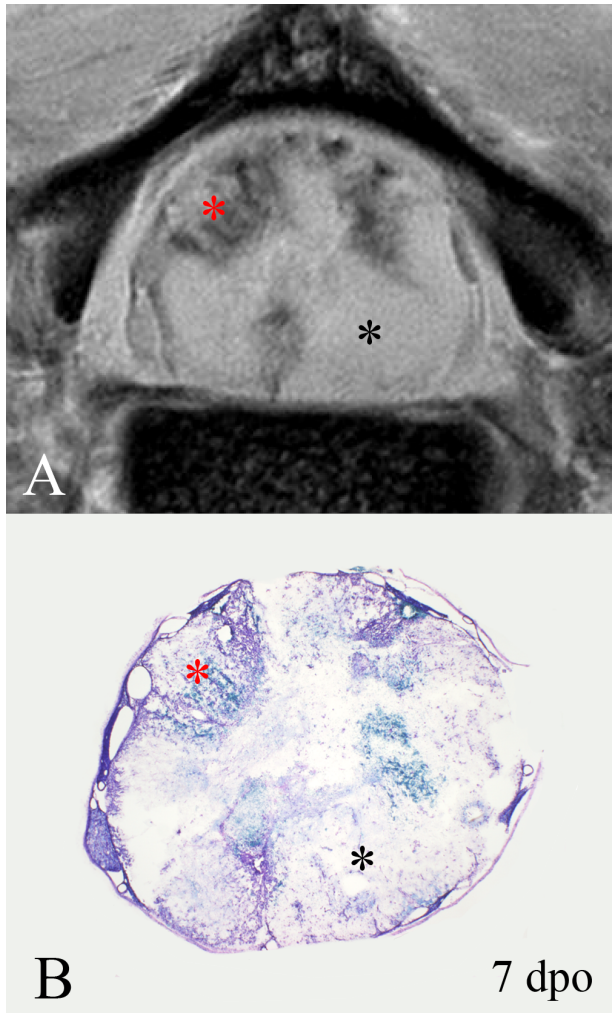

Figure 4.18: Example of an acute cord at 7 dpo with massive oedema/necrosis detected by MRI (hyper-signal, black asterisk). Some areas of haemorrhage can be seen (hypo-signal, red arrow). Again, the topographical correlation is precise. The cord volume is increased at the lesion site: the spinal cord completely fills the spinal canal. Rat n° 3676.

4.3.1.2.5 Chronic Lesions and Correlation with Locomotor Recovery. After locomotor follow-up by weekly BBB evaluations, chronically injured cords were investigated by MRI and histology, measuring lesion size, cord atrophy, and tissue sparing.

The lesion volume was calculated from the lesion surface measurements. The volumes calculated from by MRI and histology differed: the "histological" lesion volume is only approximately half the volume calculated using MRI (table 2 in the appendix, page 183). Still, all histological and MRI volumes statistically significantly correlated with the severity of the initial locomotor deficit (initial BBB score), as well as the progression of recovery (evolution of the BBB scores until the maximal achieved score). The maximal lesion/cord ratio also statistically significantly correlated with both the initial BBB score and the BBB score evolution (see appendix).

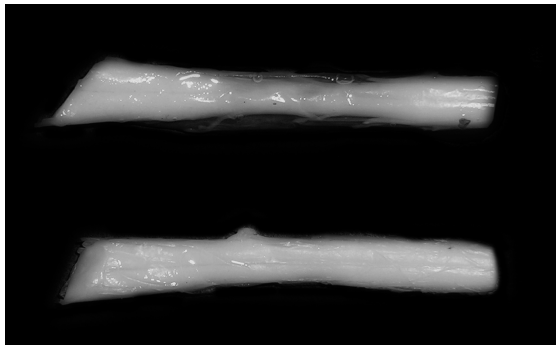

Figure 4.19: Macroscopical cord atrophy: two cords are shown in their dural envelope. Above: moderate balloon compression; below: mild compression.

Atrophy of the spinal cord can be major three months after 20 μl balloon compression injury, as indicated by histology in the combined treatment series (figure 3.8, page 57). In the BBB-followed MRI rat series, macroscopically visible atrophy varied from one rat to another (figure 4.19): in some rats, it was evident; others appeared macroscopically normal. The MR images of these chronic lesions, as opposed to the acute ones, showed that the atrophic cord did not fill the spinal canal (figure 4.20).

The minimal total surface was measured by MRI, and normalised to account for potential differences in the cords' anatomy between rats. Statistical analysis confirmed that, again, all criteria were significantly correlated with the severity of the initial locomotor deficit, as well as the progression of recovery.

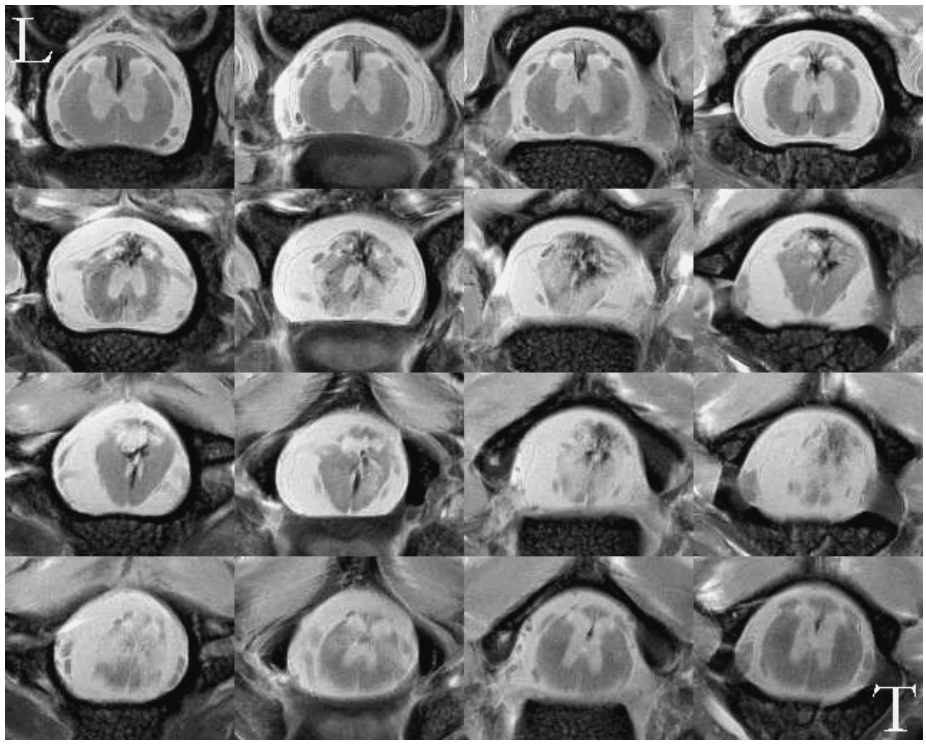

Figure 4.20: PD imaging with NA=32, showing atrophy of the spinal cord over the entire lesion length. The cord's transverse surface is decreased. There is a rhomboid deformity of the cord, a dorsal cyst (third row), and a wing shaped lesion periphery towards the thoracic cord (last row, second from the left). Rat n°3228 from the BBB series (which was not included in the complete biometry study; but histology correlated with the above MRI description), 60 dpo.

4.3.1.2.6 Spared Perilesional Parenchyma. The last evaluated criterion was the minimal amount of spared matter at the lesion centre, including the total spared matter, the spared white matter, and the ventral spared white matter (table 1, page 182). PD MRI showed preserved perilesional white matter, e.g., in figure 4.22, in the ventrolateral funiculi. However, the differentiation of normal and abnormal tissue was not always clear. A newly developed IR MR sequence was used to suppress the intact white matter's signal, as confirmed by histology (figures 4.23 and 4.24). During morphometry, *before* histology, the increased sensitivity of this sequence was found to be of significant use (1) in the detection of white matter sparing, especially around the lesion centre, and, (2) in the periphery, to confirm the abnormal nature of slight PD hyper-signal by increasing contrast (figure 4.23).

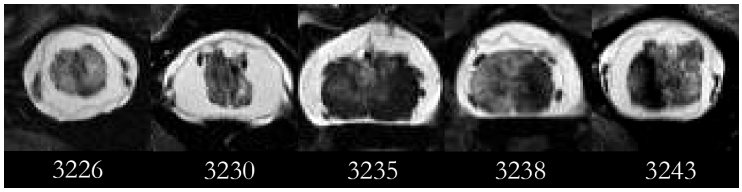

Figure 4.21: Maximal lesion extent in the five rats of the morphometry series, as assessed by IR imaging. 60 dpo.

Figure 4.21 compares the extent of the lesion and of white matter preservation in all five cords, using the IR MR section where the lesion extent was maximal.

Again, all morphometric criteria describing the quantity of spared tissue were significantly correlated with the severity of the initial locomotor deficit, as well as with the progression of recovery.

4.3.1.2.7 Graphic Illustration of the Morphometric Parameters. The graphic illustrations showing the evolution of the cord's surface and the lesion extent, as measured by MRI and histology, are shown in figure 4.25. The **volume of the lesion** is illustrated by the shaded area under the lower curve, and visual correlation with the locomotor performance (BBB curve) is excellent. The extent of **cord atrophy** is illustrated in figure 4.25: the evolution of the upper curve (cord surface measurements) from left to right illustrates the evolution of the total cord surface from the lumbar to the thoracic area. Figure 4.26 shows the total amount of **spared tissue** from the lumbar to the thoracic cord; the latter can however already be appreciated in figure 4.25: it corresponds to the total surface which is not covered by the transparent lesion surface graph.

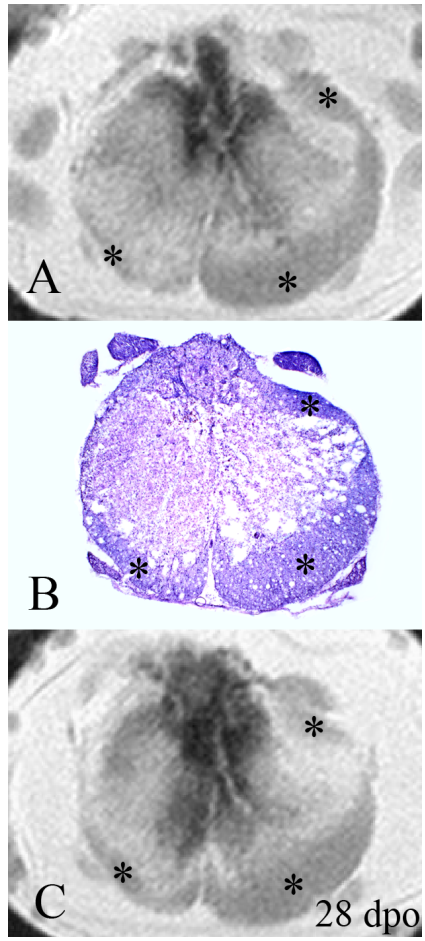

Figure 4.22: Correlation of histology and PD MRI at the lesion site. MRI shows that the ventral and ventrolateral funiculi are partially spared by the lesion at this level (asterisks). 28 dpo.
The slice thickness is significantly different between MRI (1000 μm) and histology (15 μm). The histological section (B) corresponds to the "junctional zone" of the two MR images, as demonstrated by the topography of the lesion, including central hypo-signal/haemosiderin deposits seen at high magnification, and of white matter sparing.

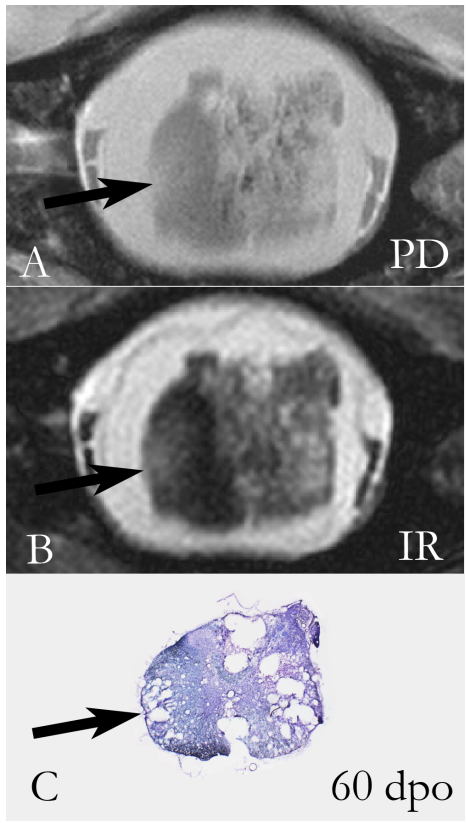

Figure 4.23: White matter damage detected by IR MRI. In this sample section (rat n° 3243), the IR sequence shows a white matter lesion in the right lateral funiculus much more clearly than the standard PD acquisition. Note, however, that the "static" comparison of PD and IR in this figure is still insufficient to represent the synergy that can be obtained by the concomitant analysis of PD32 and IR images.

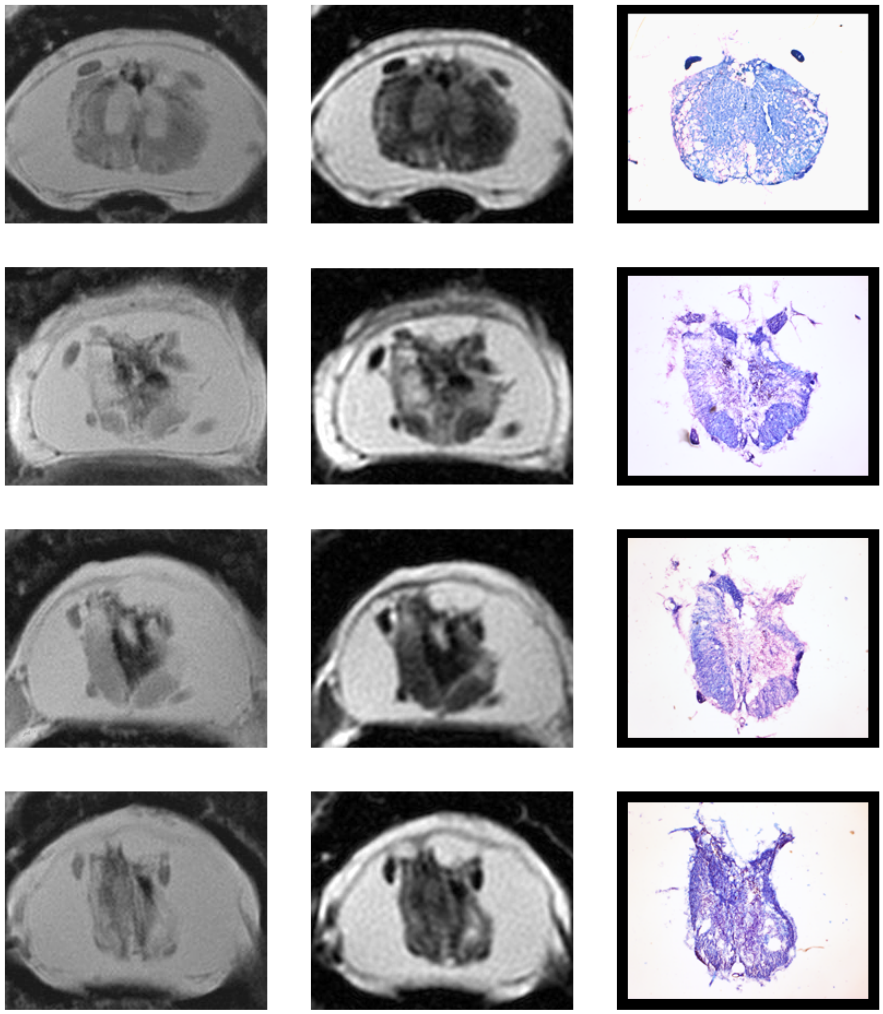

Figure 4.24: Correlation of PD (left) and IR (middle) MRI with histology (right) in rat n°3230 (part I, caudal).

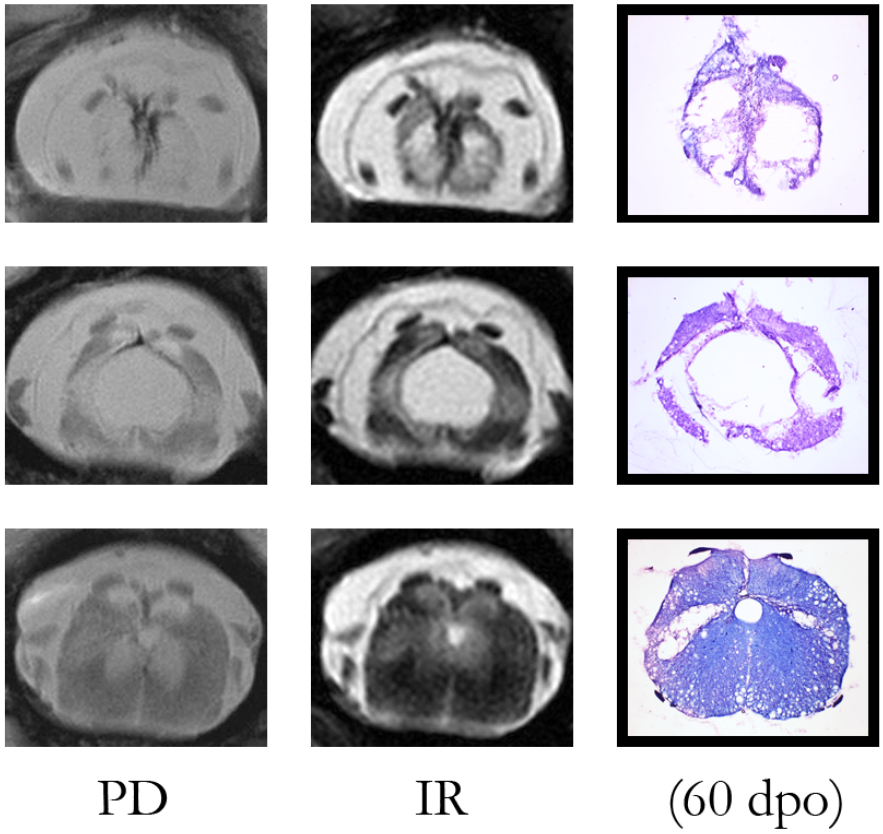

Figure 4.24: Correlation of PD (left) and IR (middle) MRI with histology (right) in rat n°3230 (part II, rostral). Note the significant cord atrophy, compared to the size of the spinal canal, the cord deformation, the presence of a liquid filled cyst (very hyper-intense), extensive necrosis (hyper-intense) with haemorrhage (hypo-intensities), and, last but not least, white matter sparing. In each section, the detection of white matter sparing is enhanced by IR MRI. Even slight vacuolation of the white matter can be detected by IR MRI (see last row).

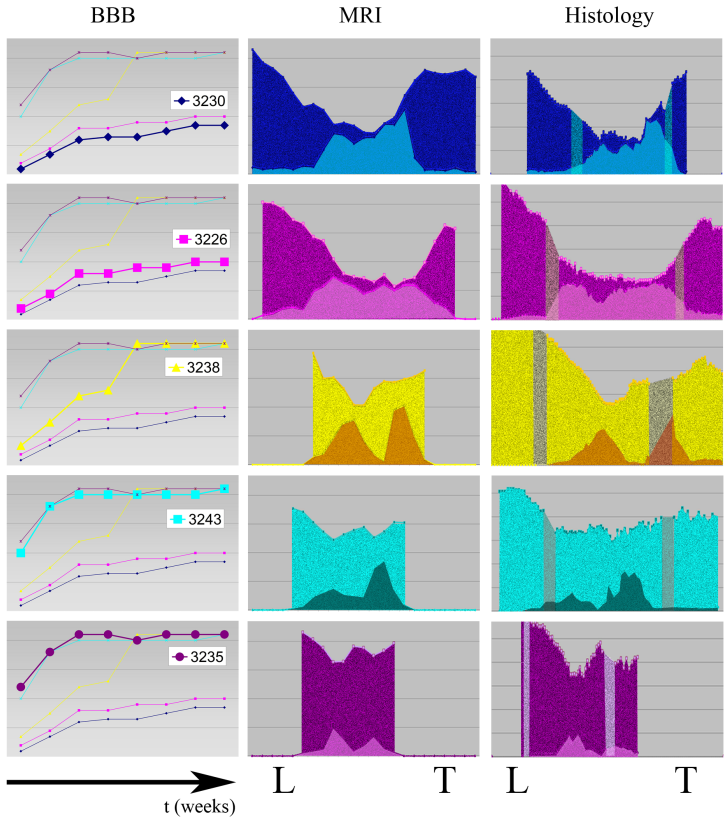

Figure 4.25: Morphometry 1: cord atrophy, lesion size, and spared parenchyma. Comparison of the five morphometric graphs (cord surface and lesion surface), from lumbar (L) to thoracic (T). Refer to page 83 et seq for scales, which have been omitted in both figures 4.25 and 4.26 for the sake of clarity, but are the same for all MRI graphs and for all histology graphs.
The upper curve corresponds to the total cord surface, and the lower curves to the lesion surface. The coloured area therefore corresponds to the cord volume, the transparent surface under the lower curve to the lesion volume, and the coloured area which is not covered by the transparent area to the preserved parenchyma.

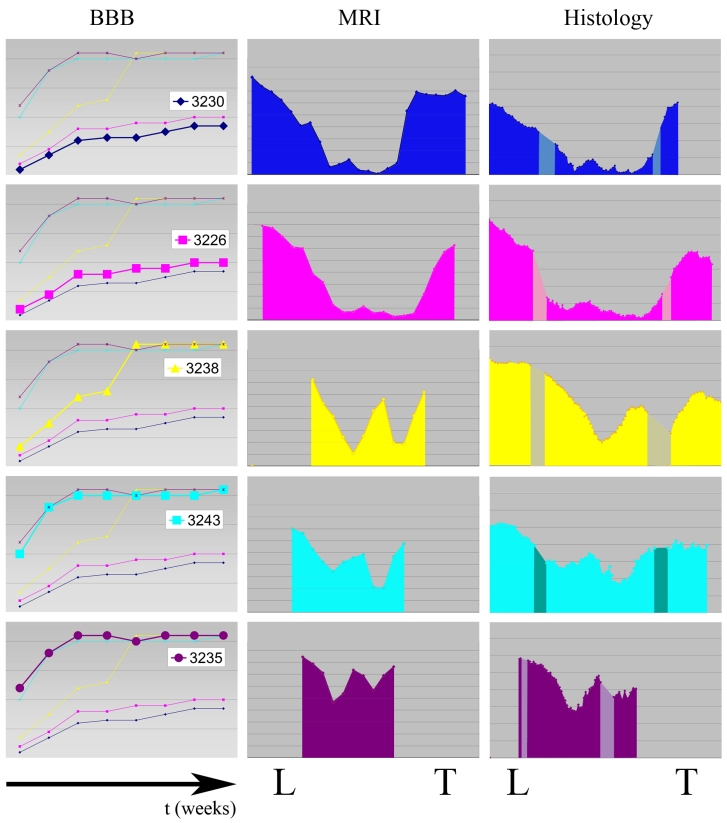

Figure 4.26: Morphometry 2: total spared parenchyma. Comparison of the five morphometric graphs, from lumbar (L) to thoracic (T).
The curve represents the total surface of preserved parenchyma (cord surface − lesion surface), over the entire length of the lesion; this corresponds in fact to the coloured area which is not covered by the transparent area in figure 4.25.
(In both figures figure 4.25 and figure 4.26, measurements were only taken on those sections that showed a lesion, and, due to tissue loss at the ends of the cord blocks, gaps occurred in the histological measurements, as illustrated by the differently shaded coloured areas, where the curves correspond to direct lines drawn between the two adjacent values.)

4.3.1.2.8 Correlation between asymmetric recovery and asymmetric lesions. Several rats showed initially asymmetrical locomotor recovery. This could be explained by asymmetry of the lesion, clearly depicted by the MR images.

Rat n° 3238 recovered more rapidly on the left side, and PD images confirmed that the lesion was more severe on the right side. The anterior and lateral funiculi were almost completely spared at the lesion centre (figure 4.27).

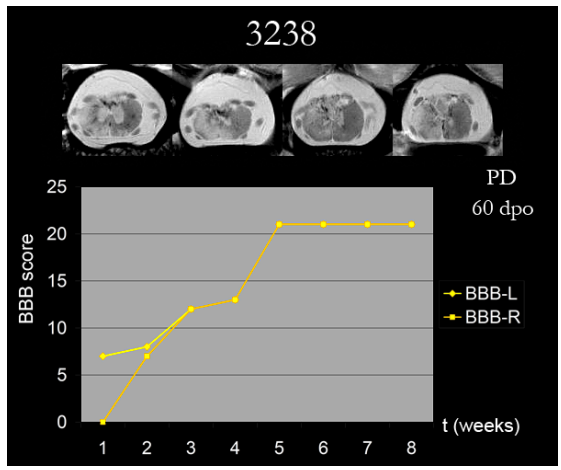

Figure 4.27: Axial PD images, maximal lesion extent, n°3238, with a clear predominance of the lesion on the right side and extensive white matter sparing on the left side, especially in the ventral and lateral funiculi. The BBB graph shows initial low performance of the right hindlimb, then progressive recovery to a normal BBB score (21).

Rat n° 3226 also recovered more rapidly on the left side, though at a lower level of locomotor performance. The right half of the cord was entirely necrotic at the lesion centre, whereas limited tissue sparing could be detected in the left ventral funiculi (IR MRI, figure 4.28); this was confirmed by histology. Note again the extent of the lesion and the degree of atrophy in this spinal cord—the rat still showed significant recovery of hind limb function (BBB = 10: limited stepping).

4.3.1.2.9 Evaluation of Rapid, "Low-Resolution" MRI .

With a reduction in acquisition time, from 8-10 hours (NA = 32) to 25 or 50 minutes (NA = 4 and 8, respectively), image quality remained very good (figure 4.29. The difference between NA = 4 and NA = 8 images consisted in a lower signal to noise ratio for NA = 4 PD MRI.

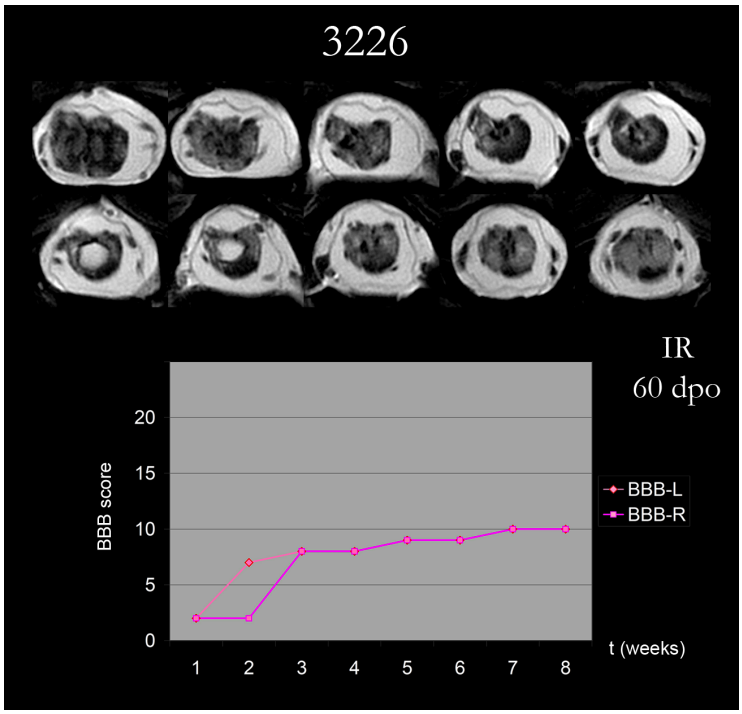

Figure 4.28: Axial IR images, n° 3226. Over the entire lesion length, limited tissue sparing can be detected in the left ventral funiculus. BBB score evolution: the left hindlimb recovered rapidly, the right one more slowly. The final score of 10 indicates the capacity for limited stepping.

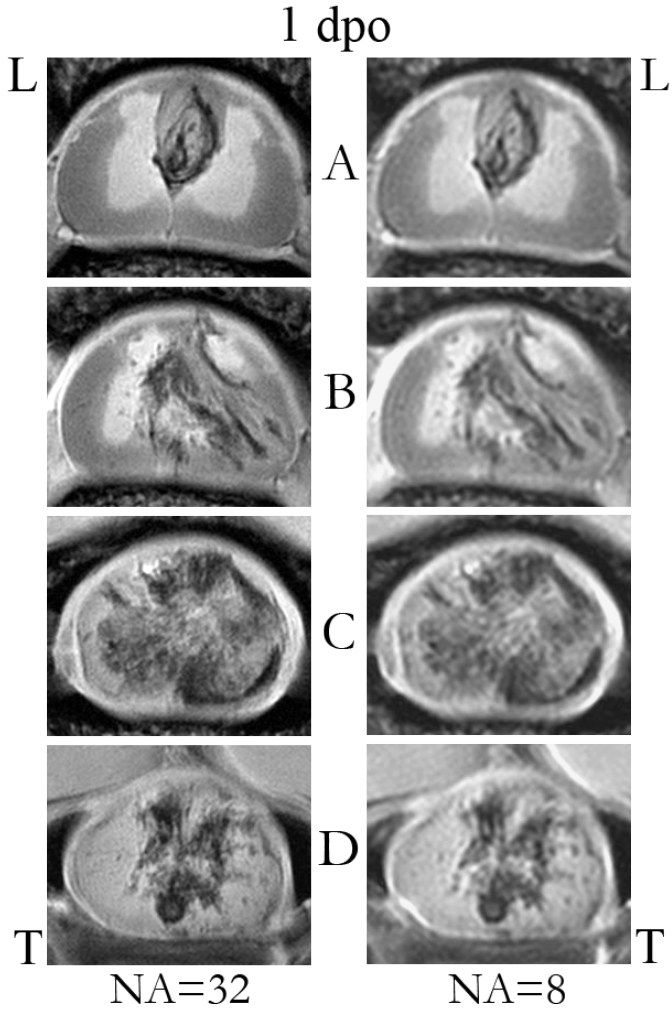

Figure 4.29: Comparison of the highest resolution (PD, NA=32) to a lower resolution (PD, NA=8). MRI sections are the same as in figure 4.16 page 94. Acquisition time was 8-10 hours for NA=32 and 50 minutes for NA=8. Resolution was 37.8 x 37.8 μm^2 for NA=32 and 68 x 68 μm^2 for NA=8.

4.3.2 Human Spinal Cord Injury

4.3.2.1 Pathology of the lesion site (C5)

The initial pathological examination of the spine was performed in 1972, seven months after the traumatic event, i.e. at the transition from the sub-acute to the chronic state of traumatic spinal cord injury [176]. The spine showed a compression fracture of the fifth cervical vertebra and a corresponding, severe, cervical spinal cord lesion with very little tissue preservation (figure 4.30); therefore, the white matter tracts were completely interrupted at the cervical level.

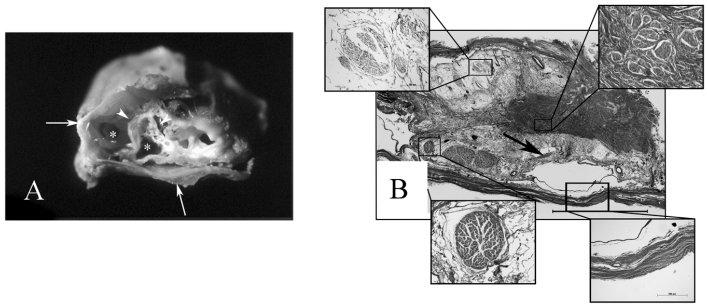

Figure 4.30: (A) Macroscopic view of cervical spinal cord at C5, seven months after trauma, showing cavitation of the lesion site (asterisks), trabeculae (arrowheads), and a thickened dura mater (arrows). (B) Histopathology of the lesion site (Picro-Mallory stain), confirming the macroscopic appearance. The structure of the spinal cord is severely disrupted; *no white matter sparing* can be demonstrated. Typical scarring processes are seen, including astrogliosis in the only detectable region of residual spinal cord tissue (arrow; detail not shown), macrophage and Schwann cell invasion associated with limited axonal growth in a collagenous scar (upper right box), and a thickened dura mater (lower right box). Nerve roots are included in the subarachnoid space, filling most of the canal (e.g., upper and lower left boxes). From publication 3, page 185.

4.3.2.2 MRI of the high thoracic spinal cord sample

The high resolution, proton-density weighted MRI (figures 4.31A and 4.32) depicts the spinal cord's microanatomy: the clearly delineated central, butterfly-shaped grey matter showed overall higher signal intensity than the surrounding white matter, where the funiculi and the white commissure are normally heavily myelinated. The tract of Lissauer, poorly myelinated and located between the apex of the dorsal horn

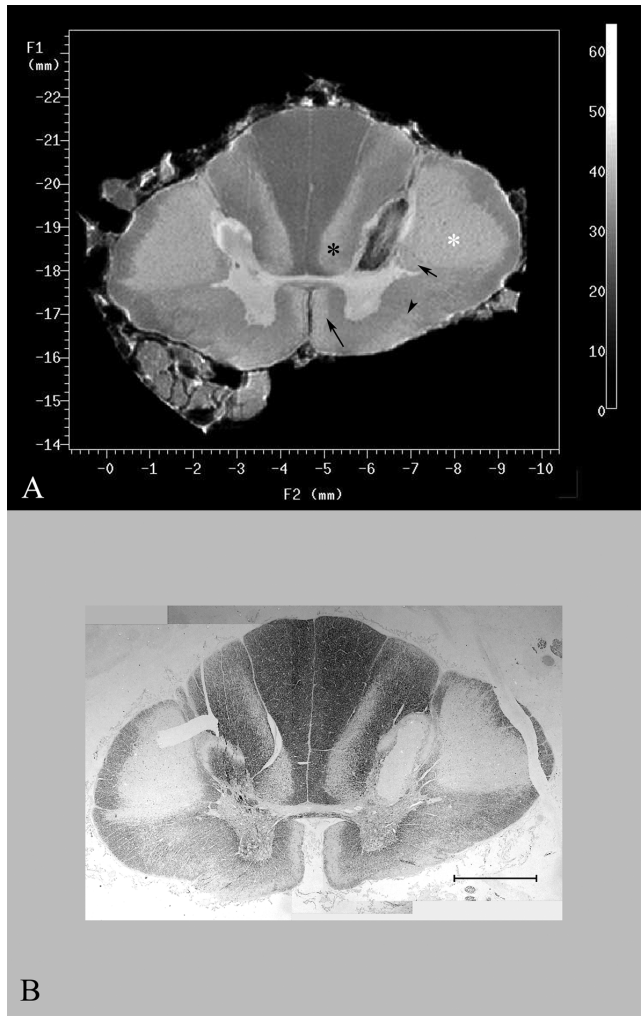

Figure 4.31: Transverse sections of the high thoracic spinal cord specimen. (A): 9.4T PD MRI. Lateral corticospinal tract: white asterisk; lateral reticulospinal tract: short arrow; anterior cortico- and reticulospinal tracts: long arrow; lateral vestibulospinal tract: arrowhead; fasciculus interfascicularis (comma tract): black asterisk. The scales indicate the original size of the cord. (B): Histological reconstruction using neurofilament immunhistochemistry for axons, demonstrating axonal loss in the hyper-intense areas shown in (A). From publication 3, page 185.

grey matter and the dorsal root entry zone, also appeared as a region of hyper-intense signal.

Abnormal hypersignal was seen in territories normally occupied by descending tracts, including very slight signal differences, as in the asymmetrically hyper-intense lateral reticulospinal tract. A scattered, slightly hyper-intense signal could be observed in the antero-lateral and the lateral column, anterior to the lateral corticospinal tract: this corresponds to the reticulospinal and descending propriospinal fibre bundles.

In the left dorsal horn, a large oval-shaped area of low signal intensity could be observed extending from laminae III to VI. Within this area, the signal was heterogeneously hyper-intense (figure 4.31A).

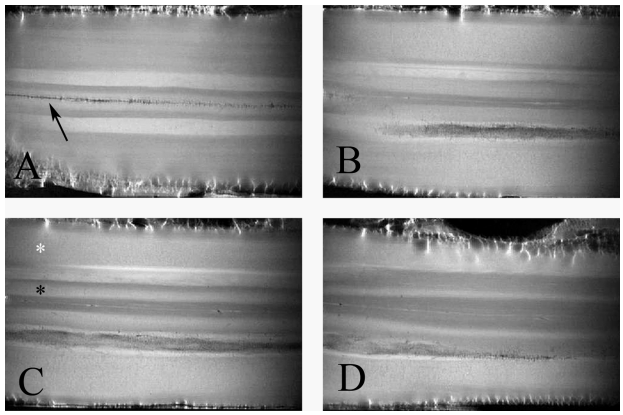

Figure 4.32: MRI sections in the horizontal (frontal) plane, from anterior (A) to posterior (D). All slices show the longitudinal organization of the spinal cord, including the hyper-intense area of Wallerian degeneration and the longitudinal extension of dorsal horn cavity. The laterally fading MR signal is due to the weakening magnetic field at the extremities. (A) Beginning at the mid-line and moving laterally, the hyper-intense, degenerating anterior corticospinal tract (long arrow), followed by white matter, then by the even more intense signal of the anterior horn grey matter, and finally by the antero-lateral white matter. (B) The heterogeneously hypo-intense dorsal horn lesion in the region of lamina VI. (C) Hyper-intense signal in the comma tract of Schultze (fasciculus interfascicularis, black asterisk) and the lateral corticospinal tract (white asterisk). From publication 3, page 185.

The longitudinal views demonstrate the continuity of these signal abnormalities along the entire longitudinal axis of the tissue sample (coronal sample views, figure 4.32, and complete coronal and sagittal views, refer to the CD).

4.3.2.3 Histology and immunohistochemistry of the high thoracic spinal cord sample

The histological stains indicated bilateral loss of axons and myelin as well as astrocytic hyperplasia in the major descending nerve fibre pathways of the white matter (not shown). In the grey matter, a unilateral area of cavitation in the dorsal horn extended from lamina III to lamina VI. The Berlin Blue stain revealed the presence of only a few scattered foamy haemosiderin-containing macrophages in and around the cavitation but its sensitivity may be insufficient to demonstrate all haemosiderin.

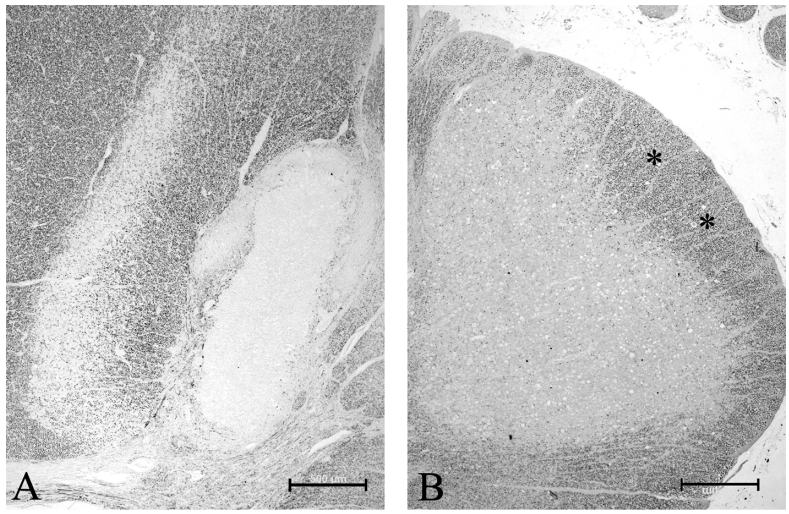

Figure 4.33: NF-200 immunohistochemistry of the descending fibre tracts. Higher magnification view showing axonal loss (NF) in the dorsal columns (fasciculus interfascicularis) and the dorsal horn lesion with adjacent normal axon staining in the ascending dorsal column tracts (A), and in the area normally occupied by the lateral corticospinal tract axons. The adjacent (ascending) posterior spinocerebellar tract (asterisks) also shows a normal NF staining pattern (B). From publication 3, page 185.

200kDa neurofilament (NF) immunohistochemistry showed an almost complete bilateral loss of axons (Wallerian degeneration) in the descending nerve fibre pathways, precisely in the areas indicated by MRI (figures 4.31B and 4.33). Higher magnification views show axonal loss (NF) in the dorsal columns (fasciculus interfascicularis) and the dorsal horn lesion with adjacent normal axon staining in the ascending dorsal column tracts (figure 4.33A), and in the area

normally occupied by the lateral corticospinal tract axons. The extent of Wallerian degeneration was well demarcated by the presence of intact axons in the adjacent areas, such as the posterior spinocerebellar tracts (asterisks), which show a normal NF staining pattern (figure 4.33B). The slight asymmetry of the lateral reticulospinal tract detected by MRI was confirmed by the NF immunohistochemistry.

In the grey matter, the well circumscribed oval lesion in the left dorsal horn was completely devoid of NF-positive axons (figure 4.33A). The loss of NF staining in the white matter was associated with a heavy loss of myelin, as shown by MBP staining.

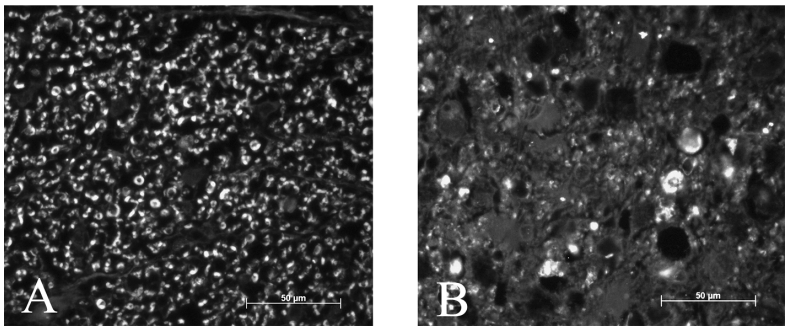

Figure 4.34: MBP immunohistochemistry. (A) Normal MBP immunofluorescence pattern (ascending tracts of the dorsal column). (B) Abnormal myelin staining pattern in the lateral corticospinal tracts, confirming Wallerian degeneration. Myelin sheaths have degenerated and myelin debris can be detected within phagocytic macrophages. From publication 3, page 185.

Myelin Basic Protein (MBP) immunohistochemistry: In the intact tracts of the dorsal column, a normal white matter MBP immunofluorescence pattern was detected (figure 4.34A). In the lateral cortico-spinal tracts, the myelin staining pattern was abnormal: few intact myelin sheaths could be detected; the sheaths have degenerated and myelin debris could be seen within phagocytic macrophages (figure 4.34B), confirming Wallerian degeneration.

Vimentin immunohistochemistry: Although immunohistochemistry for HLA-DR and CD68 as markers for inflammatory cells proved unsuccessful in this

cord sample, reactive cells could be readily identified by vimentin immunohistochemistry. White matter regions lacking NF and MBP immunoreactivity were intensely vimentin immunoreactive (figure 4.35A–E), and populated with rounded phagocytic macrophages, as well as populations of large stellate cells (reactive astrocytes, figure 4.35C). In the ovoid lesion in the left dorsal horn, strongly stained vimentin-positive cell bodies and processes were densely packed, including rounded phagocytic macrophages.

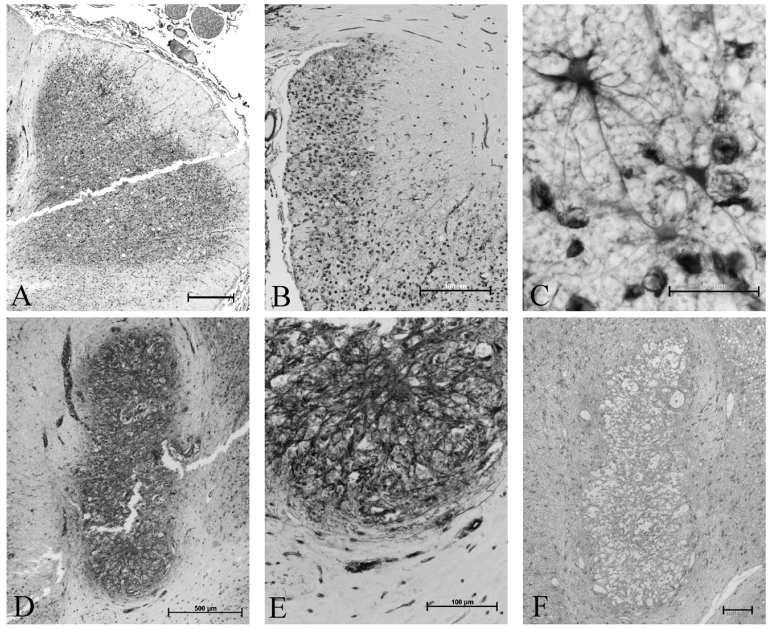

Figure 4.35: Immunohistochemistry for vimentin (A-E) and GFAP (F). (A) lateral corticospinal tract, (B) anterior corticospinal and reticulospinal tracts, adjacent to a white matter fibre tract. (C) High magnification in the degenerating lateral corticospinal tract (C). (D,E) The dorsal horn cavitation. (F) Astrocytes surrounding the dorsal horn lesion. From publication 3, page 185.

Glial Fibrillary Astrocytic Protein (GFAP) immunohistochemistry: Long and tapering, intimately interwoven processes (figure 4.35E) were derived from the GFAP-positive, hypertrophic astrocytes that were detected around the circumference of the cavitation (figure 4.35F).

4.4 Discussion

In this investigation, *post-mortem* high resolution MRI has shown its potential to depict the extent of traumatic spinal cord lesions and white matter sparing, and to predict locomotor outcome in the experimental setting.

4.4.1 Compression Injury of the Rat Spinal Cord

Since the beginning of the 1990s, gross correlations between functional recovery, histology including white matter sparing [233], and MRI [98] had been established. At that time, MRI analysis was limited by the very low image resolution to approximate qualitative assessments of lesion extent and parenchymal sparing [142], and it could only be correlated with gross functional assessments, unlike the detailed BBB score which is now available and validated.

Years later, in a correlative study of human and experimental SCI, Metz and colleagues used experimental *in vivo* 4.7 T MRI, 3 months after rat SCI. They showed (histologically) that tissue bridges across the lesion site, especially when ventrally located, were associated with better recovery, as rated by the BBB scale. Statistically significant correlations could however only be established with cord atrophy (in fact, cord diameter) and lesion length. Weak correlations were seen with MRI-measured lesion surface and lesion volume[1]. The *post-mortem* histological analysis showed a correlation of the BBB scores with the degree of white matter sparing [218].

The image quality that could be obtained in the present work is superior to most of the published rat spinal cord MR images, even at 9.4 T [34], and matched only by *post-mortem* MRI of the isolated spinal cord at exceptionally high magnetic field strengths (17.6 T [25, 322]). *Post-SCI* locomotor function in the rat has never been correlated with MRI of the present resolution. Our findings therefore significantly extend the previously published data. The spinal cord was depicted in its anatomic environment. Standard acquisition sequences, like PD weighted imaging, detected different lesion components and precisely showed the lesion topography at different survival delays. A new IR technique enhanced the detection of white matter sparing. Lesion size, cord atrophy, and spared spinal cord parenchyma, in particular of white matter, could be quantified using image analysis and morphometry. The established morphometric parameters correlated highly significantly with BBB-assessed locomotor function.

[1] This investigation was based on *longitudinal* images

4.4.1.1 Rat Spinal Cord Injury MRI: Technical Aspects

The main sequences used in the present study were PD weighted. PD parameters have been shown to determine most of the contrast in *ex vivo* MRI of the fresh rat spinal cord [34] and it has been shown in human material that 9.4 T PD imaging offers excellent contrast and resolution in fixed CNS tissue [31]. The present technique yielded significantly better image quality, in terms of spatial resolution, than that shown in the literature [34], due to a number of factors, including longer acquisition times (table 1.1, page 6). Comparable SCI MR image quality has been obtained *ex vivo* with a 17.6 T magnet and an acquisition time of 3.5 hours for a pixel size (resolution) of 23x23 μm^2 [322]. The image quality is very similar to the present "PD32" images; the slightly higher spatial resolution does not provide any additional information[2]. Furthermore, in the cited study, MRI and histology are correlated on longitudinal sections. Although this approach does show many aspects of the cord lesion, it does not provide the same type of information as the correlation of transverse imaging and histology: the appreciation of the maximal transverse extension of the cord lesion is crucial for estimating white matter tract damage and preservation.

In addition to the high resolution images (PD32), more rapid acquisitions were used in order to determine the "diagnostic efficiency" of the technique. Reduction of the NA to 8 or even 4 (PD8, PD4) still demonstrated pathological changes in sufficient detail and with a sufficient signal to noise ratio to estimate lesion severity, define its microanatomical topography, and detect different lesion components[3]. In particular, despite their lower resolution, the IR images suppressing normal white matter increase precision because of higher contrast between normal and abnormal tissue.

4.4.1.2 MRI *versus* Histology

In comparison to histology, high resolution *ex vivo* MRI can depict the spinal cord in the axial and horizontal planes, without physically sectioning it, and leaves the tissue intact for further analysis. The technique is much less laborious than histology. The acquisition of proton density images is rapid: 8–10 hours for high resolution, 50 or 25 minutes for "low" resolution, at NA=32, NA=8, and NA=4, respectively. No manpower is needed once the acquisition is initiated (e.g., over night for the long

[2]Note that, physically, MR image resolution is restricted to about 10x10 μm^2 to 20x20 μm^2, and this is independent of the magnetic field strength. But, because higher fields increase the signal to noise ratio, a 20x20 μm^2 resolution image can be obtained more rapidly, with a lower NA, on a very high field instrument.

[3]Refer to the original files on the CD for all jpeg-overviews. Note that the latter show even slightly reduced image resolution compared to single MRI sections.

acquisitions). Up to 8 (PD8) or 15 (PD4) cords can be scanned over a normal work day, with sufficient resolution for many potential applications.

Furthermore, MRI of the cord-spine block shows the cord in its anatomical setting. The vertebral level of the lesion centre and the adjacent intact parenchyma can be located. This is useful, for example, for lesions which are close to the lumbar central pattern generator: the lesion is anatomically depicted, and histological, immunohistochemical and other analyses can be efficiently directed towards the centre of interest, be it the lesion centre itself, the immediately sub-lesional cord, or even the supra-lesional cord. Additionally, imaging of the spine block can detect vertebral displacements [122]—which may occur after the laminectomies that are ubiquitous in SCI research—or other spine deformations; these deformities may influence locomotor behaviour [personal observation]. In the balloon model, the presence or absence of spinal cord lesions at the insertion site of the balloon can be shown. And, last but not least, since the cord remains in the spinal canal, pathological changes can be topographically located with respect to the anterior or posterior, right or left parts of the cord. Histologically, this is not always straightforward in severely altered, chronic and atrophic cords (see above). With *"in situ"* MRI, there is no need for spinal cord manipulation, no distortion of the sections (i.e., the lesion anatomy is stable), and no tissue freezing (which appears to influence the volume of the lesions, see below).

Aside from these advantages that distinguish MRI from histological tissue analysis, the microanatomical assessment of the lesion and of white matter sparing is at least equivalent. Because the key white matter tracts involved in basic locomotion are rather diffusely distributed in the spinal cord funiculi, the image quality obtained with the present MRI resolution appears sufficient—especially when supplemented by the newly developed inversion-recovery sequence. The interpretation of the MRI data did however remain somewhat subjective, and the estimation of white matter sparing varied slightly even with the same investigator. But this was also observed for histology, where, in addition, the tissue manipulation artefacts and other physical problems hindered the correct interpretation of white matter preservation.

During this study, the new IR sequence reduced ambiguous interpretations to the minimum, and the absence of tissue manipulation may compensate for the lower resolution of MRI, as compared to histology.

4.4.1.3 Lesion Components and Evolution

Basic histopathological changes were detected by PD imaging: oedema, necrosis, and scarring processes were hyper-intense, but not distinguishable from one another—neither on PD weighted images, nor on any other tested acquisition sequence, includ-

ing an experimental water suppression IR sequence. Haemorrhage was hypo-intense from the first post-operative day (extravasation of red blood cells, probably containing deoxyhaemoglobin) to several months after injury (haemosiderin deposits), confirming the literature [105, 141, 322]. Acute haemorrhage and hemosiderin deposits occurred mainly in the central regions, due to the greater fragility of the central grey matter [165].

As few as two to three rats par survival delay were sufficient to describe sequential pathological events at different stages of SCI. Over the first days, following 20 μl balloon compression, PD MRI and correlative histology showed an oedematous and necrotic lesion, with variable degrees of haemorrhage in the central parts. Severe cord swelling was observed over the first week: the subarachnoid spaces in the spinal canal virtually disappeared due to the increase in cord volume. At later survival delays, over the first post-operative month, cord swelling resolved and left place to progressive scarring, leading to cord atrophy at the lesion site, described several years ago in human SCI MRI [306], as well as very recently in rat SCI MRI [83]. The observed oedema-necrosis-scarring sequence confirms previous MRI studies (with lower resolution, but with contrast injection [262]).

This illustrates one of the pathophysiological principles which had initially motivated the development of a closed canal lesion technique in our laboratory: during more than a week after the injury, the swollen spinal cord completely fills the spinal canal. It cannot expand beyond the bone limits. Therefore, the setting in which acute secondary physiopathological changes occur in the closed-canal after balloon compression differ from "open" lesion techniques, like the widely employed weight drop model, or the clip compression technique, where the spinal cord is "decompressed" at the lesion site by the large laminectomies that are necessary to perform the surgery. This may influence functional recovery [90, 279] and must be accounted for in studies of the pathophysiology of SCI.

After the resolution of the acute oedematous state, the progressive stabilisation of the lesion site by scarring processes resulted in a clear delimitation of the definitively injured and the preserved tissue. It appears that cord atrophy and white matter preservation can be usefully assessed after at least one month. This is in keeping with the behavioural stabilisation, which occurs over approximately one month after balloon compression injury.

4.4.1.4 Correlation with Locomotor Function

The correlation of MRI findings, in particular white matter sparing and morphometric lesion characteristics, with locomotor performance was studied two months after injury, when locomotor recovery had reached its plateau, and when the lesion

was expected to be morphologically stable.

As described above, all morphometric parameters showed significant correlation not only with the initial locomotor deficit, but also with the evolution of the locomotor recovery over time[4]. The different parameters corresponded to evaluations of the severity of the lesion from three different angles:

The lesion size was calculated from the surface measurements of the lesion. Its volume correlated significantly with locomotor recovery.

Strikingly, the volume calculated by histology and MRI differed (table 2, appendix, page 183): the calculated MRI lesion volume was about double, compared to the histological data, despite the close morphological correlation between the two. In a previous study [142], MRI consistently produced longer estimates of lesion length than did histology. Therefore, shrinkage of the tissue during the freezing process for histological analysis is the most probable reason for this difference, and MRI is likely to be superior to standard histology for accurate volumetry, because of less tissue handling.

Cord atrophy was also found to be significantly correlated with the severity of the behavioural deficit and the evolution of recovery.

Before statistical analysis, normalisations had been developed in order to account for potential anatomical differences between rats. These could be differences in cord volume—accounted for by the ratio dividing the minimal cord surface by the maximal (i.e., normal lumbar) cord surface—or even for potential anatomical differences in the cord and canal size—accounted for by the division of the previous ratio by the highest cord/canal surface ratio, the latter corresponding to the maximal extent of the cord in the individual rat's spinal canal. However, as the lesion intensities were very different in the present study, absolute atrophy (as estimated by the smallest cord surface observed in a single cord) was already highly statistically significantly associated with the degree of recovery. Therefore, no atrophy normalisation was shown to be superior to the latter. Nonetheless, these normalisations might be useful in investigations with less differences in lesion severity.

Spared perilesional parenchyma was measured: total preserved parenchyma, total preserved white matter, and total preserved ventral white matter. Again, all of these correlated highly significantly with the behavioural evaluation. More

[4]Note however that the interpretation of the recovery curve remains limited by the non-linearity of the BBB scale.

similar lesions than the ones in the present study may be necessary in order to detect differences in their potential to predict locomotor function.

Besides these morphometric quantifications, the *anatomical* assessment of the lesion extent is crucial, because the different funiculi contain different tracts with different effects on locomotion and recovery (see above). In the present investigation, the preservation of ventral, ventrolateral, and dorsolateral funiculi was associated with rapid recovery of normal basic open field locomotion (rat n°3235). Even moderate unilateral sparing in the lateral cord (rat n°3238) was sufficient for functional recovery up to the maximal score of 21. Moderate locomotor recovery, observed in rats n°3226 and n°3230, was associated with minimal sparing in the ventral funiculus. This confirms the role of these funiculi in locomotor recovery.

It is intriguing that unilateral predominance of a lesion may still ultimately result in bilateral functional recovery and symmetrical locomotion, even when a complete hemicord is severely injured (e.g., rat n°3238, see page 106). In a more severe lesion (rat n°3226, see page 107), the very limited ventral funiculus white matter sparing was strictly unilateral, but finally associated with symmetrical locomotor recovery, i.e. limited stepping (BBB = 10). This indicates that, after a recovery period (of approximately one month), allowing for restructuration of the long tracts' CPG input, the lumbar locomotor CPG may be activated by unilaterally preserved long tracts. In the literature, there is evidence for this kind of functional compensation after unilateral lesions of the spinal motor systems, including partial bilateral recovery observed after unilaterally severe lesions [12, 272]. For example, the moderate locomotor impairment observed after unilateral ventrolateral spinal cord lesions disappears in the long run, indicating that the functional contribution of unilateral ventrolateral pathways may be subserved by remaining pathways [321]. In addition, recovery observed after a cord section sparing left ventral and lateral funiculus fibres can be abolished by the secondary transection of lumbar commissural interneurons, which are known to play a role in the coordination of the bilateral neuronal activity in the lumbar CPG [151]. Thus, plasticity of the ventral and the lateral column long tracts may mediate recovery of locomotor CPG function *via* the commissural interneurons. This is substantiated by the fact that, e.g., the reticulospinal tract synapses on the central pattern generator, and the ipsilateral reticular formation monosynaptically activates lumbar commissural interneurons (in the cat [170]).

In addition, long tract plasticity above the lesion may play a role *via* new intraspinal circuits [19]: the newly established connections above the lesion are linked to the sub-lesional CPG *via* ascending and descending axons that establish propriospinal connections between the cervical and lumbar enlargments, and that are known to also run in the ventrolateral funiculus, and with commissural and ipsilat-

eral connections [249].

The present investigation showed that the newly developed inversion recovery MRI acquisition sequence increased examiners' confidence in the detection of spared white matter and the delimitation of the lesion, as compared to non-IR PD MRI. If this was to be substantiated by a double blinded study of the assessment of white matter sparing by different examiners, PD-IR MRI could prove to be particularly relevant for the prediction of locomotor recovery.

In conclusion, the role of the ventral and lateral funiculi in motor recovery and the potential of the long white matter tracts for functionally relevant plasticity are confirmed. The described MRI technique is useful in the investigation of regional white matter sparing after experimental rat SCI.

4.4.2 Human *post-mortem* MRI

The present study shows that MRI has, with increasing resolution, the potential to detect pathological changes missed with current clinical imaging. This includes the demonstration of white matter tract degeneration by standard sequences: the demyelinated tracts appear hyper-intense in PD weighted MRI [127, 221].

In addition, the CNS tissue studied in the present investigation had been preserved since 1972, i.e., for 3 decades, in formalin. This indicates the usefulness of SCI and other CNS tissue banks, and the potential benefit of the application of modern analytic techniques to fixed CNS tissue.

Chapter 5

Conclusions and Perspectives

THE PRESENT WORK HAS INVESTIGATED PRESERVED TISSUE after SCI from two different angles. First, we have explored strategies to recruit preserved tissue after SCI *via* spared white matter tracts, propriospinal afferents, or direct pharmacological neuromodulation, in order to enhance recovery. Second, we have assessed preserved tissue by a novel MRI technique, validated by histology, and correlated morphometric parameters and regional white matter sparing with spontaneous locomotor recovery.

5.1 Experimental Therapeutic Strategies

The BWSTT and rTMS studies open a series of encouraging perspectives. The first shows that BWSTT may be particularly useful after incomplete SCI, indicates a potential benefit of early treatment, and establishes the rat model as useful to study BWSTT. The second suggests that rTMS may trigger adaptive changes in the sublesional spinal cord *via* monoaminergic descending systems. Third, despite as yet inconclusive results, the combined rTMS-pharmacotherapy approach merits further investigation.

Repetitive TMS emerges as a therapeutic modality with significant potential after SCI. Some effort will be required to precisely define its usefulness and indications. It would therefore be of major interest to build on the present experience and the lessons learned along the following lines:

In a clinical perspective: Knowing the excellent tolerance of rTMS, the present results and those of the study of rTMS in human SCI cited earlier [28], as well

as the development of new, more efficient stimulation protocols, it would be interesting to conduct a pilot study of rTMS in the clinical setting. Chronic incompletely injured SCI patients, who have been clinically stable for at least six months, could be treated using the so-called theta-burst protocol, which has been shown to induce durable neuronal excitability changes [162].

From the experimental perspective: The study of rTMS should be pursued, but refined by an adapted methodology, to make the detection of experimental treatment effects on locomotor recovery more reliable. White matter sparing and locomotor recovery must be reproducible. The experimental strategy may, for example, be supplemented by high resolution MRI to assess the topographical reproducibility of the lesion. In order to confirm the proposed physiological mechanisms, the processes underlying locomotion, spontaneous functional recovery, and treatment effects on the CPG should be analysed using histological and biomolecular analyses (see above).

5.2 MRI in Spinal Cord Injury Research

MRI after SCI in the rat has been described before, but neither at the present resolution, nor with comparable lesion follow-up or locomotor recovery correlation. For the microanatomical assessment of compressive SCI, 9.4 T *post-mortem* MRI using standard acquisition sequences appears valid.

From a technical viewpoint, the present investigation demonstrates that magnetic field strength is important, but certainly not the only aspect that needs to be addressed when aiming for high quality imaging and "diagnostic" efficiency. First of all, many factors influence image resolution, like acquisition time, the homogeneity of the magnetic field, etc. (table 1.1, page 6). Second, using appropriate acquisition sequences, certain specific pathological aspects of CNS injury can be usefully depicted, like white matter sparing with IR imaging, even with relatively low image resolution. Depending on the question to be answered, MRI can be very efficient with the correct choice of imaging parameters.

Therefore, increasing field strengths to even higher values, which would require significant technological investment, might not be useful, because increasing resolution to a truly histological level is unrealistic even with the highest available field strengths [265]: physically, the maximal possible resolution is limited to approximately 20x20 μm^2. Conversely, the development and refinement of acquisition sequences with specific functions appears as a more promising perspective in the short term. This could potentially, for example, more accurately distinguish lesion com-

ponents (like oedema and necrosis or gliosis), or more precisely define lesioned fibre tracts using complementary techniques like diffusion weighted imaging.

One particular setting is the comparison of experimental groups in the study of experimental treatment strategies. With the demonstrated potential to predict locomotor recovery, without physically handling the fragile specimen, and without physically sectioning the excised spinal cord, the described MRI technique may prove to be a particularly useful tool to assess lesion homogeneity in current SCI research.

Limitations of the present investigation include the fact that the spinal cord lesions in the behavioural MRI study were of very different severities (which also explains the clear statistical differences, despite the small group size). The potential of the technique to detect slighter variations of white matter sparing anatomy and quantity, as well as slighter differences between morphometric parameters, should be investigated in the future. Also, automated detection of white matter sparing by image fusion of PD and IR images represents an area of potential significant progress, which might accelerate the quantification of white matter sparing.

Beyond the correlation with behavioural recovery and the role of white matter sparing and lesion severity, which have been provided by the present work, many other perspectives exist for rodent MRI in experimental SCI research. In the various experimental compressive or contusive SCI models [22, 214, 256], histopathology and topography of injury may be quite different [64]. These different techniques have never been precisely compared in detail, neither for lesion topography, nor for the sequence of the pathological events. The efficiency of *post-mortem* (as well as *in vivo*) MRI may, in the future, contribute to this kind of comparison.

In the present correlative *ex vivo* MRI and locomotor recovery investigation, lesions had to be assessed at a single time point, at the chronic stage. The lesions could not be followed by MRI over time. There was no immediate correlation of one lesion's evolution with the corresponding recovery curve. The transferral of the technique to the *in vivo* setting holds a lot of promise for rodent CNS imaging, and its practicability has been shown for the rat spinal cord, with progressively increasing resolution [122, 218, 33, 102, 227, 84, 322, 291, 292].

Many other potential applications of *in vivo* rat SCI MRI are in the process of being developed to complement current research strategies [30]. Diffusion weighted imaging (DWI) has been used to estimate the preservation of white matter *post-mortem* at 9.4 T [274]. It may depict the disruption of white matter tracts in areas that appear normal with conventional MR sequences [118]. Fibre tract tracing [32] or dti tractography [84] may significantly add to the interpretation of the role of different fibre tracts in recovery. The development of "smart" contrast agents (tissue specific or activated by the presence of certain substances) and of paramagnetic tracers which can be ingested by living cells allows to localise these cells in the or-

ganism they are injected into [291, 292]. Certain substances can be detected with spectroscopy [240]. And, last but not least, functional MRI is being developed in rodents [310].

In the future, *in vivo* analysis of experimental SCI may show relevance to the clinical situation, where, for example, haemorrhage is a clear prognostic indicator for lesion severity [220]. This may be confirmed in the rat by correlative MRI and locomotor follow-up *in vivo*.

At the present time, however, in order to obtain useful *in vivo* image resolutions, either invasive techniques like coil implantations [35], or sophisticated, very high field magnets [25, 265, 322] are needed, in addition to respiratory and heart-rate coupling and anaesthesia in these small rodents, not without a certain mortality [322]. The quality of all published *in vivo* MR images remains clearly inferior to the images obtained by *post-mortem* PD32 MRI. Furthermore, until the writing of this thesis, the usefulness of morphological *in vivo* MRI has neither been firmly established for the microanatomical follow-up of the lesion, nor for locomotor correlation—even if certain authors have described *in vivo* behavioural and MRI follow-up over two months, showing the expected lesion filling by scar tissue [227], and although some gross correlation of diffusion weighted SCI imaging with behaviour has been made [236].

Many of the above mentioned MRI sequences are used in the human situation, and the continuous development of novel acquisition sequences provides the clinician with highly sophisticated tools to assess neurological patients. It is therefore of note that an almost abandoned acquisition sequence from the beginnings of clinical MRI, i.e. PD weighted imaging, appears useful with sufficient field strength (and other favourable technical parameters) in the appreciation of a key clinical aspect of human SCI—the degeneration of the long white matter tracts. In SCI patients, the precise assessment of the integrity of the white matter tracts would be a diagnostic and prognostic advance. The present investigation gives an outlook on the potential of high resolution MRI: higher power magnets will probably be used in human MRI in the future. 9.4 T MRI is already being investigated for imaging of the human nervous system [5]. Although, in the nearer future, the resolution of *in vivo* human MRI will only approximate the resolution that has been obtained *post-mortem* for technical reasons (see table 1.1, page 6)—the expected increase in resolution imaging should still allow a certain degree of white matter tract assessment, in particular when standard techniques, like PD imaging, are combined with more recent sequences, like dti tractography.

5.3 Conclusion

This investigation confirms and extends the notion that preserved tissue after spinal cord injury plays a key role in spontaneous locomotor recovery after traumatic SCI.

The therapeutic potential of the locomotor CPG has been demonstrated in the rat. The beneficial effects of rTMS should be investigated in more detail in the experimental setting, and rapid clinical investigation appears feasible.

The assessment of preserved tissue, and particularly the spared white matter tracts, is crucial in those investigations that depend on the observation of locomotor recovery after experimental SCI, and the usefulness of *post-mortem* 9.4 T MRI has been demonstrated. From the experimental perspective, many potential developments exist, including the refinement of the technique, its behavioural validation for only moderately different spinal cord lesions, and transfer to the *in vivo* setting. In a clinical perspective, ameliorations of MR image quality can be expected and are likely to provide caretakers with essential anatomical information in the foreseeable future.

Part III

Reference List

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Part IV

Appendix

Complementary Information

ASIA Classification

The ASIA classification of Spinal Cord Injuries is a clinical evaluation scale first devised in 1982, which has been regularly updated since then. In its present state, it is officially called the "International Standard for Neurological Classification of Spinal Cord Injuries", although it is still usually referred to as "the ASIA scale" [295].

The 2006 version of the ASIA scoring sheets follows; on the second page, the grades (A–E) are explained.

Patient Name _____

Examiner Name _____ Date/Time of Exam_____

ASIA
American Spinal Injury Association

STANDARD NEUROLOGICAL CLASSIFICATION OF SPINAL CORD INJURY

ISCOS

MOTOR

KEY MUSCLES (scoring on reverse side)

	R	L	
C5	☐	☐	Elbow flexors
C6	☐	☐	Wrist extensors
C7	☐	☐	Elbow extensors
C8	☐	☐	Finger flexors (distal phalanx of middle finger)
T1	☐	☐	Finger abductors (little finger)

UPPER LIMB TOTAL (MAXIMUM) ☐ (25) + ☐ (25) = ☐ (50)

Comments:

L2	☐	☐	Hip flexors
L3	☐	☐	Knee extensors
L4	☐	☐	Ankle dorsiflexors
L5	☐	☐	Long toe extensors
S1	☐	☐	Ankle plantar flexors

Voluntary anal contraction (Yes/No) ☐

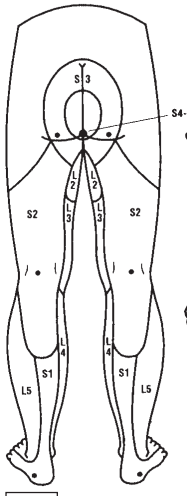
LOWER LIMB TOTAL (MAXIMUM) ☐ (25) + ☐ (25) = ☐ (50)

SENSORY

KEY SENSORY POINTS

	LIGHT TOUCH R	LIGHT TOUCH L	PIN PRICK R	PIN PRICK L
C2				
C3				
C4				
C5				
C6				
C7				
C8				
T1				
T2				
T3				
T4				
T5				
T6				
T7				
T8				
T9				
T10				
T11				
T12				
L1				
L2				
L3				
L4				
L5				
S1				
S2				
S3				
S4-5				

0 = absent
1 = impaired
2 = normal
NT = not testable

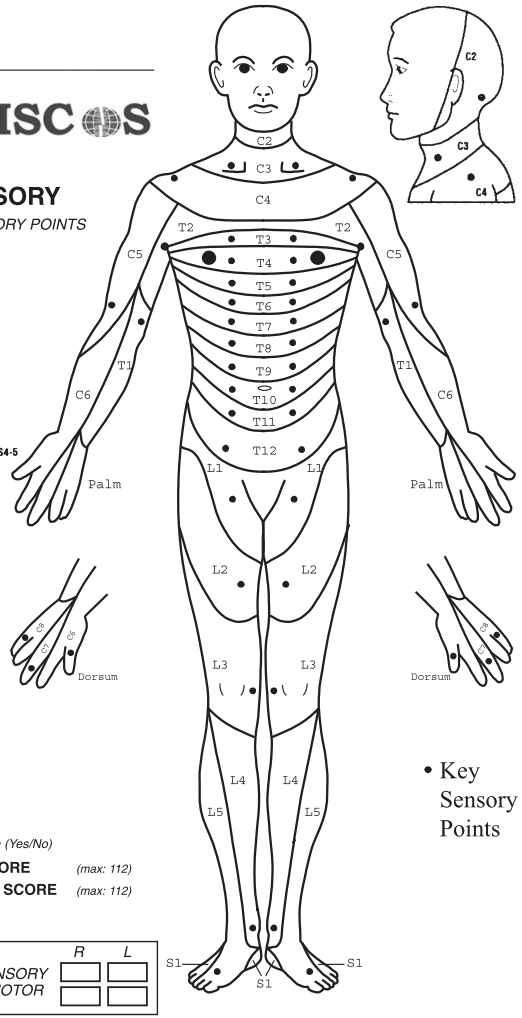


Any anal sensation (Yes/No) ☐

TOTALS (MAXIMUM) ☐ (56) + ☐ (56) = ☐ (56) (56)

☐ + ☐ = ☐ **PIN PRICK SCORE** (max: 112)

☐ **LIGHT TOUCH SCORE** (max: 112)



• Key Sensory Points

NEUROLOGICAL LEVEL	SENSORY R	SENSORY L	COMPLETE OR INCOMPLETE?	ZONE OF PARTIAL PRESERVATION	SENSORY R	SENSORY L
The most caudal segment with normal function	☐	☐	Incomplete = Any sensory or motor function in S4-S5	Caudal extent of partially innervated segments	☐	☐
MOTOR	☐	☐	ASIA IMPAIRMENT SCALE ☐	MOTOR	☐	☐

REV 03/06

MUSCLE GRADING

0 total paralysis

1 palpable or visible contraction

2 active movement, full range of motion, gravity eliminated

3 active movement, full range of motion, against gravity

4 active movement, full range of motion, against gravity and provides some resistance

5 active movement, full range of motion, against gravity and provides normal resistance

5* muscle able to exert, in examiner's judgement, sufficient resistance to be considered normal if identifiable inhibiting factors were not present

NT not testable. Patient unable to reliably exert effort or muscle unavailable for testing due to factors such as immobilization, pain on effort or contracture.

ASIA IMPAIRMENT SCALE

☐ **A = Complete**: No motor or sensory function is preserved in the sacral segments S4-S5.

☐ **B = Incomplete:** Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5.

☐ **C = Incomplete:** Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3.

☐ **D = Incomplete:** Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more.

☐ **E = Normal:** Motor and sensory function are normal.

CLINICAL SYNDROMES (OPTIONAL)

☐ Central Cord
☐ Brown-Sequard
☐ Anterior Cord
☐ Conus Medullaris
☐ Cauda Equina

STEPS IN CLASSIFICATION

The following order is recommended in determining the classification of individuals with SCI.

1. Determine sensory levels for right and left sides.
2. Determine motor levels for right and left sides.
   Note: in regions where there is no myotome to test, the motor level is presumed to be the same as the sensory level.
3. Determine the single neurological level.
   This is the lowest segment where motor and sensory function is normal on both sides, and is the most cephalad of the sensory and motor levels determined in steps 1 and 2.
4. Determine whether the injury is Complete or Incomplete (sacral sparing).
   If voluntary anal contraction = **No** *AND all S4-5 sensory scores* = **0** *AND any anal sensation* = **No**, *then injury is COMPLETE. Otherwise injury is incomplete.*
5. Determine ASIA Impairment Scale (AIS) Grade:

Is injury <u>Complete</u>? If **YES**, AIS=A Record ZPP (For ZPP record lowest dermatome or myotome on each side with some (non-zero score) preservation)

NO ↓

Is injury motor <u>incomplete</u>? If **NO**, AIS=B (Yes=voluntary anal contraction OR motor function more than three levels below the motor level on a given side.)

YES ↓

Are <u>at least</u> half of the key muscles below the (single) <u>neurological</u> level graded 3 or better?

NO ↓ AIS=C

YES ↓ AIS=D

If sensation and motor function is normal in all segments, AIS=E
Note: AIS E is used in follow up testing when an individual with a documented SCI has recovered normal function. If at initial testing no deficits are found, the individual is neurologically intact; the ASIA Impairment Scale does not apply.

The Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) [21]

0 No observable hind limb (HL) movement

1 Slight movement of one or two joints, usually the hip and/or knee

2 Extensive movement of one joint or Extensive movement of one joint and slight movement of one other joint

3 Extensive movement of two joints

4 Slight movement of all three joints of the HL

5 Slight movement of two joints and extensive movement of the third

6 Extensive movement of two joints and slight movement of the third

7 Extensive movement of all three joints of the HL

8 Sweeping with no weight support or plantar placement of the paw with no weight support

9 Plantar placement of the paw with weight support in stance only or occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping

10 Occasional weight support plantar steps, no FL-HL coordination

11 Frequent to consistent weight supported plantar steps and no FL-HL coordination

12 Frequent to consistent weight supported plantar steps and occasional FL-HL coordination

13 Frequent to consistent weight supported plantar steps and frequent FL-HL coordination

14 Consistent weight supported plantar steps, consistent FL-HL coordination; and predominant paw position during locomotion is rotated (internally or externally) at initial contact and lift off; or frequent plantar stepping, consistent FL-HL coordination and occasional dorsal stepping

15 Consistent plantar stepping and consistent FL-HL coordination; no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact

16 Consistent plantar stepping and consistent FL-HL coordination during gait; frequent toe clearance during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off

17 Consistent plantar stepping and consistent FL-HL coordination during gait; frequent toe clearance; predominant paw position is parallel at initial contact and lift off

18 Consistent plantar stepping and consistent FL-HL coordination during gait; consistent toe clearance; predominant paw position is parallel at initial contact and rotated at lift off

19 Consistent plantar stepping and consistent FL-HL coordination during gait; consistent toe clearance; predominant paw position is parallel at initial contact and at lift off; and tail is down part or all of the time

20 Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel at initial contact and lift off; trunk instability; tail consistently up

21 Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel throughout stance; consistent trunk stability; tail consistently up

Supplementary Material

Individual BBB score evolution in the first combined treatment series

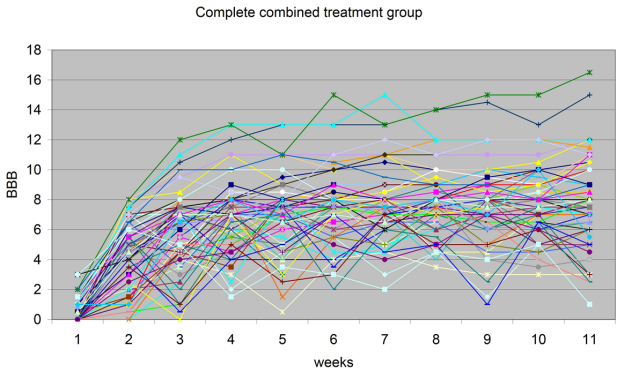


Figure 1: Single BBB score evolutions in the combined treatment series.

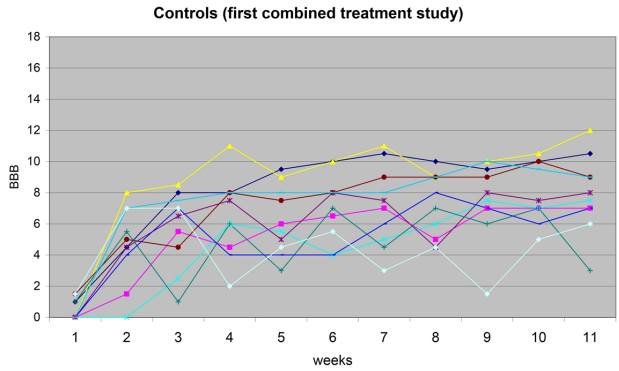


Figure 2: Control group (I)

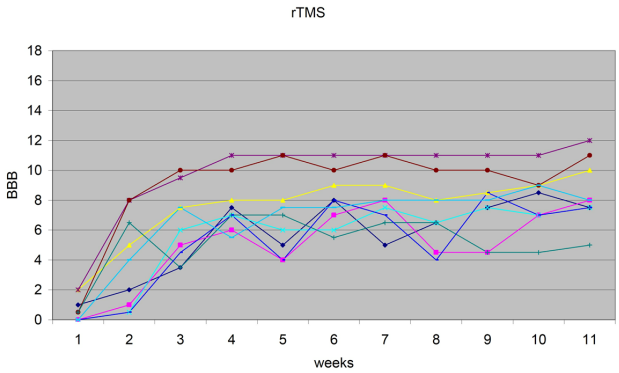


Figure 3: rTMS group (I)

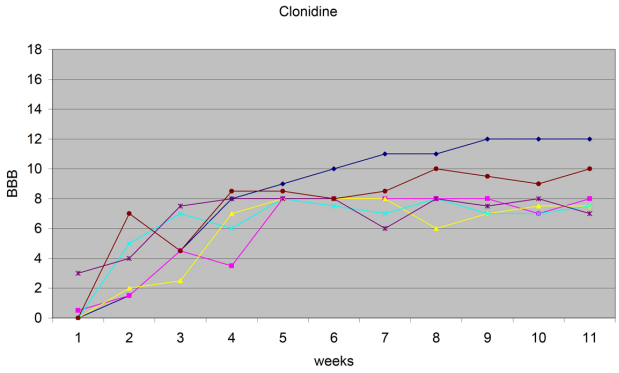


Figure 4: Clonidine group (I)

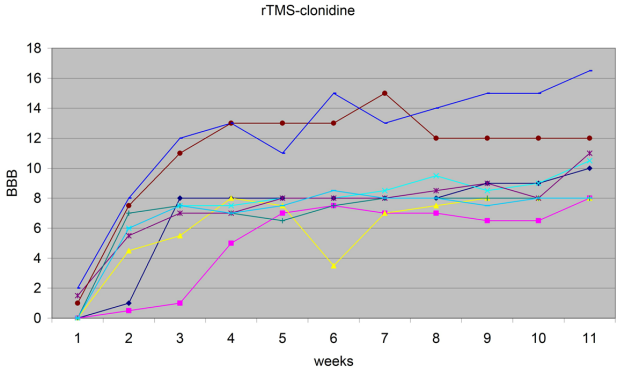


Figure 5: rTMS-Clonidine group (I)

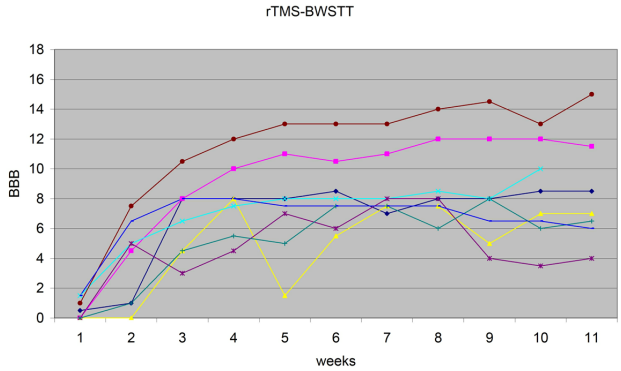


Figure 6: BWSTT group.

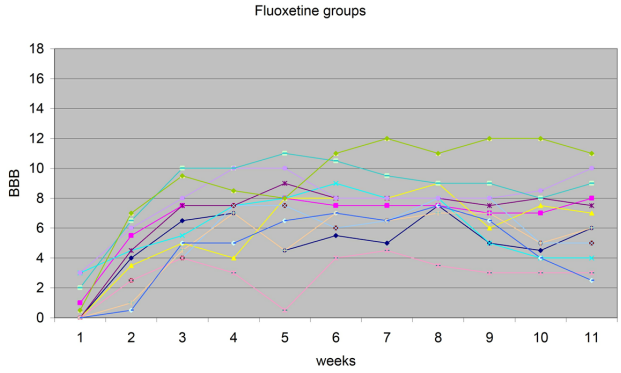


Figure 7: Fluoxetine and rTMS-fluoxetine groups (I).

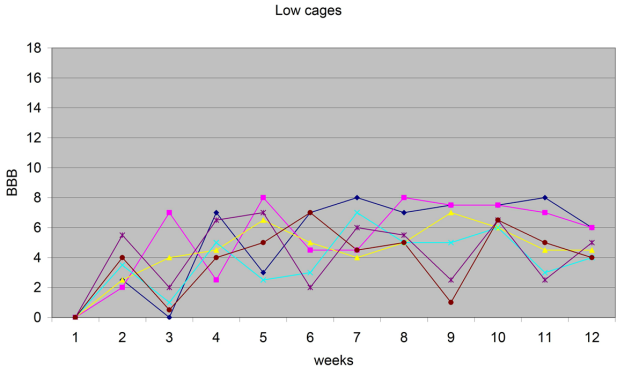


Figure 8: Control rats in smaller cages (I).

Data Analysing Possible Causes for High Behavioural Variability Observed in the First Combined Treatment Group

Correlation of Lesion Level and Behavioural Outcome

As a behavioural parameter, the mean plateau BBB scores were used (weeks 5–11, i.e. over the entire period when locomotor behaviour was stabilised).

The level of the lesion centre (as estimated by the naked eye) was assessed in two ways: by counting the vertebrae after opening the spinal canal when the spinal cords were dissected, and by measuring the distance between the the lesion and the obex (indentation at the junction of the spinal cord and the brain stem). The results are depicted in the graphs. No correlation could be found.

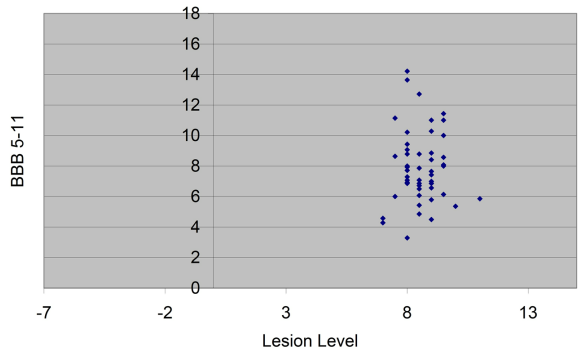


Figure 9: The mean plateau BBB score as a function of the segmental thoracic lesion level

This analysis confirms, on the other hand, that the lesion level was relatively precise at T8-T9, as expected, even though there were a few lesions in T7 and T10–11, but which had resulted in very similar locomotor scores.

The picture does not change much for the lesion-obex distance. Note that the correlation between that distance and the segmental level is imprecise, which might be due to the limitations of the simple inspection in detecting the centre of the lesion, or to anatomical differences between rats of different body sizes.

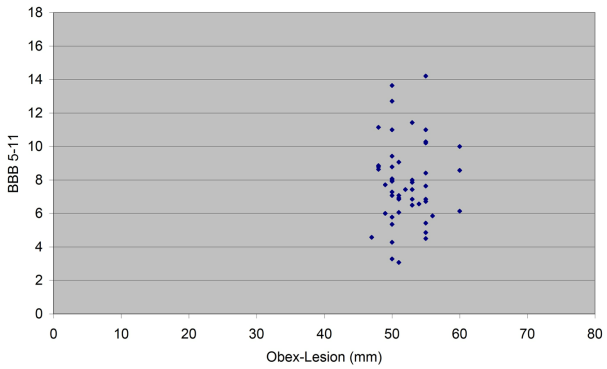


Figure 10: The mean plateau BBB score as a function of the distance between the obex and the lesion

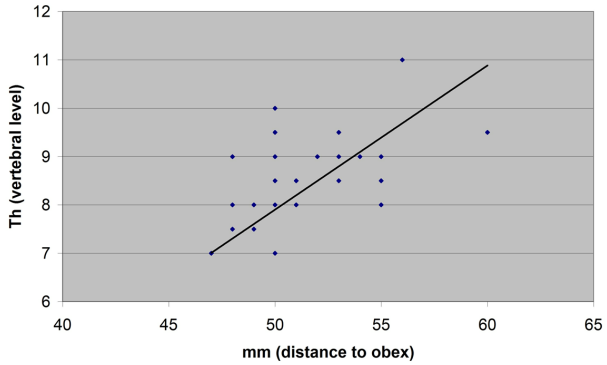


Figure 11: Correlation between the segmental lesion level and the obex-lesion distance. The origin of the regression line is at 0mm and level Th"−7" (7 cervical vertebrae).

BBB and Body Weight

Furthermore, no correlation was seen between locomotor scores and body weight:

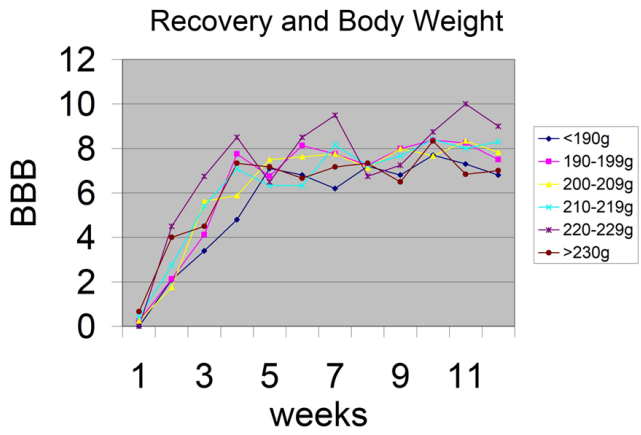


Figure 12: The BBB scores as a function of the rat's body weight (10g intervals)

Individual BBB score evolution in the second combined treatment series

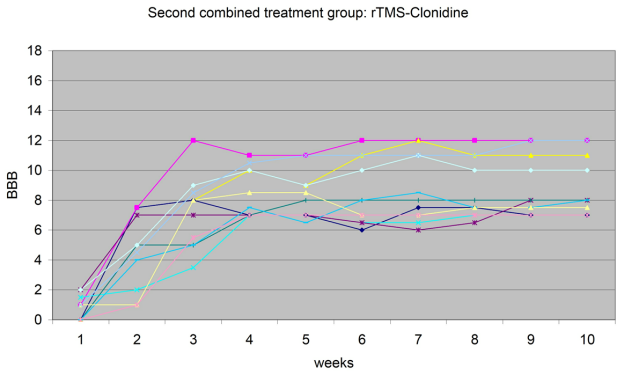


Figure 13: Single BBB score evolutions in the adapted-method/combined-treatement series.

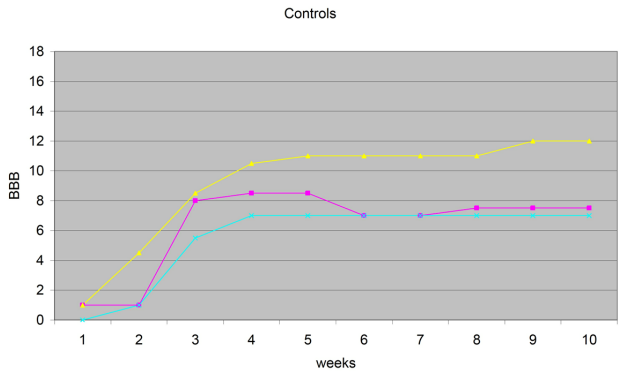


Figure 14: Control group (II).

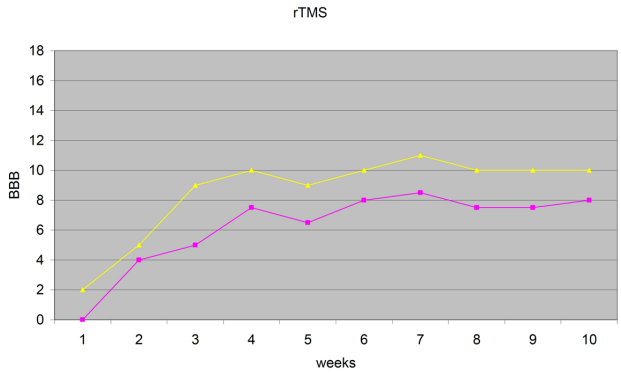


Figure 15: rTMS group (II).

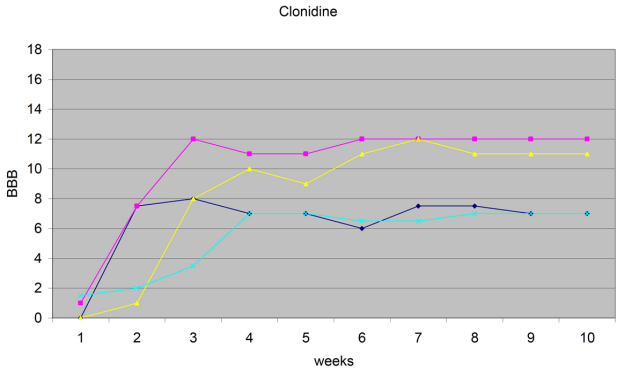


Figure 16: Clonidine group (II).

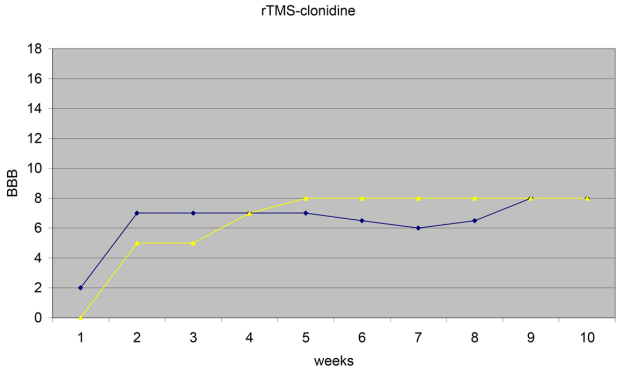


Figure 17: rTMS-clonidine group (II).

Morphometric Data

Tables 1 and 2 show measurements and calculations describing:

1. the **lesion size**: "X1", the maximal lesion/cord ratio in each cord, is an estimation of the maximal lesion extension in the transverse plane; calculated lesion volumes are shown ("X2" to "X4": histology("H"), low and high resolution PD MRI (PD8/32)), as well as their ratios.

2. **cord atrophy**, as estimated by simple surface measurements and ratios using T2 MRI ("X5", the minimal cord surface) and ratios ("X6" and "X6"', atrophy ratio and normalised atrophy ratio, respectively).

3. **sparing of cord parenchyma** ("X7"–"X11"), total parenchyma, total white matter, or ventral white matter.

X1	Maximal value of the "lesion/cord" ratio (histology)
X2	Lesion volume (MRI, PD, low resolution; μm^3)
X3	Lesion volume (MRI, PD, high resolution; μm^3)
X4	Lesion volume (histology; μm^3)
X5	Minimal cord surface (MRI, T2; pixels)
X6	Minimal value of the "atrophy ratio" (MRI, T2)
X6'	"Normalised atrophy ratio" (MRI, T2)
X7	Minimal spared ventral white matter (MRI, PD, high resolution; pixels)
X8	Minimal spared white matter surface (MRI, PD, high resolution; pixels)
X9	Minimal spared white matter surface (histology, μm^2)
X10	Minimal spared surface (grey + white; histology, μm^2)
X11	Minimal spared ventral white matter surface (histology, μm^2)

Table 1: Morphometric parameters.

Table 3 shows that all parameters statistically significantly predicted both the initial locomotor deficit, as measured by the BBB scale at 4 days post-operatively, and the evolution on the BBB scale until reaching the maximal score of 21 (i.e., the slope of the score curve).

Figures 18 and 19 show the extent of the lesion, as illustrated by the ratio of the histological lesion surface measurement over the total cord surface. Among the five studied rats, the graphical correlation with the degree of recovery is, again, excellent; however, for the correct interpretation of this parameter, total cord atrophy (see above) has to be taken into account.

Parameter	3230	3226	3238	3243	3235
X1	0.96	0.90	0.49	0.48	0.25
X2 (PD8)	23027520000	27152128000	16655648000	11874432000	7398400000
X3 (PD32)	22334198040	22387065120	15445760400	10740590280	5126677920
X4(H)	11787767193	14918652254	7062439171	8053691658	2897332688
H/PD8	0.51	0.55	0.42	0.68	0.39
H/PD32	0.53	0.67	0.46	0.75	0.57
X5	529	406	770	843	1180
X6	0.26	0.24	0.36	0.40	0.48
X6'	0.42	0.34	0.50	0.59	0.65
X7	50	165	466	639	1334
X8	50	221	671	904	1671
X9	95253	149575	419097	565030	1947274
X10	95253	149575	1363826	1689445	2700742
X11	38926	80548	254782	389082	1356914

Table 2: Morphometric data. "H"=histology; "PD8" and "PD32": low (nt=8) and high (nt=32) resolution PD MRI, respectively. "X1": lesion/cord ratio (histology); "X2" to "X4": lesion volumetry in μm^3 (measured directly (histology) or calculated from pixel size (PD MRI); "X5": minimal cord surface (T2, pixels); "X6": "atrophy ratio" (minimal cord surface/maximal cord in the same cord); "X6' ": normalised atrophy ratio (atrophy ratio divided by the highest cord/canal surface ratio in the same rat); "X7"=minimal ventral white matter sparing (PD32, pixels); "X8"=minimal white matter sparing (PD32, pixels); "X9"=minimal white matter sparing (histology, μm^2); "X10"=minimal total matter sparing (histology, μm^2); "X11"=minimal ventral white matter sparing (histology, μm^2).

Variable	Initial BBB score	BBB score slope
X1	$p<0.0009$	$p<0.0001$
X2	$p<0.0013$	$p<0.0001$
X3	$p<0.0011$	$p<0.0001$
X4	$p<0.0033$	$p<0.0001$
X5	$p<0.0024$	$p<0.0001$
X6	$p<0.0012$	$p<0.0001$
X6'	$p<0.0016$	$p<0.0001$
X7	$p<0.0033$	$p<0.0001$
X8	$p<0.0022$	$p<0.0001$
X9	$p<0.0100$	$p<0.0001$
X10	$p<0.0012$	$p<0.0001$
X11	$p<0.0098$	$p<0.0001$

Table 3: Statistical significance of the different morphometric parameters.

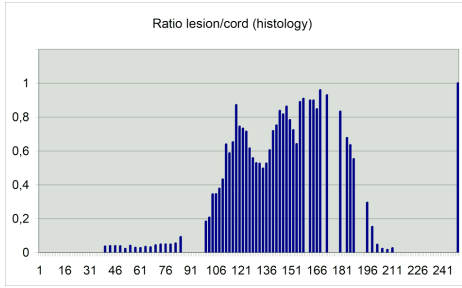


Figure 18: Histology. Ratio of the lesion surface over the total cord surface. This parameter does not directly reflect the amount of spared matter, as the ratio is influenced by the degree of cord atrophy: with the same ratio, there will be a greater absolute amount of spared matter in a larger cord than in a thinner cord.

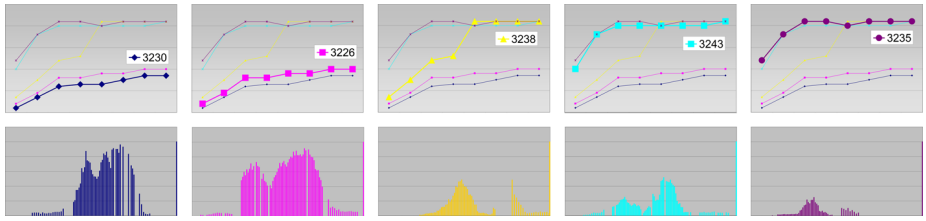


Figure 19: The lesion/cord ratio in each histological section. The BBB evolution is correlated with the relative lesion extent over the section. However, this figure also illustrates the importance of taking cord atrophy into account when interpreting recovery using this ratio. E. g., the maximal lesion/cord ratio in rats n° 3238 and 3243 is very similar (approximately 50%); the behavioural recovery is however much more rapid in rat n°3243; in the latter, the cord atrophy was inferior to that of rat n° 3238, and, thus, the total amount of residual tissue was higher.

Publication List

The pdf files of the published articles can be found on the CD.

Publication (1)

Multon et al, Journal of Neurotrauma, 2003 Aug;20(8):699–706

The effect of treadmill training on motor recovery after a partial spinal cord compression-injury in the adult rat.

Publication (2)

Poirrier et al, Journal of Neuroscience Research, 2004 Jan 15;75(2):253–61

Repetitive transcranial magnetic stimulation improves open field locomotor recovery after low but not high thoracic spinal cord compression-injury in adult rats.

Publication (3)

Scholtes et al, Neurosurgery, 2006, Sep;59(3):671–678; "Editor's Choice".

Correlation of postmortem 9.4 tesla magnetic resonance imaging and immunohistopathology of the human thoracic spinal cord 7 months after traumatic cervical spine injury.

Publication (4)

Scholtes et al, submitted to Journal of Neuroscience Methods

Rapid, *post-mortem* 9.4T MRI of spinal cord injury: correlation with histology and survival times.

Publication (5)

Scholtes et al, submitted to BMC Neuroscience

Post-mortem 9.4 tesla magnetic resonance imaging after experimental incomplete spinal cord injury in the rat: morphometric and anatomic correlates of spontaneous behavioural recovery.