DEPARTMENT OF NEUROSCIENCE
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Experimental spinal cord injury

Methodological and neuroimmunological contributions with some historical background

Matthew Joseph Fraidakis, MD

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Dedicated to my parents

”Plato is my friend, but the truth is also my friend.
If I had to choose between the two,
I would opt for the truth.”

Alexandros of Tralles*

Byzantine Greek physician from Tralles in Minor Asia and brother of *Anthemius of Tralles*, the geometer and famous architect of the magnificent St.Sophia Church in Constantinople. (*)

ABSTRACT

Spinal cord injury (SCI) is an incurable neurotraumatic catastrophe that afflicts mostly young individuals with resultant functional impairment of varying degrees of severity. The single pharmacologic treatment option at present is systemic methylprednisolone administration within 8 hours postinjury oftentimes accompanied by neurosurgical interventions. As a rule, SCI becomes a chronic condition with significant handicap for the patient and socioeconomic repercussions for the affected families and health care system. Important discoveries in the field of central nervous system regeneration since the early 80's have led to diverse potential therapeutic approaches for neuroprotection and repair. Unfortunately, most envisioned treatment apporaches would only be applicable at the acute and subacute stages of SCI, thereby excluding the large patient base with chronic SCI. In the first part of this thesis work the methodological aspects of a neurosurgical treatment protocol in a rat model of acute and chronic SCI were explored. In complete spinal cord transection experiments in rat, the acute and chronic spinal cord lesions were characterized with high-resolution magnetic resonance technology. A microneurosurgical 'repair' protocol was employed in both acute and chronic (at 2, 4 or 8 months postinjury) SCI. Behavioral evaluation of the operated animals with standard locomotor behavior tests and two novel behavioral tests, developed by the author, the Bipedal test and the Head-scratch test, demonstrated a statistically significant recovery for those animals that were subjected to the microsurgical reconstruction protocol. Partial functional recovery and histologically verifiable axonal regeneration was achieved in rats with both acute and chronic SCI. In the second part of this thesis work the neuroinflammatory and neuroimmunological correlates of peripheral and central nervous system injury were studied in mice. In one set of KO mice (TNFa, STAT4, STAT6) and their corresponding wild type controls, behavioral recovery and axonal regeneration were evaluated after spinal cord overhemisection. In another set of KO mice (STAT4, STAT6, IFNγ, IFNγR and IRF1) and their corresponding wild type controls inflammatory and glial cell reactions were assessed after unilateral facial nerve transection lesions. The results suggest a positive role for the T_H2 subset of the adaptive immune response in anatomic recovery after SCI. Finally, in this thesis work the historical origins of the 'inhibitory white matter hypothesis' were researched shedding light on the pioneering work of Lugaro. Future treatments will have to address the complexity of SCI with a multipronged approach in order to effect the appropriate type and degree of immunomodulation, achieve neuroprotection, and promote collateral sprouting and axonal regeneration ultimately resulting in tissue repair and functional recovery. This thesis suggests: 1) that complete, long-standing SCI can be amenable to therapy by demonstrating that the functional incapacity of experimental chronic paraplegia in rat is partially reversible, and 2) that judicious modulation of the immune response after SCI may have a role to play in axonal regeneration after SCI.

LIST OF PUBLICATIONS

The following papers are included in this thesis and will be referred to by their Roman numerals:

I Cheng, H., **Fraidakis, M.**, Blombäck, B,, Lapchak, P., Hoffer, B., Olson, L. Characterization of a fibrin glue-GDNF slow-release preparation. *Cell Transplantation* **1**:53-61 (1998).

II **Fraidakis, M.**, Klason, T., Cheng, H., Olson, L., Spenger, C. High-resolution MRI of intact and transected rat spinal cord. *Experimental Neurology* **153**:299-312 (1998).

III Lidman, O., **Fraidakis, M.**, Lycke, N., Olson, L., Olsson, T., Piehl, F. Facial nerve lesion response: strain differences but no involvement of IFN-γ, STAT4 or STAT6. *Neuroreport* **13**:1589-1593 (2002).

IV **Fraidakis, M.J.**, Spenger, C., Olson, L. Partial repair of chronic paraplegia in rat. *Experimental Neurology* **188**:33-42 (2004).

V **Fraidakis, M.J.**, Kiyotani, T., Pernold, K., Bergström, J., Olson, L. Functional recovery and axonal regeneration after spinal cord injury in TNF, STAT4 and STAT6 KO mice. *Neuroreport* **18**:185-189 (2007).

VI **Fraidakis, M.J.** Lugaro's forgotten legacy: the hypothesis of negative neurotropism. *Journal of the History of the Neurosciences* **19**:239-252 (2010).

CONTENTS

ABBREVIATIONS

5-HT *5-H*ydroxy*T*ryptamine
AAD *A*cute *A*xonal *D*egeneration
AANS/CNS *A*merican *A*ssociation of *N*eurological *S*urgeons/*C*ongress of *N*eurological *S*urgeons
AD *A*lzheimer's *D*isease
ADEM *A*cute *D*isseminated *E*ncephalo*M*yelitis
AFC *A*ntibody *F*orming *C*ells
AIDP *A*cute *I*nflammatory *D*emyelinating *P*olyradiculoneuropathy
AIS *A*SIA *I*mpairement *S*cale
ALS *A*myotrophic *L*ateral *S*clerosis
AMPA DL-alpha-*A*mino-3-hydroxy-5-*M*ethylisoxazol-*P*ropionic *A*cid
APC *A*ntigen *P*resenting *C*ells
ARDS *A*dult *R*espiratory *D*istress *S*yndrome
ASIA *A*merican *S*pinal *I*njury *A*ssociation
BBB *B*lood-*B*rain *B*arrier
BBB *B*asso-*B*eattie-*B*resnahan score
BDNF *B*rain *D*erived *N*eurotrophic *F*actor
BNB *B*lood-*N*erve *B*arrier
BT *B*ipedal *T*est
CBS *C*ombined *B*ehavioural *S*core
CD *C*luster of *D*ifferentiation
CDR *C*omplementarity *D*etermining *R*egion
CIDP *C*hronic *I*nflammatory *D*emyelinating *P*olyradiculoneuropathy
CNS *C*entral *N*ervous *S*ystem
CNTF *C*iliary *N*euro*T*rophic *F*actor
CP *C*ontract *P*lacing
CPG *C*entral *P*attern *G*enerator
CSF *C*erebro*S*pinal *F*luid
CSPG *C*hondroitin *S*ulphate *P*roteo*G*lycan
CST *C*ortico*S*pinal *T*ract
CT *C*omputer-assisted *T*omography
CT1 *C*ardio*T*rophin1
CTL *C*ytotoxic *T L*ymphocyte
DREZ *D*orsal *R*oot *E*ntry *Z*one
DRG *D*orsal *R*oot *G*anglia
DSD *D*etrusor-*S*phincter *D*yssynergia
DTI *D*iffusion *T*ensor *I*maging
DVT *D*eep *V*ein *T*hrombosis
EAE *E*xperimental *A*llergic (or *A*utoimmune) *E*ncephalomyelitis
EAM *E*xperimental *A*utoimmune *M*yasthenia
EAN *E*xperimental *A*utoimmune *N*euritis
ECM *E*xtra*C*ellular *M*atrix
FDA *F*ederal *D*rug *A*dministration
FES *F*unctional *E*lectric *S*timulation

FGF	*F*ibroblast *G*rowth *F*actor
FLAIR	*FL*uid *A*ttenuation *I*nversion *R*ecovery
FG	*F*luoro*G*old
FR	*F*luoro*R*uby
GDNF	*G*lial cell *D*erived *N*eurotrophic *F*actor
GFAP	*G*lial *F*ibrillary *A*cidic *P*rotein
GM-CSF	*G*ranulocyte *M*onocyte-*C*olony *S*timulating *F*actor
HLA	*H*uman *L*eucocyte *A*ntigen
HSP	*H*eat *S*hock *P*rotein
IFN	*I*nter*F*ero*N*
IGF1	*I*nsulin-like *G*rowth *F*actor 1
IL	*I*nter*L*eukin
IL1R	*I*nter*L*eukin *1 R*eceptor
iNOS	*i*nducible *N*itric *O*xide *S*ynthase
IRF1	*I*nterferon *R*egulatory *F*actor 1
JNK	*J*un *N*-terminal *K*inases
KO	*K*nock-*O*ut
LCMV	*L*ymphocytic *C*horio*M*eningitis *V*irus
LEMS	*L*ower *E*xtremity *M*otor (ASIA) *S*core
LIF	*L*eukæmia *I*nhibitory *F*actor
LMEM	*L*inear *M*ixed *E*ffects *M*odel
LPS	*L*ipo*P*oly*S*accharide (or *Endotoxin*)
LRR	*L*eucine *R*ich *R*epeat
MAG	*M*yelin-*A*ssociated *G*lycoprotein
MAI	*M*yelin-*A*ssociated *I*nhibitor
MBP	*M*yelin *B*asic *P*rotein
M-CSF	*M*onocyte-*C*olony *S*timulating *F*actor
MEMRI	*M*anganese-*E*nhanced *MRI*
MEP	*M*otor *E*voked *P*otentials
MHC	*M*ajor *H*istocompatibility *C*omplex
MOG	*M*yelin *O*ligodendrocyte *G*lycoprotein
MP	*M*ethyl*P*rednisolone
MRI	*M*agnetic *R*esonance *I*maging
MS	*M*ultiple *S*clerosis
NACSIS	*N*ational *A*cute *S*pinal *C*ord *I*njury *S*tudy
NAD	*N* icotinamide *A*denine *D*inucleotide
NGF	*N*erve *G*rowth *F*actor
NgR	*Nog*o *R*eceptor
NK	*N*atural *K*iller cells
NMDA	*N-M*ethyl-D-*A*spartate
NT	*N*euro*T*rophin
OECs	*O*lfactory *E*nsheathing *C*ells
OMgp	*O*ligodendrocyte *M*yelin *g*lyco*p*rotein
PNS	*P*eripheral *N*ervous *S*ystem
PAF	*P*latelet *A*ctivating *F*actor
PAMPs	*P*athogen *A*ssociated *M*olecular *P*atterns
PCD	*P*rogrammed *C*ell *D*eath
PD	*P*arkinson's *D*isease

PE	*P*ulmonary *E*mbolism
PG	*P*rosta*G*landin
PLP	*P*roteo*L*ipid *P*rotein
PPCM	*P*rogressive *P*ost-traumatic *C*ystic *M*yelopathy
PPMM	*P*rogressive *P*ost-traumatic *M*yelomalacic *M*yelopathy
QST	*Q*uantitative *S*ensory *T*esting
RARE	*R*apid *A*cquisition *R*elaxation *E*nhancement
ROI	*R*eactive *O*xygen *I*ntermediates
RNI	*R*eactive *N*itrogen *I*ntermediates
RTN	*Re**T**i*culo*N*e
SAC	*S*pace *A*vailable for the *C*ord
SCBF	*S*pinal *C*ord *B*lood *F*low
SCI	*S*pinal *C*ord *I*njury
SCID	*S*evere *C*ombined *I*mmuno*D*eficiency
SCIWORA	*S*pinal *C*ord *I*njury *W*ithout *R*adiographic *A*bnormalities
SCIWORET	*S*pinal *C*ord *I*njury *W*ithout *R*adiographic *E*vidence of *T*rauma
SD	*S*prague *D*awley
SIRS	*S*ystemic *I*nflammatory *R*espons*e* *S*yndrome
SLE	*S*ystemic *L*upus *E*rythematosus
SSEP	*S*omato*S*ensory *E*voked *P*otentials
STAT	*S*ignal *T*ransducers and *A*ctivators of *T*ranscription
TBI	*T*raumatic *B*rain *I*njury
TCR	*T C*ell *R*eceptor
$\boldsymbol{T_{EFF}}$	*T* *EFFECTOR* cells
TGF	*T*ransforming *G*rowth *F*actor
$\boldsymbol{T_{H}}$	*T* *H*ELPER cells
TIR	*T*oll/*I*L1R homologous *R*egions
TLR	*T*oll-*L*ike *R*eceptor
$\boldsymbol{T_{REG}}$	*T* *REGULATORY* cells
$\boldsymbol{iT_{REG}}$	*i*nducible T *REGULATORY* cells
$\boldsymbol{nT_{REG}}$	*n*atural T *REGULATORY* cells
TNF	*T*umor *N*ecrosis *F*actor
UEMS	*U*pper *E*xtremity *M*otor (ASIA) *S*core
UTI	*U*rinary *T*ract *I*nfections
VCAM	*V*ascular *C*ell *A*dhesion *M*olecule
VIP	*V*asoactive *I*ntestinal *P*eptide
WT	*W*ild *T*ype
ZPP	*Z*one of *P*artial *P*reservation

HISTORICAL BACKGROUND

On ne connaît bien une science que lorsqu'on en connaît l'histoire. *Auguste Comte*

The first medical record of spinal cord injury (SCI) as a morbid condition with a grave prognosis can be retrieved from an egyptian papyrus acquired by the British egyptologist Edwin Smith in Luxor in 1862, and thus christened the *Edwin Smith Surgical Papyrus* (Breasted, 1930; Clagett, 1989). Its content is believed to have been contributed by at least three authors who lived between the Third (c. 3000-2500 B.C.) and Twelfth Dynasties (around 1650 B.C.), the first of which presumably being ***Imhotep***, a high priest of the Old kingdom era whose legend had grown so much with the centuries that by the time Egypt came under Greek rule (4^{th} century B.C.) he was worshiped as god (Caton, 1904; Garry, 1932; Forbes, 1940; Sigerist, 1961; Clagett, 1989). Therein diseases or injuries are classified as ailments 'to be treated', 'to contend with' or 'not to be treated', with spinal cord injury unequivocally deemed among the latter (Breasted, 1930). Treatable spinal injuries are described as well. In case 29, bandage with a piece of fresh meat is offered as treatment to a 'gaping wound in a cervical vertebra', while in case 30 a bandage regimen of fresh meat and honey is recommended for treating 'a sprain in a cervical vertebra'. In both figure cases the spinal cord was spared. But in case 31, a case of dislocation of a cervical vertebra, one reads (Breasted, 1930):

> If you examinest a man having a dislocation in a vertebra of his neck, shouldst thou find him unconscious of his two arms and his two legs on account of it, while his phallus is erected on account of it, and urine drops from his member without his knowing it; his flesh has received wind; his two eyes are bloodshot; it is a dislocation of a vertebra of his neck extending to his backbone which causes him to be unconscious of his two arms and two legs. If, however, the middle vertebra of his neck is dislocated, it is an emissio seminis which befalls his phallus. Thou shouldst say concerning him: "One having a dislocation in a vertebra of his neck, while he is unconscious of his two legs and his two arms and his urine dribbles. An ailment not to be treated".

Finally, in case 33, the same dismal prognosis awaits someone whose 'falling head downward has caused that one vertebra crush into the next one' (Breasted, 1930). This is definitely the first description of a *burst fracture* of the spine.

Homer offers many examples of violent deaths in *Iliad*, some of which must have certainly led to penetrating spinal cord injury, but it is in *Odysseia* that we find the first documentation in greek literature of traumatic, nonpenetrating fatal spinal cord injury in the intriguing story of Elpenor's death (Homer, 1967; Warren, 1970):

> A youth there was, Elpenor was he nam'd,
> Nor much for sense, nor much for courage fam'd;
> The youngest of our band, a vulgar soul
> Born but to banquet, and to drain the bowl.
> He, hot and careless, on a turret's height
> With sleep repair'd the long debauch of night:
> The sudden tumult stirr'd him where he lay,
> And down he hasten'd, but forgot the way;
> Full endlong from the roof the sleeper fell,

And *snapt the spinal joint*, and walk'd in hell.

The old egyptian aphorisms might have been known to the first Greek founders of medicine as an empirical science during the 6^{th}-3^{rd} centuries B.C. As far as we know, ***Alcmæon*** of Croton in Magna Græcia (6^{th}-5^{th} centuries B.C.), philosopher and physician, was the first to acknowledge the brain as the 'command centre' of the human body (Soury, 1899):

... εν τω εγκεφαλω ειναι το ηγεμονικον. (Alcmæon, *Fragm.*)
[*transl.* … the governing faculty is in the brain]

... Αλκμαιων την κεφαλην, εν η εστι το ηγεμονικον. (PseudoPlutarch, *Placita V*)
[*tansl.* …according to Alcmæon the command center is in the head]

He believed it to be the centre of sensation and mentation, yet strongly insisting on the distinction between these two (Soury, 1899).

...ως ετερον ον το φρονειν και το αισθανεσθαι. (Theophr., *De sensu et sensibil.*)
[*transl.* ...to think is different than to feel]

He also believed the brain to be intimately linked to the sensory organs through open channels which he called 'πορoι' (*gr.* πορος: hollow channel, passage), what we now call the *sensory nerves*. [In greek texts, from Homer's epic poetry through the medical writings of Alcmæon, Hippocrates, Herophilus, Erasistratus, Celsus, Aretæus, Galenus and even Rufus from Ephesos, the term 'nerve' (*gr.* νευρον) referred to or encompassed the 'tendons'.] These 'πορoι' conveyed the external stimuli to the brain (Soury, 1899; Codellas, 1932; Souques, 1936).

... απασας δε τας αισθησεις συνηρτησθαι προς τον εγκεφαλον...επιλαμβανεσθαι γαρ τους πορους δι' ων αι αισθησεις. (Theophr., *De sensu et sensibil.*) [*transl.* … all senses are connected with the brain…since the passages through which the sensations (pass) are occupied.]

But Alcmæon was by no means only a theoretician, but the first Greek medical experimentalist who performed the earliest recorded dissections –and possibly vivisections– and attributed disease to organic causes affecting parts of the body, such as the blood, the *spinal cord* or the brain (Soury, 1899; Souques, 1936).

...αιμα η μυελον η εγκεφαλον. (Alcmæon, *Fragm)* [*transl.* …blood or spinal marrow or brain]

He also wrote the first book on Physiology, "On Nature", much admired by Aristotle and his disciple Theophrastus (McHenry, 1969; Lloyd, 1970). Alcmæon's *cerebrocentric* view of the brain were very probably transmitted by Anaxagoras (500-428 B.C.), a confrère of Socrates, to ***Hippocrates*** of Cos (c. 469-379 B.C.) and from him to his disciples (Lloyd, 1970). The Hippocratic theory that when disease afflicts the brain it leads to disorders such as paralysis and epilepsy was radical for a time and culture where demons provided a perfect alibi for the theodicy of priests, the incompetence of charlatans

and the ignorance of the common man. In the *Corpus Hippocraticum* one reads (Hippocrates, ed. 1952, 1988):

> Men ought to know that from nothing else but the brain come joys, delights, laughter and sports, and sorrows, griefs, despondency, and lamentations. And by this, in an especial manner, we acquire wisdom and knowledge, and see and hear and know what are foul and what are fair, what are bad and what are good, what are sweet, and what are unsavory…And by the same organ we become mad and delirious, and fears and terrors assail us… All these things we endure from the brain.

Hippocrates and his students were astute clinicians. They were also skilled amateur neurosurgeons who practised *trephination* judiciously, used various techniques and designed *fine tools* that would spare the meninges during the process (Hippocrates, ed. 1952, 1988; Arnott *et al.*, 2002; Kshettry *et al.*, 2007). Indeed, they went to great lengths to assure that the *diploë* was fractured before burring a hole on the cranium, by rubbing on it a special black ointment that would penetrate and expose any cracks when wiped off (Hippocrates, ed. 1952, 1988; Thomson, 1938; Majno, 1975). They were also aware that children were more susceptible to skull trauma and knew the reasons for it. As we can read in the treatise *On Injuries of the Head* their practical skills were advanced (Hippocrates, ed. 1952, 1988):

> … if you cannot discover whether the bone be broken, contused, or both the one and the other, nor can see the truth of the matter, you must dissolve the jetblack ointment, and fill the wound with it when this dissolved, and apply a linen rag smeared with oil, and then a cataplasm of the *maza* with a bandage; and on the next day, having cleaned out the wound, scrape the bone with the *raspatory*. And if the bone is not sound but fractured and contused, the rest of it which is scraped will be white; but the fracture and contusion, having imbibed the preparation, will appear black, while the rest of the bone is white. And *you must again scrape more deeply the fracture where it appears black*; and if you thus remove the fissure, and cause it to disappear, you may conclude that there has been a contusion of the bone to a greater or less extent, which has occasioned the fracture that has disappeared under the raspatory; but it is less dangerous and a matter of less consequence when the fissure has been effaced. But if the fracture extend deep, and do not seem likely to disappear when scraped, such an accident requires *trepanning*. But having performed this operation, you must apply the other treatment to the wound… if the flesh which surrounds the wound be ill cured… it gets into a febrile state, and becomes much inflamed… but the wound should be made to suppurate as quickly as possible; for, thus the parts surrrounding the wound would be the least disposed to inflammation… The bones of children are thinner and softer, for this reason, that they contain more blood [than those of the adults]; and they are porous and spongy, and neither dense nor hard. And when wounded to a similar or inferior degree by weapons of the same or even of an inferior power, the bone of a young person more quickly suppurates, and that in less time than the bone of an older person; and in acccidents which are to prove fatal, the younger person will die sooner than the elder… With regard to trepanning, when there is a necessity for it, the following particulars should be known… you must not at once saw the bone to the meninx; for it is not proper that the membranes should be laid bare and exposed to injuries for a length of time, as in the end it may become fungous. And there is another danger if you saw the bone down to the meninx and remove it at once, lest in the act of sawing you should wound the meninx. And if you wish to saw at once down to the membrane, and then remove the bone… you must saw the bone … with a *serrated trepan*, and in doing so must frequently take out the trepan and examine with a *sound* [specillum] and otherwise along the tract of the instrument… you must take care where you apply the trepan, and see that you do so only where the bone appears to be particularly thick… but if you use a *perforator* you must not penetrate to the membrane… but must leave a thin scale of bone…

By account of historical record, the Hippocratics were also the first to hint at a crossed somatotopic brain representation, a rather straightforward conclusion they drew while

treating patients who had suffered a depressed skull fracture with ensuing contralateral paralysis and/or contralateral seizures (Hippocrates, ed. 1952, 1988; Sigerist, 1961; Clarke, 1963; Edelstein, 1967; Lockhorst, 1982, 1996). In fact, they were first to describe the 'right hemiplegic-aphasic syndrome' as a result of injury to the left hemisphere (Vinken and Bruyn, 1969; Lockhorst, 1982, 1996; Vulliemoz *et al.*, 2005). To quote again from the *Corpus Hippocraticum* (Hippocrates, ed. 1952, 1988, *On Injuries of the Head*):

> When a person has sustained a mortal wound on the head, which cannot be cured, nor his life preserved, you may form an opinion of his approaching dissolution, and foretell what is to happen from the following symptoms which a person experiences… And, for the most part, convulsions seize the other side of the body; for, if the wound be situated on the left side, the convulsions will seize the right side of the body; or if the wound be on the right side of the head, the convulsion attacks the left side of the body. And some become *apoplectic*.

As regards the spinal column, Hippocrates gave impressive descriptions of its anatomical structure, was aware of the fine differences between vertebræ from different spinal levels, and devoted many fabulous paragraphs on treatments of gibbosity, vertebral fractures and vertebral dislocations. Hippocratics pioneered the medical treatment of spinal injuries and made use of two different traction benches, the *Hippocratic ladder* and the *Hippocratic board* or contraptions that applied pressure on the back of the patient such as the *scamnum*, in order to reduce vertebral fractures and fracture dislocations and treat spinal deformities (Loeser, 1970; Sanan and Rengachary, 1996). Hippocrates also described how certain fractures with displacement of the vertebral body could be deadly or lead to paralysis because of the accompanying "spinal marrow" damage as opposed to uncomplicated spinous process or other vertebral fractures that have a milder prognosis (Hippocrates, ed. 1952, 1988). In *On the articulations* we read (Hippocrates, ed. 1952, 1988):

> In the first place, the structure of the spine should be known, for this knowledge is requisite in many diseases. Wherefore, on the side turned to the belly the vertebræ are in a regular line, and are united together by a pulpy and nervous band of connection, originating from the cartilages, and extending to the spinal marrow. There are certain other nervous cords which decussate [crossed ligaments], are attached [to the vertebræ] and are extended from both sides of them… On the opposite side the vertebræ are connected together by a *ginglymoid* articulation. Common cords [ligaments] are extended to all parts, both those within and those without. There is an ossseous process from the posterior party of all and each of the vertebræ, whether greater or smaller; and upon these processes there are cartilaginous epiphyses… The ribs are united to them [to the vertebræ and their processes], having their heads inclined rather to the inside than the out, and everyone of them is articulated with the vertebræ… The space between the ribs and the processes of the vertebræ is filled on both sides by muscles, which arise from the neck and extend to the loins… The spine longitudinally, is a straight line slightly curved; from the os sacrum… the spine inclines backward, for the bladder, the organs of generation, and the loose portion of the rectum, are situated there [sacral cyphosis]. From this, to the attachment of the diaphragm, the spine inclines inward, and this portion alone, from the internal parts, gives origin to the muscles, which are called *psoæ* [lumbar lordosis]. From this to the great vertebra which is above the top of the shoulders [C7], it is convex behind lengthways [thoracic cyphosis]; but it is more in appearance than it really is, for the spinous processes are highest in the middle [thoracic zone], and less so above and below. The region of the neck is convex before [cervical lordosis]… And the spinal marrow would suffer, if from the displacement of the vertebræ it were to be bent even to a small extent; for the displaced vertebra would compress the spinal marrow, if it did not break it; and if compressed and strangled, it would induce insensibility of many great and important parts, so that the physician need not give himself any concern about rectifying the displacement of the vertebra, accompanied as it is by many

other ill consequences of a serious nature… On the main, it should be known, respecting the accidents that I have briefly described, that diplacements forward are of fatal and injurious nature; but that displacements backward, for the most part, do not prove fatal, nor occasion retention of urine nor torpor of the limbs… but displacements forward produce both these bad effects, and many others in addition. And truly they are more apt to lose the power of their legs and arms, to have torpor of the body, and retention of urine, those who experience no displacement either forward or backward, but merely a violent concussion along the spine, while those who have displacement backward are least subject to these symptoms.

And in *Instruments of reduction* it is reiterated (Hippocrates, 1952, 1988):

Displacement of the spine, if inward, threatens immediate death, attended with retention of urine and loss of sensibility. Outward, the accident is free from most of these bad effects, much more so when there is merely concussion without displacement; the effects in the former case being confined to the spot affected, whereas in the latter they are further communicated to the whole body, and are of a mortal caharacter… Displacements [of the vertebræ] from a fall rarely admit of being rectified, and those above the diaphragm are most difficult to rectify.

Herophilus of Chalcedon in Asia Minor (c. 335/325-280/255 B.C.), a graduate of the Hippocratic school of Cos, who worked in Ptolemaic Alexandria and became a pioneer anatomist and neuroanatomist of antiquity, is rightly considered to be the Father of Anatomy –as Hippocrates is the Father of Medicine– with Erasistratus of Keos, a close second (Finlayson, 1893; Dobson, 1925, 1927; von Staden, 1989). It is believed that they were the only physicians to perform human dissections until the Rennaissance –in fact Galen might have had access to a corpse or two. Herophilus, who, thanks to the lax regulations of the enlightened Ptolemies on cadaveric dissections, might have performed more than hundred dissections according to his own account, was the first to anatomically view the human nervous system *holistically*, comprised of the brain (*gr*. εγκεφαλος, i.e. the cerebrum, its ventricles and the cerebellum), the spinal cord, their coverings –of which he identified the dura mater and the pia mater– and the cranial and other peripheral nerves (Finlayson, 1893; Dobson, 1925, 1927; Singer, 1957; Clarke and O'Malley, 1968; McHenry, 1969; Lloyd, 1973; von Staden 1989). As the forefather of neurophysiology, he distinguished sensory from motor nerves, described parts of the eye and understood the function of the optic nerve, which, as far as we know, Alcmæon had been the first to dissect more than two centuries before Herophilus (Finlayson, 1893; Dobson, 1925; Singer, 1957; McHenry, 1969; Lloyd, 1973; von Staden 1989). Moreover, since Herophilus knew that the motor nerves arose from the brain and spinal cord, he implied a centrifugal flow of command mediated by the peripheral nerves resulting in *voluntary motion* (McHenry, 1969; von Staden 1989):

... the neura [nerves] that make voluntary movement possible have their origin in the cerebrum and spinal cord. (Rufus Ephesius, *De anatomica partium hominis*)

Arguably, Herophilus' understanding of the neurophysiology of motility was extraordinary for his time.

For his part, ***Erasistratus*** (c. 310-250 B.C.) was virtually the Father of Comparative Neuroanatomy who, while also working in Alexandria, brilliantly correlated cerebellum size to ability for speed and cerebral surface convolution complexity to intelligence

(Finlayson, 1893; Dobson, 1927; Perrin, 1958; Keele, 1961; McHenry, 1969; Lloyd, 1973). As of the latter achievement, we should add that the brain 'coils', i.e. the cortical convolutions (the *gyri*), were first described by ***Praxagoras*** of Kos (c. 340 B.C.), a contemporary of Aristoteles, also from the Hippocratic school and a teacher of Herophilus, but it was Erasistratus (c. 260 B.C.) who speculated on their significance in the mental faculties and, rather inopportunely, noted their resemblance to the intestinal coils (Cardwell, 1905, 1906; Pearce, 2003). This hypothesis would reemerge thanks to Thomas Willis and his dissections of fish, birds and various mammals, only 2000 years later! Erasistratus was also a proponent of the *theory of pneuma*, i.e. that *pneuma* (i.e. air of variable temperature; *gr.* πνευμα<πνεω: to blow) flowed through the nerves to and fro the ventricles, a position also held by other notable Greek physicians, such as ***Philistion*** from Locris and ***Diocles*** from Karystos, contemporaries of Hippocrates and Plato, respectively (Moon, 1923a, 1923b). ***Athenæus*** from Attaleia, a Greek physician who worked in Rome in the 1st century A.D., founded his own school of Pneumatists by slightly modifying Erasistratus' notion of *pneuma* (Moon, 1923a, 1923b; Quin, 1993). The general theory* of *pneuma*, is not just a historical footnote and not irrelevant to our subject, as will be seen later, but the *first theory of brain function*, and the primordium from which the science of modern neurophysiology sprang two millenia later. Indeed, it held for a longer time than the teachings of Aristoteles or Galenus.

It was the Greek master physician ***Galen*** (gr. *Γαληνος*, c. 129/130-200 A.D., or *Clarissimus* [Lat. glorious] Galenus for the revering Romans) of Pergamon in Asia Minor, working in Alexandria, Pergamon and Rome, who first experimented with various cordotomy lesions in apes, among many other animals which fell to his scrutiny, namely donkeys, cattle, sheep, pigs, cats, dogs, weasels, monkeys of many kinds, and even an elephant (Prendergast, 1930). However, the Barbary ape was his favorite experimental subject due to its close anatomical and *behavioral* resemblance to man. This is why Vesalius was deceived for quite a long time before he finally understood the reason why his own findings, which derived from human cadaveric dissection, often differed from Galen's (Singer, 1952; Cushing, 1962). Galen's anatomical knowledge of the central and peripheral structures of the nervous system was astonishing and it was he who introduced the concepts of dermatomyotomal innervation and topographical diagnosis ('localization') in clinical neurology (Kempf, 1904; Walsh, 1927; Singer, 1949; Sarton, 1954; Galen, ed. 1962). Much of this knowledge he acquired by performing cordotomy experiments, i.e. sectioning the cord at different levels and depths, and correlating spinal level of injury to specific neurological outcome, somatosensory and vegetative (Kempf, 1904; Galen, ed. 1962, 1968, 1973, 1976; Creutz, 1931; Sarton, 1954; Singer, 1949, 1956, 1957; Lloyd, 1973). He also performed cord hemisections and observed the homolaterality of the functional deficit (Kempf, 1904; Sarton, 1954; Galen, ed. 1976). As mentioned before, the Hippocratics had observed the crossed symptomatology after brain trauma. Galen, who never lost an opportunity to laud Hippocrates, further established by clinical observation and animal experimentation the decussation of sensorimotor pathways in his effort to explain the pathophysiology of "apoplexy" [**gr.* αποπληξια; a rather broad term already in use in the *Corpus Hippocraticum* referring to either paralysis of

the whole body, obviously accompanied by loss of consciousness, or paralysis of members of the body, with or without loss of consciousness, its modern day equivalent being *stroke* –a term with a narrower significance] or cerebral trauma and the resultant contralateral paralysis and/or postapoplectic convulsive crises, which begin as partial clonic seizures contralaterally to the brain lesion (Kempf, 1904; Galen, ed. 1973, 1976; Creutz, 1931; McHenry, 1969; O'Leary and Goldring, 1976). He was, moreover, the first to diagnose brachial plexus injury and propose a treatment (Kempf, 1904; Singer, 1949; Sarton, 1954; Galen, ed. 1976). Here are two riveting excerpts from *De locis affectis*, one of his later works composed after 192 A.D. where an excellent differential diagnostic approach is elaborated and a comprehensive symptomatology of spinal cord injury is offered (Galen, ed. 1976):

Watch now how I will explain the therapy which I found by the knowledge of the affected part…A patient who applied some medicine to three fingers of his hand told us that these fingers has already lost all feeling for thirty days, whereas the motion of the fingers had remained intact, and that the applied drug had not helped at all…I searched for the reason why nothing had helped this man by inquiring about the preceding symptoms…and asked whether he had not received a blow in the upper parts [of his spine]. He told me…he had been hit at the upper midriff…after he fell from his carriage on the way to Rome it did not take long before his fingers started to suffer. I could now prove that the next nerve behind the seventh vertebra had become displaced and had maintained an inflammatory scarring condition…Later I told him to stop applying the medicine to the fingers, I put it mainly on the area of the spine which corresponded to the origin of the affected part [i.e. the nerve roots of the brachial plexus]. And so it happened…that the fingers of the hand were cured by the application of medicine to the spine…

In effect, if the spinal cord is completely damaged at the level of a certain vertebra, all parts of the body below become paralyzed. When only one side of the spinal cord has been damaged but the other side remains intacts, only parts exactly corresponding [to the injured side of the cord] are paralyzed. However, if the root of a nerve is affected and the other parts below the damaged area are not involved, only those parts will be paralyzed to which the nerve spreads. When you have learned this exactly you will not harm the affected limbs by neglecting the spine but turn your attention to the affected part and treat it…But those whose spinal nerves become affected require application of the medicine to the vertebral column. When a patient sustains an injury by a fall from a high place or by a severe blow and the spine is seriously affected, an inflammation frequently extends to several organs and damages not only the muscles but also the bladder. Those whose bladder is involved have urinary retention. In some cases not only the urine but also the stools are completely retained, since the intestines are evidently involved.

Among other lasting achievements, Galen maintained the distinction between motor and sensory nerves of Herophilus and generalized it to motor and sensory pathways, which he believed terminated in the cerebellum and cerebrum respectively (Sarton, 1954; Siegel, 1970). He even extended that dichotomy of pathways by describing the autonomic ganglia, the rami communicantes, the sympathetic trunk chain and 'sympathetic pathways' –though Galen's notion of 'sympathy' (as opposed to his notion of 'idiopathy') hardly corresponds to the sympathetic branch of the autonomic nervous system (Sarton, 1954; Siegel, 1970). He also described the *cerebral aqueduct* (πορος) in *De usu partium* (a discovery misappropriated to ***Sylvius***, the Parisian teacher, and later castigator, of Vesalius) and believed the 4th to be the most important of the ventricles for the circulation of *pneuma* (πνευμα ζωτικον; which is indeed true but for the circulation of the cerebrospinal fluid) (Sarton, 1954; Galen, ed. 1968; Quin, 1993; Rocca, 1997). Finally, it is worth mentioning and further testimony to his acumen that he conceived of the capacity

of the cerebrum for change and evolution probably led to this observation by his dissections of different species (Creutz, 1931; Sarton, 1954; Siegel, 1970). Galen understood the capacity of the CNS for change not only phylogenetically but also ontogenetically (in today's terminology *plasticity* and *neuroplasticity*, coined by Lugaro and Minea respectively) since he realized the importance of rehabilitation in the prognosis of CNS injury in general, reflecting a therapeutic attitude in sharp contrast to the aforementioned egyptian aphorism (Lugaro, 1899, 1909; Minea, 1909; Vinken and Bruyn, 1969; Buchtel, 1978; Berlucchi, 2002; Jones, 2004; Berlucchi and Buchtel, 2009; Fraidakis, 2010). His proposals on physiotherapy after 'apoplexy' or speech therapy for aphasics are still valid today (Galen, ed. 1968, 1973, 1976; Creutz, 1931; Vinken and Bruyn, 1969; McHenry, 1969). On the subject of plasticity one is tempted to offer Galen's majesty verbatim from *De usu partium* (Galen, ed. 1968):

In substance the encephalon is very like the nerves, of which it was meant to be the source, except that it is softer, and this was proper for a part that was to receive all sensations, form all images and apprehend all ideas. For a substance easily altered is most suitable for such actions and affections, and a softer substance is always more easily altered than one that is harder. This is the reason why the encephalon is softer than the nerves, but since there must be two kinds of nerve, as I have said before, the encephalon itself was also given a twofold nature, that is, the anterior part is softer than the remaining hard part, which are called *encranium* [cerebrum] and *parencephalis* [cerebellum] by anatomists.

It must be mentioned here that another Greek physician, ***Aretæus*** of Cappadocia (81-138 A.D.), who worked in Syria, Alexandria and Rome, around the time of Galen's prominence, explicitly mentioned the *decussation* of neural pathways and localized it above the cervicomedullary junction (Aretæus, ed. 1997). Aretæus clearly contrasted the contralateral paralysis caused by a cerebral trauma, and the ipsilateral paralysis caused by a spinal or meningeal lesion even at a level as high as the craniovertebral joint (Aretæus, ed. 1997; Hude, 1923; Creutz, 1931; Couch and Couch, 1935; Kudlien, 1963). Aretæus and Galen do not make reference to each other in their writings, so it is possible that they had either reached the same conclusion independently or more likely that they simply carried along in their writings knowledge that was common among greek physicians since the time of Hippocrates, albeit more explicitly (Aretæus, ed. 1997; Hude, 1923; Creutz, 1931; Sarton, 1954). Aretæus writes in (Περι αιτιων και σημειων των χρονιων παθων, Book A, VII) *On the causes and signs of chronic diseases*:

If, now, the head [cranium] suffers at its lowest part, such as at the meninge of the spinal cord, then the homonymous [homolateral] and related parts are paralysed, those on the rightside if [the lesion is] on the right and those on the left side if [the lesion is] on the left. But if the head is afflicted on its right side, then suffer the parts of the left side of the body, and if it is afflicted on its left side then suffer the parts of the right side of the body. The reason for this is the crossing-over [επαλλαγη] of the origins of the nerves; for the nerves on the right do not course on the right side up to their termination, but each of them from its site of origin passes onto the other side, thus crossing-over each other in the form of the letter χ. [*translation from original by MF*]

Aretæus also makes the distinction between the clinical entities "apoplexy", "paralysis", "paresis" and "paraplegia" (Περι αιτιων και σημειων των χρονιων παθων, Book A, VII):

Apoplexy, paraplegia, paresis and paralysis, they are all of the same sort, because they all signify abnormality in movement, tactile sense, or both, and sometimes even the cognitive functions and the other senses. Apoplexy is the paralysis of the whole body, the senses, the intellect and movement… *Paraplegia* is a paralysis of the tactile sense and locomotion of a part of the body, the arm or the leg. Paralysis constitutes mainly a paresis of movement and energy [ενεργειης; here signifying 'strength,capacity for action']. If, though, only the tactile sense is deficient, which is rare, then this is called *anæsthesia*, rather than paresis. [*translation from original by MF*]

This distinction and terminology are largely still in use today, with the exception that the term 'paraplegia' has acquired by consensus a more specific but erroneous meaning, which is not faithful to that imparted by the greek preposition "παρα" ["παρα" does not refer to the lower limbs but rather to parts 'on the side', e.g. 'parasagittal'; "κατα" is the preposition that would carry this meaning, as exemplified by the antonyms 'cataplexy' and 'apoplexy']. Thus, Aretæus' use of the term 'paraplegia' encompasses primarily the current terms 'monoplegia' or 'hemiplegia' (Aretaios, ed. 1997).

The term 'hemiplegia', but again with a different import than today, appears first in the writings of ***Paul*** of Ægina (625-690 A.D.), a famous physician, surgeon and medical writer of the Byzantine era also trained in the Alexandrian school (7th century A.D.). For Paul of Ægina too, 'paraplegia' is equivalent to what neurologists mean today by 'hemiplegia' (Paul of Ægina, ed. 1844; Creutz, 1931). It is also in Paul's work that we find the first mention of the procedure of *nerve suture*, although it must have been practised by earlier Greek physicians (Adams, 1864; Creutz, 1931). It is believed, however, that he was among the first to have practised *decompressive laminectomy* in acute SCI (Paul of Ægina, 1844). Finally, another great Byzantine physician whose writings eloquently treated the subject of spinal cord anatomy and pathology was ***Oreibasius*** (325-403 A.D.). Oreibasius clearly described the effects of lesions at different cord levels and made improvements to the Hippocratic bench (Creutz, 1931; Sanan and Rengachary, 1996). During the Middle ages in Europe, the golden era of Islamic science and medicine and into the first centuries of the Western Renaissance, medical knowledge concerning the spine and spinal cord and management of their injuries barely advanced from the standards of Byzantine medicine.

Many dark eons would pass until the next breakthroughs, at a more fundamental level of investigation this time, through the classic experiments of some of the forefathers of neuroscience. In neuroanatomy, Andreas ***Vesalius*** (1514-1564) improved on the Galenic anatomical foundations simply by masterfully depicting in his *Fabrica* the "human anatomy" that Galen had not had the chance to observe and describe (Singer, 1952). However, he still revered Galen and remained at best an agnosticist when not convinced by a galenic doctrine, for example, the theory of the animal spirits emanating from the ventricular system (Singer, 1952; Cushing, 1962). Vesalius found that the optic and peripheral nerves were not hollow tubes that funneled *pneuma*, as Erasistratus and the later Pneumatists maintained, and though this might have led him to become sceptical on how the animals spirits flow through them to and fro the brain, he did not offer any alternative hypothesis of brain function (Vesalius, 1543; Moon, 1923a, 1923b; Quin, 1993; Singer, 1952). On the contrary, Thomas ***Willis*** (1621-1675), was both a talented

comparative neuroanatomist with a penchant for denominating anatomical structures and at the same time an insightful thinker. It is in *Cerebri Anatome*, which he authored with major contributions from a group of prominent natural scientists from Oxford, the so called *Virtuosi*, that the terms *hemisphere*, *corpus striatum*, *peduncle*, *pyramid* and *reflexion* (from which *reflex* was derived) are first seen (Feindel, 1962; Willis ed. 1965). His greatest legacy is that he pronounced the role of the cerebral parenchyma in brain function instead of the ventricles which he dismissed as ”empty space” (Willis ed. 1965; Isler, 1968). His next major contribution was the distinction of brain parenchyma in gray and white matter and the assumption that each had different functions! Going up against 1500 years of galenic tradition was bold enough, so Willis did not altogether reject the *theory of pneuma*. He still believed that the brain produced animal spirits, only not in the ventricles, and that from the gray matter of the ”cortical spires” through the white matter of the *corpus callosum* and the *striæ* of the *corpus striatum*, they reached the *medulla* and then via the peripheral nerves the muscles and other organs (Feindel, 1962; Willis ed. 1965; Isler, 1968). Considering what Willis meant with ’corpus callosum’ and ’medulla’ (the whole mass of subcortical white matter and everything from diencephalon to hippuris, respectively) his *odology* was not only revolutionary but remarkably ’on the right track’.

In the domain of neurophysiology, Robert ***Whytt*** (1711-1766), the first modern neurophysiologist, described the salient physiological features of muscle fibers. Among other results, he discovered that stimulation of exposed muscle resulted in a *refractory period of excitation* till the muscle could be restimulated (Whytt, 1763, 1768). Luigi ***Galvani*** (1737-1798) and his gifted pupil ***Aldini***, after methodical experimentation on frogs, sheep and even decapitated humans, proposed the existence of an *electric fluid* (“*fluido elettrico*”) that was secreted in the brain and ran centrifugally to the muscles via the nerves (Brazier, 1984). We see here the vestiges of the *theory of the pneuma*, which is indicative of how the notion of pneuma gradually transmogrified from air to fluid to electric current. Presumably, Galvani used the word *fluid* to invoke a medium with the capacities of the old *pneuma*,that by tradition was thought to originate from the ventricles, which of course were later found to be fluid-filled. It is also reminiscent of ***Descartes***’ (1596-1650) iatromechanistic view of the animal body as a *hydraulic system*, inspired, as is believed, by the hydraulic automatons in the royal gardens of St. Germain in Paris (Butterfield, 1949). The inspired experiments of ***Bell*** (1774-1842) and ***Magendie*** (1783-1855), finally and conclusively confirmed Herophilus’ hypothesis of the existence of separate, structurally and functionally, motor and sensory nerves, and distinguished the spinal roots in motor (anterior) and sensory (posterior), while ***Hall***’s animal experiments led to the discovery of the *reflex arc* and the central role of the spinal cord and medulla oblongata in this ”excito-motory system” (Bell, 1811, 1830, 1966; Magendie, 1822; Hall, 1833; Brazier, 1984, 1987). A century later the *muscle stretch reflex*, which Descartes had postulated depended on hydraulics, would be described in detail by ***Sherrington*** and ***Lidell*** (Sherrington, 1906; Lidell and Sherrington, 1924; Lidell, 1960; Brazier, 1987). While on the subject of motor nerves and muscle reflexes we should mention that ***Krause*** and ***Kühne*** were probably the first to describe the motor end plate with the aid of a microscope (Kühne, 1862; Krause, 1863).

In the domain of neurohistology, Antony ***van Leeuvenhoek*** (1632-1723) using a prototype microscope made in 1674 the first microscopic observations of the retina and the optic nerve, and found the latter to be devoid of hollow space, something that first Galen had argued about against the Erasistrateans (Galen, 1968). Although ***Fontana*** (1730-1805) might have been the first to describe myelinated peripheral nerve fibers in 1781, the eponymous glial cell of the peripheral nervous system (PNS) was identified by Theodor ***Schwann*** (1810-1882). This prompted him to formulate *his* version of the *Cell Theory* in 1839 (Fontana, 1778; Clarke and Bearn, 1972; Finger, 1994; Waxman *et al.*, 1995). I say 'prompted' and not 'inspired', since his botanist friend Matthias ***Schleiden*** (1804-1881) had previously observed the cellular architecture of plants and had come up with a Cell Theory of his own, which Schwann, a zoologist, simply extended to the animal kingdom (Waxman *et al.*, 1995). But the seeds of the Cell Theory had been sown earlier by the Czech Jan Evangelista ***Purkyně*** (1787-1869), who in 1837 described his own namesake cells in the cerebellar cortex, coined the term 'protoplasm' and was among the first to observe with a microscope the neuraxon and its myelin sheath (Henry, 1953; Posner, 1969).

It has been postulated that nerve regeneration was first observed serendipitously by William Cumberland ***Cruikshank*** (1745-1800) in 1776, who at the time worked as John Hunter's dissector, while conducting vagus nerve transection experiments in dogs (Cruikshank, 1795). Nerve regeneration and degeneration were subsequently studied by Fontana, Haighton, Prévost, Flourens, Burdach, Steinrück, Valentin, Müller, Nasse, Remak, Günther, Waller, Schön, Schiff, Bruch, Lent, Philipeaux, Vulpian, and Ranvier. Two schools of thought thus emerged, the "dualists" and the "monists" (Fontana, 1778; Haighton, 1795; Prévost 1827; Valentin, 1836, 1839; Burdach, 1837; Müller, 1838, 1842; Remak, 1838; Nasse, 1839; Waller, 1850, 1852; Schiff, 1854; Bruch, 1855; Lent, 1856; Philipeaux and Vulpian, 1859a, 1859b, Vulpian, 1866; Ranvier, 1878). The *dualists* believed that *nerve fibre* and *nerve soma* were two different 'nerve elements' and that nerve fibres arose from the union of individual Schwann cells. From the *dualist* camp originated the 'reunionist' regeneration theory* and its derivatives. The *monists*, who were initially a quiet minority, comprised mainly by Waller and ***Remak***, believed instead that nerve fibers stemmed from the nerve cell body, from which they also depended for trophic support, and should therefore be acknowledged as the forerunners of the *Neuron Doctrine* (Ochs, 1975, 1977). From their monistic position arose *monogenism*, the hypothesis of nerve regeneration that eventually prevailed.

A lasting eponym came with August Volney ***Waller*** (1816-1870), an English histologist and physiologist, who in 1849 described the degeneration of the distal stump of a peripheral nerve after its "division" (Waller, 1850). As he duly notes in his paper, others before him had observed degenerative changes in the distal portion of a ligated (Steinrück) or transected (Günther, Schön and Nasse) sciatic nerve of a frog, while ***Burdach*** and ***Valentin*** had not found any alterations in the severed nerve fibers of the sciatic of the frog above or below a ligature (Burdach, 1837; Steinrück, 1838; Valentin, 1839; Günther and Schön, 1940; Nasse, 1839; Waller, 1850; Howell and Huber, 1892).

So, arguably, the first microscopic descriptions of '*Wallerian degeneration*' were made by **Nasse** in 1839 and ***Günther*** and ***Schön*** in 1940, while ***Steinrück***, before them, had only remarked on the thinning of the distal portion of a ligated frog sciatic nerve, which he missattributed to "*neurolemal atrophy*" (Waller, 1850; Howell and Huber, 1892). Working in the early days of microscopy, with hardly any fixation methods available, the results of all these scientists are certainly commendable. Waller, himself working with a frog model of glossopharyngeal and hypoglossal transection and using caustic potash "which dissolves all tissues except the nerves" and distilled water to "render it [the tissue fragment] more transparent", gave nonetheless accurate descriptions of the degeneration over time, and concluded on the glossopharyngeal (Waller, 1850):

> About the 20th day the medullary particles [myelin] are completely reduced to a granular state … we find the presence of the nerve merely indicated by numerous black granules, generally arranged in a row like the beads of a necklace. In their arrangement it is easy to detect the wavy direction characteristic of the nerves. They are still contained in the tubular membrane, which is but very faintly distinguished, probably from the loss of the medulla and from atrophy of its tissue… These granules may be detected within the papillary nerves for a considerable period of time. I have seen them apparently unaltered in the papillæ [of the frog tongue tissue bit] *upwards of five months* after division of the nerve, *reunion not having taking place* [*italics by MF*].

This last phrase would qualify Waller as the first *monogenist* and opponent of the erroneous hypothesis of nerve regeneration, that of *autogenesis* in its various versions (*reunionist* or *polygenesis* or *catenary* theory*). Indeed, Waller's subsequent notable studies on nerve regeneration, again using frogs as experimental animals, attest to that, since he clearly described regeneration of "new, thinner fibers (one-quarter to one-eighth the normal diameter)" from above the level of nerve transaction (Waller, 1852). According to ***Vulpian***'s (1826-1887) *catenary theory**, after the section of a peripheral nerve with its neuraxons and the ensuing degeneration of the distal segments of the neuraxons, regeneration takes place *in situ* inside the distal stump of the cut nerve by the coalescence of Schwann cells arrayed in a linear chain (Lat. *catena*) and the *newly formed* distal axonal segments then join and fuse (Fr. *réunion*) with the *old* neuroaxons of the proximal stump of the cut nerve (Philipeaux and Vulpian, 1859a, 1859b; Vulpian, 1866). To this ***Ranvier*** countered, with Forssman, Cajal, Marinesco, Lugaro and others later concurring, that regeneration is achieved by invading axonal sprouts from the proximal stump into the distal stump, the so called theory* of *monogenism* (Waller, 1852; Ranvier, 1878; Forssman, 1898).

The discovery of the *reazione nera* by Camillo ***Golgi*** in 1873 and its successful modification and application by Cajal –the method passed on to him, as well as the Weigert-Pal method, by the Spanish psychiatrist from Valencia and amateur histologist Luis Simarro in 1887– ushered into a period of monumental revelations in the field of neurohistology (Golgi, 1886, 1907, 1908; Pannese, 1996; Cimino, 1999; Mazzarello, 1999; López-Muñoz *et al.*, 2006; Torres-Fernández, 2006; De Carlos and Borrell, 2007). Fervent antagonism ensued between *reticularists*, headlined by Golgi, Tanzi, ***Deiters***, ***Gerlach***, ***Kölliker*** (converted later), ***Held***, and *connectionists*, notably ***Waller***, ***von Gudden***, ***Forel***, ***His***, ***Van Gehuchten***, ***Sherrington***, ***Auerbach***, ***Lugaro*** and of course Cajal (Waller, 1850, 1852; Kölliker, 1852, 1853, 1863, 1867, 1891, 1899, 1905; Deiters,

1865; von Gudden, 1870; Gerlach, 1871, 1872; Golgi, 1886, 1907, 1908; His, 1883, 1886, 1889; Forel, 1887, 1937; Van Gehuchten, 1891; Van Gehuchten and Martin, 1891; Lugaro, 1894, 1904, 1906a, 1906b, 1906c; Held, 1897; Sherrington, 1897; Auerbach, 1898a, 1898b; Nissl, 1903; Cajal, 1907, 1910, 1917, 1928, 1954; Mott, 1909; Bielschowsky, 1902, 1904, 1928; McMenemey, 1953; Wright, 1953; Van der Loos, 1967; Shepherd, 1991; De Felipe and Jones, 1992; Jones, 1994; Cimino, 1999; Mazzarello, 1999; Bennett, 2002; Azmitia, 2002, 2007; Cowan and Kandel, 2002; López-Muñoz *et al*., 2006; Torres-Fernández, 2006; De Carlos and Borrell, 2007). Eventually, the *Neuron Doctrine* was officially formulated in 1891 by ***von Waldeyer-Hartz***, who also coined the term *neuron*, while ***Sherrington***, who like Auerbach, rejected the continuity between axonal terminals and dendrites gave us the term *synapsis* (von Waldeyer-Hartz, 1891; Sherrington, 1897; Cowan and Kandel, 2002). Cajal probably felt that von Waldeyer-Hartz was overly credited for the *Neuron Doctrine*, and expressed his chagrin as follows (Cajal, 1954):

> Professor Waldeyer, to whom poorly informed persons attribute the *neuron theory*, supported it with the prestige of his authority but did not contribute a single personal observation. He limited himself to a short, brilliant exposition (1891) of the objective proofs adduced by His, Kölliker, Retzius, Van Gehuchten and myself, and he invented the fortunate term of 'neuron'.

The combination of personal talent and the 'black reaction' from Pavia proved fortuitous for ***Cajal*** who in 1890 identified the *cone of growth* and later enunciated with van Gehuchten the law of *dynamic* (or *functional*) *polarization* –according to which impulses travel from dendrites to soma and from soma to axon, hence the arrows in Cajal's later drawings– and the rudiments of his *neurotropic hypothesis* based on developmental studies of the chick retina (Cajal, 1892a, 1892b; Lugaro, 1894, 1899, 1904, 1906a, 1906b, 1906c, 1909; Cajal, 1905, 1910, 1917, 1928, 1929, 1954; McMenemey, 1953; De Felipe and Jones, 1991; De Felipe and Jones, 1992; Mazzarello, 1999). For the record, it must be said that the term *neurotropism* was coined by ***Forssman*** in 1898 to denominate the tendency of the severed nerve fibers of a nerve to enter the distal stump (Forssman, 1898). Moreover, Forssman was probably the first to suuggest that the neurotropic stimuli for the regenerating fibers were the products of myelin decomposition (Forssman, 1898; Lugaro, 1904, 1906a, 1906b, 1906c). In 1903, Cajal made his own methodological contribution by discovering the *reduced silver nitrate impregnation* method (to which Cajal often referred as the *neurofibrillary* method, for brevity) independently from ***Bielschowsky***, who used a similar method (Bielschowsky, 1902, 1904, 1928; Cajal 1903; Doyle, 1939). Cajal and the Italians ***Perroncito***, a student of Golgi, and ***Lugaro*** among others applied it to the study of PNS regeneration where they found further evidence for the *neurotropic* hypothesis and against the *autogenesis* theory* of PNS regeneration, the latter revived and staunchly defended by Albrecht ***Bethe*** (Bethe, 1901; Lugaro, 1894, 1899, 1904, 1906a, 1906b, 1906c; Cajal, 1905, 1907, 1913, 1928; Perroncito, 1905, 1906, 1908, 1909; Marinesco and Minea 1906; Marinesco, 1906; Minea, 1909; Mazzarello, 1999).

The *neurotropic hypothesis* as elaborated by these pioneer neuroscientists was striking for its maturity. The growth cone was viewed as a robust dynamic structure, capable of: 1) responding to attractive signals via *receptors* on its surface, as hinted by Cajal, 2)

extending axonal sprouts under harsh conditions as early as one day after axotomy and even when isolated from its soma (!), 3) crossing the proximodistal gap and entering the distal stump by the second day, 4) altering its growth direction in order to rectify its course toward its target, and finally 5) reestablishing contact with its target, hence Cajal's metaphoric characterization of growth cones as "flexible battering rams" (Cajal, 1892a, 1892b, 1905, 1910, 1913, 1917, 1928, 1929; Lugaro, 1894, 1904, 1906a, 1906b, 1906c; Azmitia, 2002, 2007; López-Muñoz *et al.*, 2006; De Carlos and Borrell, 2007). With notable acuity, the Romanians ***Marinesco*** and ***Minea*** attributed to the Schwann cells in the distal stump, that proliferated and aligned themselves along the neurilemma to form the *endoneurial tubes* (or *bands of Büngner*), attractant properties, insinuating *chemotropism* as the mechanism of neurotropism in PNS regeneration and Cajal, Lugaro, Tello and others confirmed this conjecture (Marinesco, 1906; Marinesco and Minnea, 1906; Lugaro, 1904, 1906a, 1906b, 1906c; Cajal, 1910, 1913, 1928; Tello, 1911). Half a century later Sperry would essentially reiterate the *chemoaffinity hypothesis* to explain the complete and topographically correct regeneration of retinal ganglion cells' axons towards the optic tectum, after crush injury of the optic nerve of frogs (Sperry, 1963). It would be shown a few decades later that rewiring in ***Sperry***'s model was not due to *bona fide* chemoaffinity but to chemoaffinity *in default of* chemorepulsion (Bonhœffer and Huf, 1980, 1982; Gierer, 1981, 1983; Thanos and Mey, 2001; McLaughlin and O'Leary, 2005).

Thus, we arrive at the historical origins of one of the most tantalizing enigmas of neuroscience and a perennial conundrum in CNS regeneration research, the "old dogma of irregenerability of central paths" (Cajal, 1928). Long before Cajal, it had been consistently demonstrated that while regeneration was possible in the PNS after axotomy, CNS lesions resulted in *abortive sprouting*, a "fatal and inexorable fact" elevated to the status of "unimpeachable dogma" (Marinesco, 1906; Marinesco and Minea, 1906; Cajal, 1910, 1913, 1917, 1928). In order not to ignore the names and demerit the original work of these pioneer SCI researchers, mostly neurologists, that preceded or were contemporary with Cajal, I quote an homage from Cajal himself (Cajal, 1913, 1928):

> ...there have been numerous scientists who, desirous of exploring the mechanism of the degenerative reactions and of the possible reparatory acts of the central nerve paths, have studied the histological alterations, either of men suffering from traumatic myelitis, sclerosis, secondary degenerations, commotions, etc. or else of animals that had been traumatized or submitted experimentally to poisonings, local anæmias and infections, etc. It would be a long list that included all the authors who have distinguished themselves by some original contribution in this difficult field. We may recall the following... who have worked almost exclusively on traumatized animals. Among the older authors it is important to remember Dentan (1873), Eichhorst and Naunyn (1874), Schiefferdecker (1876), Kahler (1884), Homen (1885), Lowenthal (1885), Ziegler (1888), Coën (1888), Barbacci (1891), Kerestszeghy and Hanns (1892), Strœbe (1894), etc. and among the more modern Minor, Nageotte, Lugaro, Cajal, Marinesco, G. Sala and Cortese, O. Rossi, Dustin, Forster, and Jacob... Excellent descriptions of the traumatic degeneration and regeneration of spinal roots interrupted at a greater or less distance from the spinal cord have been given, not only by the older authors (Strœbe, Bethe, etc.) but also by Lugaro, Marinescco, O. Rossi, Sala and Cortese, and Dustin.

Implicit to this "old concept of the essential impossibility of regeneration" of central axons were: 1) the notion that CNS as PNS neurons have an intrinsic regeneration potential –not necessarily of the same capacity– as exemplified by the postlesional

sprouting of central axons, and 2) the fact that this purported intrinsic regeneration potential of central axons is rendered inadequate or doomed to failure by one or more undetermined factors inherent to the CNS milieu. Both of these observations had been consistently made by some of the aforementioned neurologists or pathologists, and still others in various animal species.

> Pathologists consider it an unimpeachable dogma that there is no regeneration of the central paths, and therefore that there is no restoration of the normal physiology of the interrupted conductors in the spinal cord. A vast series of anatomico-pathological experiments in animals and an enormous number of clinical cases that have been methodically followed by autopsy, serve as a foundation for this doctrine, which is universally accepted today. Nevertheless, some neurologists setting on one side the incontestable disturbing fact that functional damage is irreparable, have made known histological observations of the partial regeneration of neurones and nerve fibers. Thus Masius, Vanlair, Müller, etc. noted some time ago in lower vertebrates such as reptiles and amphibians what appeared to be positive evidence of sprouting of the axons. Similar phenomena where described in the spinal cord of mammals by Bikeles, Finckler, Strœbe, Miyake, etc., who used the old methods of histological staining. The neurofibrillary methods have placed the problem on a precise basis, and brought the solution much nearer… By means of them it has been demonstrated, beyond doubt, that there is a production of new fibers and clubs of growth in the spinal cord of tabetics (Nageotte and Marinesco) and of cones and ramified axons in the scar of spinal wounds of man and animals (Cajal, Marinesco, Lugaro, Bielschowsky, O.Rossi, G. Sala, and Cortese, Dustin, Perrero, L. Forster, etc.). These investigations, while they have brought out unquestionable signs of repair, which are comparable in principle with those of the central stump of the nerves, have also confirmed the old concept of the essential imposibility of regeneration, showing that, after a more or less considerable period of progress, the restoration is paralyzed, giving place to a process of atrophy and definitive breakdown of the nerve sprouts. [Cajal, 1928]

Cajal and the other proponents of the neurotropic hypothesis believed that the root of the problem of the irregenerability of the central paths was the lack of trophic support, contrary to the PNS environment with its Schwann cells and their rich chemotactic actions, and hypothesized that regeneration could be enhanced if central axons were provided with trophic elements *in situ* (Cajal, 1913, 1928). Though the explanation of *trophic deficit* might have been grounbreaking in its early days, by 1913 it had become conventional and experimental evidence from several labs had accumulated to back it up. Three lines of data favored the *trophic deficit* hypothesis. First, Cajal and others had shown that central axons, their sprouts or their collaterals were strongly attracted by prelesioned spinal roots replete with *bands of Büngner*, and sometimes would course extraparenchymally along the *pia mater* (even through connective scar) to get there (Cajal, 1910, 1913). Lugaro had previously made a similar observation about the regenerating lower motor neuron (peripheral) axons, exiting the anterior roots and ascending the neighbouring posterior roots under the supposed chemotropic influence of the dorsal root Schwann cells (Lugaro, 1904, 1906a, 1906b, 1906c). Second, ***Tello*** demonstrated that central axons could grow in bundles among the *bands of Büngner* of a peripheral nerve implanted in the cortex of young rabbits (Tello, 1907, 1911). Moreover, Tello showed that predegenerated peripheral nerve grafts (”*empty grafts*”) were more chemoattractive to central axons than ‘fresh’ nerve implants (Tello, 1911). However, as Cajal also admits, Lugaro was the first to attempt a peripheral nerve grafting experiment in the cerebral cortex of dogs but for methodological reasons failed to obtain central axon regeneration into the graft (Lugaro, 1904, 1906a, 1906b, 1906c; Cajal, 1928). Lugaro had not used predegenerated sciatic grafts and had not lesioned the cerebral cortex before

implantation. The explicit conclusion from the aforementioned experiments was that the intrinsic regenerative capacity of CNS neurons is more potent or efficient within a PNS milieu, especially a predegenerated one, most probably due to the existence of chemotropic signals lacking in CNS but present in the nerve implant, signals which also increased after denervation. The source of the "*neurotropic substances*", as Marinesco had hypothesized years before, was presumed to be the Schwann cells. Indeed, Tello showed that "the juice pressed from the *bands of Büngner* of the peripheral stump of the cut nerve" could *per se* attract the cerebral fibers (Cajal, 1913, 1917, 1928). Though Tello's experiments generated Cajal's praise they also provoked heavy criticism elsewhere and went largely unappreciated for decades. This line of research had to await the 80's before bearing fruit again (Sugar and Gerard, 1940; Le Gros Clark, 1943; Richardson *et al.*, 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982). The third kind of evidence supporting the *trophic deficit* hypothesis is more controversial despite bearing Cajal's stamp. Pathologists before Cajal had accurately described and distinguished the different types of scar tissue formed after spinal lesions of varying severity. Moreover, Cajal, based on his observations in spinal cord regeneration experiments, believed that the fibrous scar and meningeal cells contained or secreted "*trophic substances*" that had chemoattractive actions on regenerating axons (Cajal, 1928). He considered this a definitive argument for the *trophic deficit* hypothesis and judging from the zest of his writings on the subject seemed convinced beyond doubt of the proregenerative effects of the mesodemal –in contrast to the neuroglial– scar on CNS axons (Cajal, 1928):

> The authors who have studied the genesis of the cicatricial tissue of spinal wounds, agree, *mutatis mutandis*, that when the lesion is not very extensive the reunion of the edges of the nervous substance is effected by means of neuroglial tissue. If the interruption is total and very wide, involving important blood-vessels, ependyma, meninges, etc. two scars are formed. One of these is *internal* and covers directly the interrupted stumps of the nerve centre; it is composed of neuroglia. The other, *external*, is in the form of a sheath around the wound, into which it often penetrates in the form of a wedge. This latter scar is in continuity with the meninges, especially the *pia mater*; it is from their connective cells that it originates. The cells of the first scar are ectodermal in origin; those which produce the second belong to the mesoderm. The two scars are always separated by more or less well marked lines of demarcation. We may add that in all wide wounds which involve the ependyma there is produced in time, within the neuroglial scar and in continuity with its cavity, a more or less anfractuous cyst, at whose walls the mesodermal formation always stops… There exists a certain parallelism between the regenerative reaction of the white matter and the presence and proximity of the cicatricial mesodermal formations. Very small punctures and fine wounds which are partial, in the grey and white matter, and which are rapidly cicatricized by means of neuroglial tissue hardly ever bring about sprouting of axons; even the traumatic degenerative process is precarious and of slight extent. On the contrary, complete interruptions of the cord, with an irruption of exudates and an abundant proliferation of the mesodermal cells of the *dura* and the *pia mater*, followed by an application and even a partial penetration of the connective scar into the lips of the white matter, are apt to bring about, not only the appearance of numerous nerve sprouts, but even their active migration from the region of the cord into the connective scar, and in certain cases even into the nerve roots. It thus seems natural to conjecture that the *regenerative process of the white matter*, which is so remarkably faint and sluggish under ordinary conditions, can be powerfully *stimulated by means of active or trophic substances liberated by the mesodermal scar and diffused in the spinal wounds and their edges*. [*italics by MF*]

Cajal's observations of the seemingly positive role of mesodermal scar in periwound sprouting in the CNS as well as his explanatory hypothesis of "trophic substances" emanating from the cicatrix have been confirmed by recent findings (Batchelor *et al.*,

1999, 2000, 2002a, 2002b, 2008a, 2008b). In a striatal wound mouse model, sprouting of nigrostriatal dopaminergic fibers was seen at the wound margins, even despite the presence of inhibitory *chondroitin sulphate proteoglycans* (CSPGs), but not beyond the wound edge and into the lesion interior (Asher *et al.*, 2001; Moon *et al.*, 2000, 2001a, 2001b, 2002; Morgenstern *et al.*, 2002; Rhodes *et al.*, 2003). It was shown that this periwound sprouting spatiotemporally correlated with the establishment of a chemical gradient of neurotrophic factors, such as BDNF and GDNF produced by locally activated microgliacytes and macrophages, that peaked at the lesion edge, thus suggesting a trophic factor-dependence of sprouting (Batchelor *et al.*, 1999, 2000, 2002a, 2002b; Satake *et al.*, 2008b). A causal relationship between trophic factor gradient and dopaminergic fiber sprouting was further supported by a marked decrease in sprouting if the striatal lesion was followed by local infusion of antisense oligonucleotides (Batchelor *et al.*, 2002a, 2002b). It was finally hinted that neurotrophic factors may additionally render the wound site more permissive by downregulating the synthesis of CSPG (Batchelor *et al.*, 1999, 2000, 2002a, 2002b, 2008a, 2008b; Moon *et al.*, 2002). Finally, the molecular mechanisms of astroglial scar relative nonpermissiveness for axonal regeneration has been adequately elucidated during the last 30 years (Reier and Houlé, 1988; Silver, 1993, 1994; Davies et al 1997, 1998, 1999; Stichel *et al.*, 1999b, 1999c, 1999d; Bruce *et al*, 2000; Moon *et al.*, 2000, 2001a, 2001b, 2002, 2003; Morgenstern *et al.*, 2002; Yick *et al.*, 2000; Asher *et al.*, 2001; Rauch *et al.*,2001; Bradbury *et al.*, 2002; Jones *et al.*, 2002, 2003; Rhodes *et al.*, 2003; Buss *et al.*, 2004, 2007, 2009; Chau *et al.*, 2004; Matsui and Oohira, 2004; Silver and Miller, 2004; Ribotta *et al.*, 2004; Caggiano *et al.*, 2005; Klapka *et al.*, 2005; Steinmetz *et al.*, 2005; Fawcett, 2006; Mingorance *et al.*, 2006; Busch and Silver, 2007; Cafferty *et al.*, 2007; Lingor *et al.*, 2007; Schiwy *et al.*, 2009).

While on the subject of mesodermal and neuroglial scar one should make special mention of the discovery of neuroglia and of the microgliacyte. The first to demonstrate the presence of neuroglia in the CNS was ***Keuffel***, who clearly described, after removal of the nervous tissue from spinal cord sections, a meshwork of supporting tissue that he thought was outbranchings from the pia mater (Keuffel, 1811). ***Arnold*** and ***Virchow*** repeated these early observations (Arnold, 1845; Virchow, 1846). Indeed, it was Virchow who coined the term *neuroglia* for this new type of granular tissue enmeshed in the central nervous system (Virchow, 1860). That neuroglia consisted of cells was first postulated by ***Deiters***, while the possibility of existence of more than one types of neuroglial cells was suggested by ***Lewis*** (Dieters, 1865; Lewis, 1878). The different metallic stains used at the time critically influenced that era's notions of neuroglia. Golgi's impregnation method helped reveal the cellular composition of neuroglia but Weigert's method led many early histologists to view neuroglia as a *syncytium* (Golgi, 1886; Weigert, 1890; Reinke, 1897; Eurich, 1897a, 1897b, 1898; Whitwell, 1898; Robertson, 1899; Held, 1903; Hardesty, 1904; Lugaro, 1907; Mazzarello, 1999). It is logical but also ironic that the concepts of *neuron* and *neuroglia* would suffer parallel fates at the hands of neurohistologists using the same set of staining techniques. Thus, a controversy came about over the syncytial or nonsyncytial constitution of neuroglia –reminiscent of the *reticularist-connectionist* debate– with several *reticularists* being partisans of the syncytial hypothesis. This quandary was eventually resolved with the aid of electron

microscopy (Weigert, 1890; Reinke, 1897; Eurich, 1897a, 1897b, 1898; Whitwell, 1898; Robertson, 1897, 1898, 1899, 1900a, 1900b; Held, 1903; Hardesty, 1904; Mori and Leblond, 1969). ***Robertson*** was the first to oppose ***Weigert's*** *syncytial hypothesis* and also the first to claim that neuroglial cells undergo a physiological turn-over with a life cycle that could extend for several years (Weigert, 1890; Robertson, 1897, 1898, 1900a, 1900b; Messier *et al.*, 1958; Smart and Leblond, 1961). He also experimented with a novel staining method that made use of platinous oxide and resulted in selective staining of a CNS cellular element that differed from the neuronal and neuroglial cells known at the time (Robertson, 1899, 1900a). He named this distinct nonneuronal CNS element *mesoglia* for he believed it to be of mesodermal origin and he even reported that following CNS injury mesoglial cells exhibited morphological changes and phagocytic activity (Robertson, 1899, 1900a, 1900b). Thus, Robertson's mesoglia definitely encompassed microglia and probably even oligodendroglia (Robertson, 1899; Penfield, 1924). Essentially, what Robertson was describing for the first time thanks to his *platinum method* was a 'third element of the brain', a discovery that was later misattributed to Cajal, hence the misnomer "third element of Cajal" (Lugaro, 1907; Cerletti, 1908). The riddle of the 'third element' was broken some years later by del Rio Hortega, yet a mortified Cajal demanded Don Pio, his former gifted assistant, to quit his post (McMenemey, 1953, del Rio Hortega, 1919). For his achievements, Robertson should be regarded as a pioneer in the study of neuroglia.

Nissl was the first to specifically identify microglia in brain sections from patients with various conditions, as a specific type of reactive neuroglia capable of migration and phagocytosis and named them *Stäbchenzellen* (i.e. rod cells) (Nissl, 1899). ***Cerletti***, ***Alzheimer***, ***Ulrich*** and ***Achúcarro*** later confirmed this finding, further described this new cell type and speculated on its embryological origin (Lugaro, 1907; Cerletti, 1908, 1909, 1912; Alzheimer, 1910; Ulrich, 1910; Achúcarro, 1909, 1910a, 1900b). Initially, Nissl and Cerletti advocated an ectodermal origin but Alzheimer and Achúcarro favored a mesodermal lineage with Nissl finally conceding. Achúcarro –who according to some historians was Cajal's favorite student– boldly suggested that the *Stäbchenzellen* are resident mononuclear leucocytes that migrate into the CNS via the bloodstream (Achúcarro and Gayarre, 1914). Later, ***Pio del Rio Hortega*** (1882-1945) gave the definitive histological and functional description of the microgliacyte and also supported its mesenchymal origin (del Rio Hortega, 1919, 1921; 1932; Ling and Wong, 1993; Barron, 1995). Del Rio Hortega made seminal contributions herewith thanks to his method of *silver carbonate staining* which stained both oligodendroglia and microglia (del Rio Hortega, 1919, 1921, 1932). He distinguished microglia from macroglia (astroglia and oligodendroglia) thus clarifying the arcane *third element* notion –which in reality encompassed both oligodendroglia and microglia (del Rio Hortega, 1919, 1921, 1932). He considered, like Achúcarro, the circulating monocytes as their cell of origin that seed the CNS during development (del Rio Hortega, 1919, 1921, 1932). And, finally, he witnessed in brain stab wound experiments in different species how resting ramified microglia transformed into migratory amœboid cells indistinguishable from macrophages (del Rio-Hortega, 1919, 1921, 1932).

This retrospective of the *belle époque* of spinal cord injury and regeneration research would be incomplete if it did not throw light on a neglected historical fact. Today one often reads or hears from neuroscientists that Cajal was the first to suggest an inhibitory action of the CNS white matter on axonal regeneration (Filbin, 2003). This, however, is a factoid. During the 80s, inhibitory actions of the white matter or its myelin components on axonal regeneration were proven by classic experiments from Berry and Schwab, though an inhibitory role for CNS myelin has been suggested even earlier (Kiernan, 1979; Berry, 1982; Schwab and Thoenen, 1985; Schwab and Caroni, 1988; Caroni and Schwab, 1988a, 1900b). We cannot tell for sure whether these neuroscientists were aware or not that this hypothesis had been enunciated before their time but we can say with certainty that the priority for this original hypothesis does not belong to them. Indeed, it belongs to Lugaro (**) and it is a historical fact that Cajal categorically disagreed with him!

Lugaro formulated his hypothesis of *negative neurotropism* based on his results of spinal root regeneration and cortical nerve grafting experiments in dogs (Lugaro, 1904, 1906a, 1906b, 1906c, 1909). His experiments confirmed the purported neurotropic effects of Schwann cells on regenerating anterior and posterior root peripheral nerve fibres (motor or sensory) (Lugaro, 1904, 1906a, 1906b, 1906c, 1909). In his experiment he saw that the lesioned lower motor neuron fibers of the anterior roots ascended the lesioned posterior roots under the influence of the remnant Schwann cells but once the fibers arrived at the spinal cord parenchyma (what we today call the *dorsal root entry zone*, DREZ) they were repulsed and deviated to infiltrate the *pia mater* (Lugaro, 1904, 1906a, 1906b, 1906c). He explained this finding as the result of a possible *negative neurotropic action* of the CNS white matter on peripheral axons. He even extended this hypothesis and envisioned a similar inhibitory action of peripheral myelin on central axons and designed a peripheral-to-central nerve grafting experiment to test it (Lugaro, 1906a, 1906b, 1906c). At the same time Lugaro was fully aware of the low regenerative potential of central axons and also wanted to test if the Schwann cells in the peripheral nerve implant exerted chemotropic actions on central axons as they did on peripheral. Lugaro was the first to address this question and was the first to do so with an intracerebral peripheral nerve grafting experiment in 1906, preceding Tello, Le Gros Clark and Aguayo (Lugaro, 1904, 1906a, 1906b, 1906c; Tello, 1911; Le Gros Clark, 1943; Richardson *et al.*, 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982; Friedman and Aguayo, 1985). As mentioned earlier Lugaro's sciatic-to-cortex implantation experiment did not succeed most likely for methodological reasons (he operated on only 2 dogs –apart from the other reasons mentioned above) but with a bit of symmetrical thinking he was inclined to interpret this negative result as supportive of a lack of neurotropic action or even the presence of a negative neurotropic action of the Schwann cells on the central axons (Lugaro, 1906a). Despite the limited scope of his work and some erroneous conclusions, Lugaro should be credited as the *originator* of the hypothesis of white matter inhibition of axonal regeneration and the one who inspired Tello's elegant experiments.

For his part Cajal, who had himself studied spinal root lesions extensively, disagreed with Lugaro (Cajal, 1917, 1928). Cajal offered rich descriptions of regenerating posterior root fibers and had witnessed more complex axonal trajectories than Lugaro thanks to his

superior histological know-how and different experimental conditions. In fact, Cajal not only saw posterior root regenerating fibers with a centrifugal course as Lugaro but also described regenerating fibers that entered the cord or even bifurcated and grew within it (Cajal, 1928). It was for that reason mainly that he opposed Lugaro's hypothesis of white matter negative neurotropism and attempted a more pluralistic explanation of the complex neurocladic phenomena he observed. For Cajal, 1) the *trophic deficit* hypothesis, 2) a positive neurotropism exerted by the mesodermal scar, or 3) a stochastic, nonneurotropic axonal elongation that followed the "*path of least resistance"* among the mechanical obstacles encountered, were sufficient to explain the variety of fiber trajectories (Cajal, 1928):

A singular fact that shows that there is no neurotropic influence of the spinal tissue on the new root fibres, is that sensory fibres, arriving through a root, escape from the posterior bundle to become centrifugal, taking advantage of other roots, neighbouring or distant. It is also curious to note, though this is a very rare feature, that an occasional sensory fibre, traveling along the bundles, sends out a collateral to lose itself right in the connective tissue, which it reaches after having crossed the basal membrane of the cord…the preceding observations prove that the newly-formed root fibres which penetrate into the cord proceed *along the path of least resistance*, blindly impelled by the incessant growth of the terminal point. The details of the itinerary of the fibres within the posterior bundle do not suggest the idea of the presence within the cord of orienting and attracting substances, *but neither do they imply*, as Lugaro believed, the *repulsive action of negative neurotropic stimuli*… we have called attetion to the property possessed by young sensory and motor root fibres of growing and ramifying preferably in the embryonic connective tissue, as though they found in it some substance that stimulated their metabolism and growth… an even more remarkable case of arborization of a sensory bundle fibre. In a region that is fairly distant from a spinal wound and where the sensory roots, seen along their length, are normal and very little stimulated, a certain robust conductor suddenly abandons the cord, making an angle; it then places itself on the *pia mater*, gaining in thickness and stainability and traveling towards the wound. It becomes retrograde, after describing an arc, and it finally resolves itself into a very extensive supra-pial arborization… a notable example of neurocladism through traumatic stimulation or what seems more probable, *through the influence of substances given out by the cicatricial tissue*. From all the preceding observations we may deduce the following conclusions: 1. The intraspinal portion of the posterior roots, that is, the posterior bundle, regenerates with difficulty, as Lugaro already believed. This, however, is due, *not to a negative neurotropism*, but to the absence, within the cord, of trophic materials, and to the presence of mechanical obstacles at the entrance. For this reason the majority of the sensory axons that regenerate are detained or become retrograde at the edges of the posterior bundle… There exists a certain parallelism between the regenerative reaction of the white matter and the presence and proximity of the cicatricial mesodermal formations. Very small punctures and fine wounds which are partial, in the grey and white matter, and which are rapidly cicatricized by means of neuroglial tissue hardly ever bring about sprouting of axons; even the traumatic degenerative process is precarious and of slight extent. On the contrary, complete interruptions of the cord, with an irruption of the exudates and an abundant proliferation of the mesodermal cells of the *dura* and the *pia mater*, followed by an application and even a partial penetration of the connective scar into the lips of the white matter, are apt to bring about, not only the appearance of numerous nerve sprouts, but even their active migration from the region of the cord into the connective scar, and in certain cases even into the nerve roots. It thus seems natural to conjecture that the regenerative process of the white matter, which is so remarkably faint and sluggish under ordinary conditions, can be powerfully stimulated by means of active or *trophic substances liberated by the mesodermic scar* and diffused in the spinal wounds and their edges. [*italics by MF*]

It was in the domain of neurobiology that neuroregeneration research would come of age. Viktor ***Hamburger***'s developmental biological studies in the 1930s, fabulous in their simplicity, and the follow-up work of ***Bueker***, ***Levi-Montalcini*** and ***Cohen*** provided the final proof and vindication of Cajal's neurotropic (and by extension *neurotrophic*)

hypothesis. Hamburger saw that extirpating the wing bud of a 72h chick embryo resulted in ipsilateral spinal sensory ganglion and anterior horn hypoplasia, which demonstrated a kind of biological 'spooky action at a distance' (to borrow the einsteinian dictum) stemming from an organ peripheral to the CNS (Hamburger, 1934, 1993; Brunso-Bechtold and Hamburger, 1979; Henderson *et al.*, 1980, 1983; Oppenheim and Haverkamp, 1988; Oppenheim, 1989; Oppenheim *et al.*, 1989). These results were later reproduced by Levi-Montalcini and Levi (Levi-Montalcini and Levi, 1942). Bueker and Hamburger also showed the reverse action. Removing the appendage and implanting mouse sarcoma tissue at its place, or simply adding an extra limb bud, led to hypertrophy of the associated spinal ganglia (Hamburger, 1958, 1977, 1981, 1993; Hollyday and Hamburger, 1976, 1977; Hollyday *et al.*, 1977). The presumed active substance was finally isolated, identified as a polypeptide and purified, and was to become the prototypic *neurotrophin*, nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951; Cohen *et al.*, 1954; Levi-Montalcini and Cohen, 1956; Cohen, 1959, 1960; Levi-Montalcini, 1966, 1987, 1995; Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Oppenheim and Haverkamp, 1988; Oppenheim, 1989; Oppenheim *et al.*, 1989; Lindsay, 1994). The rest of the neurotrophins, in chronological order of discovery, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5, or just NT-4), neurotrophin-6 (NT-6), and neurotrophin-7 (NT-7), are homologous to NGF and together comprise the neurotrophin family which is but one group of proteins with neurotrophic and/or neurotropic actions out of an ever expanding collection of 'growth factors', whose receptors, complex intracellular signalling pathways and pleiotropic roles in development, plasticity and regeneration of the nervous system have provided the *raison d'être* for neurobiology (Lindsay *et al.*, 1985; Davies *et al.*, 1986; Johnson *et al.*, 1986; Barde *et al.*, 1987 ; Hofer and Barde 1988; Oppenheim and Haverkamp, 1988; Oppenheim, 1989; Oppenheim *et al.*, 1989; Barde, 1990; Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990a, 1900b; Hallböök *et al.*, 1991; Davies, 1994; Lindsay, 1994; Cowan, 1995; Lewin and Barde, 1996; Bartfai and Schultzberg, 1993; Bothwell, 1995; Thoenen, 1995; Ip and Yancopoulos, 1996; Henderson *et al.*, 1993a, 1993b, 1994, 1996a; Segal and Greenberg, 1996; Ebadi *et al.*, 1997; Skaper and Walsh, 1998; McAllister *et al.* 1999; Baloh *et al.*, 2000; Butte, 2001; Kalb, 2005). Importantly, for most of them, expression, receptors and not solely trophic actions are not confined within the nervous system, so arguable they could more holistically be viewed as neurokines or cytokines (Aloe *et al.*, 1994; Levi-Montalcini *et al.*, 1996; Sariola, 2001; Aloisi, 2003; English, 2003; Spedding and Gressens, 2008).

The demonstrations by Gundersen and Barrett in the late 70's that sensory axons grown *in vitro* will turn towards a source of NGF and by Aguayo *et al.* in the early 80's that CNS axons could regenerate for long trajectories along a PNS graft and then establish functional synapses orthotopically in the CNS, beautifully complemented the work of the aforementioned pioneers and reinvigorated the SCI research field (Gundersen and Barrett, 1979; Griffin and Letourneau, 1980; Richardson *et al.*, 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982; Friedman and Aguayo, 1985). Seminal discoveries followed at a steady pace thereafter. The cloning of novel *neurotrophic factors* (GDNF subfamily of TGFβ superfamily, FGF family, neuropoietic cytokines, etc.) and the demonstration of

their potent and cell-selective actions on neuronal survival, regeneration and even neurotropism reinforced the old *trophic deficit* dogma (Cajal, 1928, 1929; Gundersen and Barrett, 1979, 1980; Richardson *et al.*, 1980; David and Aguayo, 1981; Henderson *et al.*, 1981, 1983, 1993a, 1993b, 1994, 1996a, 1993b; Benfey and Aguayo, 1982; Friedman and Aguayo, 1985; Ip and Yancopoulos, 1996; Pennica *et al.*, 1996; Treanor *et al.*, 1996; Yamamoto *et al.*, 1997; Bregman *et al.*, 1997; Galzie, 1997; Baloh *et al.*, 2000; Ford-Perriss *et al.*, 2001; Airaksinen and Saarma, 2002; Airaksinen, *et al.*, 2006). The concurrent discovery of a multitude of heterogenous *neurotropic factors* and nervous system *morphogens* by Tessier-Lavigne, Jessel, Goodman and many others truly revolutionized the neurosciences by establishing the importance of *chemotropic mechanisms* in CNS development, regeneration or plasticity (*chemoattraction* and *chemorepulsion* mediated by diffusible chemotactic *–chemoattractant* or *chemorepellent–* signals or by concentration gradients thereof, and *contact-mediated attraction/repulsion*, *surround-repulsion*, and growth cone collapse or turning, by adhesion on nondiffusible guidance cues via cell-cell or cell-ECM contact) and elucidating the complexities of growth cone dynamics, axonal sprouting, pathfinding, and fasciculation, dendritic guidance and arborisation, synaptogenesis, neuronal and glial migration, and overall pattern formation (Dodd and Jessel, 1988; Eisen, 1988; Tessier-Lavigne *et al.*, 1988; Doherty and Walsh, 1989; Lander, 1989; Müller *et al.*, 1990; Placzek *et al.*, 1990a, 1990b, 1990c; Walter *et al.*, 1990; Bargmann and Horvitz, 1991; Bixby and Harris, 1991; Davies and Cook, 1991; Gordon-Weeks, 1991; Lumsden and Cohen, 1991; Tessier-Lavigne and Placzek, 1991; Keynes and Cook, 1992, 1995; Tessier-Lavigne, 1992, 1994, 2002; Silver, 1993; Bähr and Bonhoeffer, 1994; Kennedy *et al.*, 1994; Colamarino and Tessier-Lavigne, 1995; Dodd and Schuchardt, 1995; Kennedy and Tessier-Lavigne, 1995; Ebens *et al.*, 1996; Goodman, 1996; Jay, 1996; Kolodkin, 1996, 1998; Müller *et al.*, 1996; Nieto, 1996; Püschel, 1996; Serafini *et al.*, 1996; Tessier-Lavigne and Goodman, 1996; Bähr, 1997; Faissner, 1997; Gale and Yancopoulos, 1997; Guthrie, 1997, 1999, 2000, 2001; He and Tessier-Lavigne, 1997; Ming *et al.*, 1997; Orioli and Klein , 1997; Varela-Echavarría and Guthrie, 1997; Chen *et al.*, 1998; Culotti and Merz, 1998; Flanagan and Vanderhaeghen, 1998; Fujisawa and Kitsukawa , 1998; Goodhill, 1998; Goodhill and Baier, 1998; Song *et al.*, 1998; Van Vaktor, 1998; Auld, 1999; Chisholm and Tessier-Lavigne, 1999; Holder and Klein, 1999; Joosten and Bär, 1999; Lee and Jessel, 1999; Quinn, *et al.*, 1999; Seeger and Beattie, 1999; Song and Poo, 1999; Tear, 1999a, 1999b; Van Vaktor and Lorenz, 1999a, 1999b; Wang and Tessier-Lavigne, 1999; Andersen and Bi, 2000; Brose and Tessier-Lavigne , 2000; Jessel and Sanes, 2000; Galko *et al.*, 2000; Ranscht , 2000; Song *et al.*, 2000; Löschinger *et al.*, 2000; Kalil *et al*, 2000; Kaprielian *et al.*, 2000, 2001; Korey and Van Vactor, 2000; Mason and Erskine, 2000; McFarlane, 2000; Nakamoto, 2000; Tannahill *et al.*, 2000; Zoo *et al.*, 2000; Cheng *et al.*, 2001; Dickson, 2001; Giger and Kolodkin, 2001; Jacob *et al.*, 2001; Joester and Faissner, 2001; Pasterkamp and Verhaagen, 2001; Lemke, 2001; Skaper *et al.*, 2001; Stoker, 2001; Thanos and Mey, 2001; Wilkinson, 2001; Yamaguchi, 2001; Yu and Bargmann, 2001; Dickson and Senti, 2002; Ferreira and Paganoni, 2002; Grunwald and Klein, 2002; Park *et al.*, 2002; Steward, 2002; Yamamoto *et al.*, 2002; Charron *et al.*, 2003; Dent and Gertler, 2003; Fiore and Püschel, 2003; Huber *et al.*, 2003; Jan and Jan, 2003; Thiery, 2003; Tsai *et al.*, 2003; Barton *et al.*, 2004; Chotard and Salecker, 2004; Gallo and Letourneau,

2004; Henley and Poo, 2004; Hu and Strittmatter, 2004; Ichijo *et al.*, 2004; Kim and Chiba, 2004; Kiryushko *et al.*, 2004; Koeberle and Bähr, 2004; Long *et al.*, 2004; Marillat *et al.*, 2004; Nakamoto *et al.*, 2004; Sabatier *et al.*, 2004; Schnorrer and Dickson, 2004; Steward *et al.*, 2004; Williams *et al.*, 2004; Yoshikawa and Thomas, 2004; Zou, 2004; Bovolenta, 2005; Charron and Tessier-Lavigne, 2005; Holt and Dickson, 2005; Kalil and Dent, 2005; Koprivica *et al.*, 2005; Kruger *et al.*, 2005; Maskery and Shinbrot, 2005; Masuda and Shiga, 2005; McLaughlin and O'Leary, 2005; Plachez and Richards, 2005; Salie *et al.*, 2005; Skaper, 2005; Yaron *et al.*, 2005; Chilton, 2006; Goldshmit *et al.*, 2006; Gomez and Zheng, 2006; Kennedy *et al.*, 2006; Liu *et al.*, 2006; Niclou *et al.*, 2006; Stoeckli, 2004, 2006; Endo *et al.*,2007; Moore *et al.*, 2007; Tran *et al.*, 2009; Kolodkin and Tessier-Lavigne, 2010; Petrinovic *et al.*, 2010).

Martin Berry and Schwab broke new ground in the early and mid-80's by establishing the inhibitory nature of the CNS myelin which lent credence to the *inhibitory white matter* hypothesis that had become tenable by previous sporadic experimental results of CNS myelin being nonpermissive for axonal growth, inducing repulsion or collapse of the growth cone (Kiernan, 1979; Berry, 1982; Schwab and Thoenen, 1985). Schwab also generated antibodies against the proteinaceous inhibitory component of CNS myelin, and these neutralizing antibodies were later shown to have proregenerative actions *in vitro*, *ex vivo* and *in vivo* (Caroni and Schwab, 1988a, 1998b; Schwab and Caroni, 1988; Schwab, 1990, 1996, 2002, 2006; Schnell and Schwab, 1990, 1993; Schnell *et al.*, 1994; Silver, 1994; Bregman *et al.*, 1995; Kastin and Pan, 2005; Freund *et al.*, 2007; Craveiro *et al.*, 2008; Gozenbach and Schwab, 2008). Since then several CNS myelin (myelin-associated inhibitors, MAI) and nonmyelin inhibitory components have been identified and their intriguing interactions with various tentative receptor-coreceptor complexes as well as downstream signaling cascades are currently being vigorously investigated (Luo and Raper, 1994; McKerracher *et al.*, 1994; Montag *et al.*, 1994; Mukhopadhyay *et al.*, 1994; Bartsch *etal.*, 1995; David *et al.*, 1995; Filbin, 1996, 2003, 2009; Li *et al.*, 1996; Schäfer *et al.*, 1996; Shen *et al.*,1998; Chen *et al.*, 2000; Prinjha *et al.*, 2000; GrandPré *et al.*, 2000; Tessier-Lavigne and Goodman, 2000; Wang *et al.*, 2000, 2002; Liu *et al.*, 2002; Kottis *et al.*, 2002; Domeniconi *et al.*, 2002, 2005; Wong *et al.*, 2002; Inatani *et al.*, 2003; Tang, 2003; Œrtle and Schwab, 2003; Mi *et al.*, 2004; Benson *et al.*, 2005; He and Koprivica, 2004; Karnezis *et al.*, 2004; Park *et al.*, 2005; Jokic *et al.*, 2006; Shao *et al.*,2005; Venkatesh *et al.*, 2005; Ji *et al*, 2006; Okada *et al.*, 2006; Schwab *et al.*, 2006; Wang *et al.*, 2006; Zurn and Bandtlow, 2006; Charron and Tessier-Lavigne, 2007; Chaudry and Filbin, 2007; Kubo *et al.*, 2007; Quarles, 2007; Atwal *et al.*, 2008; Giger *et al.*, 2008; Löw *et al.*, 2008; Mi, 2008; Chen *et al.*, 2008; Yang and Schnaar, 2008; Xie and Zheng, 2008; Cafferty *et al.*, 2010; Jaworski *et al.*, 2010; Kaneko *et al.*, 2010; Lee *et al.*, 2010; Llorens *et al.*, 2010). The role of the glial scar as a major physicochemical barrier for CNS regeneration was further clarified and enzymatic degradation of at least one inhibitory extracellular matrix (ECM) component was shown to be beneficial for axonal regeneration (Reier and Houlé, 1988; Silver, 1993, 1994; Davies et al 1997, 1998, 1999; Stichel *et al.*, 1999b, 1999c, 1999d; Bruce *et al*, 2000; Moon *et al.*, 2000, 2001a, 2001b, 2002, 2003; Morgenstern *et al.*, 2002; Yick *et al.*, 2000; Asher *et al.*, 2001; Rauch *et al.*,2001; Bradbury *et al.*, 2002; Jones *et al.*, 2002, 2003; Rhodes *et al.*, 2003; Buss *et*

al., 2004, 2007, 2009; Chau *et al.*, 2004; Matsui and Oohira, 2004; Silver and Miller, 2004; Ribotta *et al.*, 2004; Caggiano *et al.*, 2005; Klapka *et al.*, 2005; Steinmetz *et al.*, 2005; Fawcett, 2006; Mingorance *et al.*, 2006; Busch and Silver, 2007; Cafferty *et al.*, 2007; Lingor *et al.*, 2007; Schiwy *et al.*, 2009).

From the 70's many different tissue (embryonic or adult, neural or non-neural) grafting protocols have been developed for neural transplantation purposes and this approach was also extensively tried in SCI albeit with modest results in adult animals (Reier *et al.*, 1986, 1992, 1994, 2004; Houlé and Reier, 1988; Kunkel-Bagden and Bregman, 1990; Jakeman and Reier, 1991; Bregman *et al.*, 1993, 1997, 2002; Falci *et al.*, 1997; Ribotta *et al.*, 1997, 2000; Åkesson *et al.*, 1998, 2001; McDonald *et al.*, 1999; Coumans *et al.*, 2001; Zurita *et al.*, 2001; Bradley, 2008; Shen *et al.*, 2009). In the 90's cell transplantation approaches became more sophisticated thanks to use of Schwann cells, genetically-modified fibroblasts, and olfactory ensheathing cells (OECs) a neuroglial cell type of the olfactory bulb with peculiar characteristics (Li and Raisman, 1994, 1997; Xu *et al.*, 1995a, 1995b, 1997; Li *et al.*, 1997, 1998, 1999, 2003; Raisman, 1997, 2000, 2001; Ramon-Cueto *et al.*, 1998, 2000; Stichel *et al.*, 1999a; Barnett *et al.*, 2000; Imaizumi *et al.*, 2000; Kato *et al.*, 2000; Lu *et al.*, 2001, 2002; Jin *et al.*, 2002; Lu and Ashwell, 2002; Iannotti *et al.*, 2002, 2003; Keyvan-Fouladi *et al.*, 2003; Plant *et al.*, 2003; Sasaki *et al.*, 2004, 2006; Boyd *et al.*, 2005; Fouad *et al.*, 2005; Richter *et al.*, 2005; Ruitenberg *et al.*, 2005; Dobkin *et al.*, 2006; Pastrana *et al.*, 2006; Bradley, 2008. Transplantation of immune system cells (macrophages, T cells, dendritic cells) also began in the 90's but this approach is still controversial because results from different labs have been contradictory (Rapalino *et al.*, 1998; Moalem *et al.*, 1999, 2000; Popovich *et al.*, 1996; Jones *et al.*, 2002, 2004; Popovich *et al.*, 2006; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010; Busch *et al.*, 2009, 2010; Iannotti *et al.*, 2010). The discovery of neurogenesis and adult stem cells in the CNS provided headlines and an exciting therapeutic avenue for SCI various stem or precursor cell implantation protocols (embryonic stem cells, mesenchymal or neural stem cells, differentiarted or not, and other progenitor cells, such as neural, glial, hæmatopoietic, etc.) have already been tested in the lab and even reached clinical trials (Reynolds and Weiss, 1992; Gage *et al.*, 1995; Eriksson *et al.*, 1998; McDonald *et al.*, 1999; Shihabuddin *et al.*, 2000; Horner *et al.*, 2001; Thomson *et al.*, 2001; Cao *et al.*, 2001, 2002a, 2002b; Han *et al.*, 2002, 2004; Ogawa *et al.*, 2002; Teng *et al.*, 2002; Vroemen *et al.*, 2003; Wu *et al.*, 2003; Harper *et al.*, 2004; Hill *et al.*, 2004; Koshizuka *et al.*, 2004; Yan *et al.*, 2004; Cummings *et al.*, 2005; Faulkner and Keirstead, 2005; Howard *et al.*, 2005; Iwanami *et al.*, 2005; Keirstead *et al.*, 2005; Koda *et al.*, 2005; Mitsui *et al.*, 2005; Pallini *et al.*, 2005; Schultz, 2005; Goldman and Windrem, 2006; Karimi-Abdolrezaee *et al.*, 2006; Sohur *et al.*, 2006; Bradley, 2008; Hess and Borlongan, 2008; Ormerod *et al.*, 2008; Okano and Sawamoto, 2008; Ross and Verfaillie, 2008; Pal *et al.*, 2009).

Finally, modern molecular techniques (e.g. microarrays) allowing genomic and proteomic analysis have recently been applied in experimental models and presage an era of better understanding of neuroregeneration and plasticity (Farlow *et al.*, 2000; Carmel *et al.*, 2001; Bonilla *et al.*, 2002; Nesic *et al.*, 2002, 2005; Sun *et al.*, 2002; Bareyre and Schwab,

2003; Kaiser and Nisenbaum, 2003 ; Pan *et al.*, 2004 ; Rabert *et al.*, 2004 ; Velardo *et al.*, 2004; De Biase *et al.*, 2005; Di Giovanni *et al.*, 2005; Hashimoto *et al.*, 2005; Perreau *et al.*, 2005; Byrnes *et al.*, 2006; Pastrana *et al.*, 2006). Indeed, in the past 25 years, the SCI research field has become a hotbed for dramatic discoveries which will hopefully revolutionize clinical neurology in the years to come.

(*) Usage of the term 'theory' is not warranted in all cases of hypotheses later proven to be invalid. The term 'hypothesis' would be the correct one, however I did not opt for it everywhere in the text, since these ideas were believed for a long time to be valid 'theories' and since they are viewed historically as disproven 'theories'.

(**) Ma ad ogni modo non si può negare che, sia pure come un fenomeno rudimentale, una qualche attività rigenerativa nei centri si presenta, e perciò deve esservi un neurotropismo rigenerativo indipendente dall'azione specifica delle guaine di Schwann. Ciò premesso, il fenomeno da noi constatato, il fatto cioè che le fibre delle radici anteriori sono capaci di risalire nelle radici posteriori, ma si rifiutarono di penetrare nel midollo, si può interpretare ammettendo che i cilindrassi neoformati subiscano il chemotropismo generico delle cellule di Schwann delle radici posteriori e percio salgano lungo queste regolarmente sin dove esse ne sono fornite, ma che appena finiscono le cellule di Schwann i cilindrassi si trovino del tutto disorientati e fors' anche di fronte ad una azione neurotropica negativa che li respinge e li induce a costeggiare la sostanza nervosa centrale, infiltrandosi nella pia madre. Un'azione negative analoga è possibile che venga esercitata in contrappost o dalle cellule di Schwann sui cilindrassi nati nei centri e non destinati alla periferia, ma ai centri stessi. A risolvere questo quesito ho diretto speciali esperienze. Ma primo ho voluto riconfermare con esperimenti appositi il contegno delle fibre delle radici anteriori di fronte al midollo e alle radici posteriori . . . (320) Dalle esperienze qui esposte noi possiamo trarre le seguenti conclusioni: 1) Le fibre delle radici anteriori possono continuarsi istologicamente con quelle della branca centrale della radice posteriore. 2) Esse seguono il percorso normale sinche vi sono guaine di Schwann; giunte al midollo, deviano per penetrare nella pia madre. 3) I centri nervosi esercitano dunque un'azione neurotropica negativa sulle fibre delle radici anteriori. 4) Le cellule di Schwann, fonte del neurotropismo nella rigenerazione dei nervi periferici, non esercitano alcuna azione neurotropica, o ne esercitano una addiritura negativa,sui cilindrassi dei centri nervosi. (327) (Lugaro, 1906a).

[But in any case it cannot be denied that, even it as a rudimentary phenomenon, some kind of regenerative activity occurs in the centers, and there should be, therefore, a regenerative neurotropism independent from the specific action of Schwann sheaths. Given that, the phenomenon we have observed, i.e., the fact the fibers of the anterior roots are capable to ascend again through the posterior roots but they refuse to penetrate the spinal cord, can be interpreted by admitting that the newly formed axons experience the generic chemotropism of the Schwann cells of the posterior roots and therefore ascend along the latter unless these are not furnished, but as soon as the Schwann cells finish the axons are totally disoriented and perhaps they also face a negative neurotropic action which pushes them back and induces them to border the central neural tissue infiltrating the pia mater. An analogous negative action could be exerted by Schwann cells on the axons originating from the centers and not destined to the periphery but to the centers themselves. To solve this issue I have planned special experiments. But first I wished to further confirm with ad hoc experiments the behaviour of the fibers of the anterior roots with respect to the spinal cord and to the posterior roots . . . The following conclusions can be drawn from the experiments that are here presented: 1) The fibers of the anterior roots can be histologically in continuity with those of the central branch of the posterior root. 2) They follow their normal course until Schwann's sheaths are present; once they reach the spinal cord they are deviated to penetrate the pia mater. 3) The nervous centers exert, therefore, a negative neurotropic action on the fibers of the anterior roots. 4) Schwann cells, which are the source of neurotropism in the regeneration of peripheral nerves,do not exert any neurotropic action, or even exert a negative neurotropic action, on the axons of neural centers.] (*translation from original by MF*)

GENERAL BACKGROUND:
EPIDEMIOLOGY AND PROGNOSIS

Spinal cord pathology can have, apart from traumatic, genetic, developmental, congenital, infectious, toxic, metabolic, ischæmic, autoimmune, tumorous or idiopathic causes (Kirshblum, 2002; Lin *et al.*, 2003; Rowland, 2005). Traumatic ætiology is by far the commonest, followed by multiple sclerosis and cancer (Schwab and Bartholdi, 1996). A hard truth about traumatic spinal cord injury (SCI) is that it affects mainly young individuals (median=27 years, mean=32.6 years) (Schwab and Bartholdi, 1996; Sekhon *et al.*, 2001; McDonald and Sadowsky, 2002; Kirshblum, 2002; Dobkin, 2003; National Spinal Cord Injury Database, 2001; National Spinal Cord Injury Statistical Center, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). The majority of new injuries, approximately 70%, occur in individuals less than 40 years of age, with the greatest incidence between ages 15 to 30 years, a peak between 20 to 25 years and a strong male to female preponderance (4:1) (Schwab and Bartholdi, 1996; Kirshblum, 2002; McDonald and Sadowsky, 2002; Dobkin, 2003; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). A second peak in SCI incidence is observed in the 'elder than 65 years' age-group where underlying spinal pathology such as cervical spondylosis, ankylosing spondylitis, osteoporosis, disc degeneration, spinal stenosis or rheumatoid arthritis with its risk for catastrophic atlantoaxial subluxation, predispose the elderly to fractures or cord damage even after a trivial, low-energy collision injury, such as falls from standing or falls from bed (Lovasic, 1999; Villanueva, 2000; Kirshblum, 2002; McDonald and Sadowsky, 2002; Dobkin, 2003; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Jabbour *et al.*, 2008; van den Berg *et al.*, 2010a, 2010b). Pædiatric and especially infantile, neonatal or prenatal SCI, are rare but pose exceptional diagnostic and therapeutic challenges, short-term and long-term (Rekate *et al.*, 1999; Brown *et al.*, 2001; Hayes *et al.*, 2005; Brand *et al.*, 2006; Jagannathan *et al.*, 2006; McCall *et al.*, 2006).

The annual incidence rate in North America is estimated at 30-60 cases per million persons with about 12.000 new cases per year in the US alone (Tator and Fehlings, 1991; van den Berg *et al.*, 2010a, 2010b). Worldwide annual incidence ranges between 15-55 per million for a grand total variably estimated at 130000-200.000 (Kraus 1978; Tator *et al.*, 1993a, 1993b; Silberstein and Rabinovich, 1995; Berkowitz *et al.*, 1998; Sekhon *et al.*, 2001; National Spinal Cord Injury Database, 2001; Kirshblum, 2002; McDonald and Sadowsky, 2002; Anderson, 2004; Ackery *et al.*, 2004; National Spinal Cord Injury Statistical Center, 2005; Thuret *et al.*, 2006; Pickett *et al.*, 2006; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). This roughly translates to a new lesion every 3 minutes! Overall, prevalence is estimated to approximately 300.000 in North America, about as many in Europe, and a global figure of 2,5 million chronic sufferers (Kraus 1978; Sekhon *et al.*, 2001; National Spinal Cord Injury Database, 2001;

McDonald and Sadowsky, 2002; Anderson, 2004; Ackery *et al.*, 2004; National Spinal Cord Injury Statistical Center, 2005; Saillant *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Pickett *et al.*, 2006; Thuret *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Rossignol *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b).

Lifetime healthcare costs per SCI patient can reach 4 million dollars and assuming a near-normal life-span for surviving SCI victims, the annual management costs for all SCI patients in the US has been estimated between $ 4-7 billion (Stripling, 1990; Tator *et al.*, 1993a, 1993b; Berkowitz *et al.*, 1998; Sekhon *et al.*, 2001; McDonald and Sadowsky, 2002; Kirshblum, 2002; National Spinal Cord Injury Database, 2001; Anderson, 2004; Ackery *et al.*, 2004; National Spinal Cord Injury Statistical Center, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). The financial burden to society surpasses the abovementioned estimates if one also takes into consideration the 'loss of productivity' parameter (Anderson, 2004). Unfortunately, socioeconomic factors, such as education and income, mediate disparities in health care access and outcome among SCI patients of different ethnic backgrounds, with African-Americans bearing the brunt of inequity (Berkowitz *et al.*, 1998; Claridge *et al.*, 2006; Krause *et al.*, 2006). In the US, lack of commercial insurance was shown to be a compromising factor for maximal recovery and rehabilitation (Claridge *et al.*, 2006). For example, a large US survey showed that 20% of patients with SCI had no access to physical therapy while 45% exercised on their own without supervision by a physiotherapist (Anderson, 2004).

The main causes for traumatic SCI are *vehicular accidents* including pedestrian injuries (36-48%); *acts of violence*, mostly penetrating, missile or stabbing injuries, with regional variations in incidence, most common in the urban centers of the US, Brazil, South Africa and various war zones (5-29%); *falls*, especially among elders, and other work-related accidents, such as mining and logging (17-21%); and *high-risk sports accidents* (2-10%) such as equestrian, rock-climbing, skiing, paragliding, etc., or accidents related to other risky recreational activities such as diving, which results in roughly 1000 SCI yearly in the US with a 95% rate of tetraplegia (Tator *et al.*, 1993b; National Spinal Cord Injury Database, 2001; McDonald and Sadowsky, 2002; Kirshblum, 2002; Bird *et al.*, 2005; Cobb *et al.*, 2005; Forchheimer *et al.*, 2005; Hayes *et al.*, 2005; le Roux *et al.*, 2005; National Spinal Cord Injury Statistical Center, 2005; Saillant *et al.*, 2005; Biering-Sørensen *et al.*, 2006; Jagannathan *et al.*, 2006; DeVivo *et al.*, 2006; Gauler *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Rossignol *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). Alcohol is a factor implicated in as many as 1 out of 4 SCI in the US (Tator *et al.*, 1993b). Certain reports even suggest an association between blood alcohol concentration at the time of the injury and severity of neurological impairment after SCI (Forchheimer *et al.*, 2005). In Europe, as a consequence of a different social policy on fire-arms, sports and work-related accidents cause more SCI than acts of violence (Raineteau and Schwab, 2003). This is not just a trifle statistic, if we consider that gun-shot related SCI carry the worst prognosis (Kirshblum, 2002; Lin *et al.*, 2003). In the elderly group (age > 65 years)

falls alone account for the majority of traumatic injuries to the spinal cord, with an estimate of 63% in the Canadian population (Lovasic, 1999; Villanueva , 2000; Pickett *et al.,* 2006; Jabbour *et al.*, 2008). Neonatal SCI can be the result of invasive nursery procedures or underlying neonatal pathology, where traction/hyperextension of the cervical spine during seemingly atraumatic or breech delivery result in stretching/compression damage of the spinal cord (Brand *et al.*, 2006). It must be stressed that preexisting spinal degeneration or malformation, not only in the elderly but even in young adults, is not a negligible predisposing factor for SCI. According to one study, 10% of SCI patients suffered from preexisting cervical spondylosis (Tator, 1993b). Also, in Down syndrome patients even a minor extension cervical injury can have devastating results because of an underlying congenital abnormality of the occipito-cervical junction (Buck, 1987).

Improved retrieval systems, prehospital and in-hospital care have led to a decreasing trend for complete injuries (approximately 45-50%) and a decreased overall mortality in the acute and subacute settings (Tator *et al.*, 1993b; Devivo *et al.*, 1999; National Spinal Cord Injury Database, 2001; Sekhon *et al.*, 2001; National Spinal Cord Injury Statistical Center, 2005; Garshick *et al.*, 2005; McDonald and Sadowsky, 2002; Saillant *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Gauler *et al.*, 2006; Krell *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). Because SCI patients are often polytraumatic cases, mortality at the scene can exceed 50%, while mortality one year after SCI is only some 13% (Rowland, 2005). In-hospital mortality is still non-negligible even in major trauma centers with well-trained personnel and sophisticated imaging techniques, ranging between 4,4-16,7% among different centers (Devivo *et al.*, 1999; Kirshblum, 2002; Garshick *et al.*, 2005; Saillant *et al.*, 2005; Levi *et al.*, 2006; Kwon *et al.*, 2006). Approxmately 15% of SCI patients worsen neurologically during the period following hospital admission. Deterioration may be the result of poor management since injuries in the bony or soft tissues of the vertebral column can be missed and inadvertently lead to *in-hospital SCI* a few days to weeks after admission (Levi *et al.*, 2006; Kwon *et al.*, 2006). The most common reasons for this paradoxical fact are trivial, i.e. insufficient imaging work-up (65%) or even read-out (35%), and again the elderly are more at risk due to their unassuming injuries and predisposing conditions (Lovasic, 1999; Villanueva, 2000; Levi *et al.*, 2006; Miyanji *et al.*, 2006). Although many risks exist, deterioration during transportation is more often than not artifactual and attributable to incorrect first determination of level of injury, rather than the result of aggravation of the primary injury (Felleiter *et al.*, 2006). As for the pædiatric patients, they too can be victimized iatrogenically, because children have thin calvaria, thus external immobilization with a *Halo* brace becomes difficult and subject to a high complication rate (Hippocrates, ed. 1952; Rekate *et al.*, 1999; Brown *et al.*, 2001; Hayes *et al.*, 2005; Jagannathan *et al.*, 2006; McCall *et al.*, 2006).

The majority of SCI (c. 55%) occur in the cervical region (C1-T1) (especially in the elderly and pædiatric groups), while only around 15% in each of the remaining three regions, thoracic (T1 to T11), thoracolumbar (T11-T12 to L1-L2) and lumbosacral (L2

to S5) (Hughes, 1974; Bohlman, 1979; Ikata *et al.*, 1989; Bohlman and Ducker, 1992; Silberstein *et al.*, 1995; Schwab and Bartholdi, 1996; Devivo *et al.*, 1999; Fehlings *et al.*, 1999; Lovasic, 1999; Villanueva, 2000; National Spinal Cord Injury Database, 2001; Sekhon *et al.*, 2001; McDonald and Sadowsky, 2002; Kirshblum, 2002; National Spinal Cord Injury Statistical Center, 2005; Saillant *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Kwon *et al.*, 2006; McCall *et al.*, 2006; Miyanji *et al.*, 2006; Pickett *et al.,* 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Fawcett *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). For anatomical reasons, cervical SCI is less frequently associated with fracture as compared to thoracic or lumbar SCI (in a recent Canadian study the respective estimates were 56%, 100% and 85%), which is why thoracic injuries more often result in complete cord lesions than rostral or caudal injuries (Bohlman, 1979; Bohlman and Ducker, 1992; Tator *et al.*, 1993b; Fehlings *et al.*, 1999; Lovasic, 1999; Brown *et al.*, 2001; National Spinal Cord Injury Database, 2001; McDonald and Sadowsky, 2002; Kirshblum, 2002; Saillant *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Kwon *et al.*, 2006; Miyanji *et al.*, 2006; Pickett *et al.,* 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Fawcett *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). Missile injuries of the spine (which in one study were thoracic in 50% and cervical or lumbar each in 25%, of the cases) are associated with a high mortality and severe permanent neurological deficit even though the spine, most often, remains mechanically stable (DeVivo *et al.*, 1999, 2006; Garshick *et al.*, 2005; le Roux *et al.*, 2005; Biering-Sørensen *et al.*, 2006; Ho *et al.*, 2007). This is due to a higher energy of impaction imparted on a smaller volume of the delicate cord tissue. This is intuitive and based on the laws of physics concerning conservation of energy, and is also lucidly exemplified in the case of vehicle collision-related SCI, where a 2 vehicle-mismatch disfavors the driver or passenger of the smaller, lighter or less sturdy vehicle (Cobb *et al.*, 2005). Pædiatric SCI has increasingly unique characteristics, the younger the victim is. The natural laxity and elasticity of the spinal column of infants or children and their unique neuroanatomical correlates, predispose them to ligamentous and/or parenchymal injuries at cervical level, often without radiographic abnormalities, a condition named *SCIWORA* (Spinal Cord Injury Without Radiographic Abnormalities) (Rekate *et al.*, 1999; Brown *et al.*, 2001; Hayes *et al.*, 2005; Jagannathan *et al.*, 2006; McCall *et al.*, 2006). SCIWORA, despite the lack of bony disruption, carries a grave prognosis (Hayes *et al.*, 2005; McCall *et al.*, 2006). Moreover, it has been estimated that children younger than 9 years of age are more prone to upper cervical injuries than older children or adults, where lower cervical lesions predominate (Lovasic, 1999; Villanueva, 2000; Brown *et al.*, 2001; Hayes *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Jagannathan *et al.*, 2006; Lee *et al.*, 2006; McCall *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). *SCIWORET* (Spinal Cord Injury Without Radiographic Evidence of Trauma) is a related syndrome that is common in adults predisposed by cervical stenosis, ankylosning spondylitis or disc herniation. Since both these syndromes are essentially radiological diagnoses it is natural that advances in neuroimaging have resulted in a significant drop in their incidence. Thus, before the CT era SCIWORET incidence was around 14%, with the advent of CT it dropped to 5%, and nowadays with the widespread use of MRI in SCI management they are rarely seen

(Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b).

The American Spinal Cord Injury Association (*ASIA*) devised in 1982 an Impairment Scale (ASIA-IS, ASIA or *AIS*: A-E; worst-normal) intended for clinical use in the evaluation of patients with SCI (Dobkin, 2003; Marino *et al.*, 2003, 2004; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Ho *et al.*, 2007; Fawcett *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). This was adopted a decade later by the International Spinal Cord Society (Kirshblum, 2002; Lin *et al.*, 2003). AIS-A corresponds to complete sensorimotor loss below the lesion level, AIS-B to some sensory preservation but no motor function below the lesion level, AIS-C to some motor preservation below lesion level with the majority of muscles below 3/5 (in the standard 5 grade-neurological scale for muscle strength) and finally AIS-D to motor preservation with the majority of muscles above or equal to 3/5 (3 corresponds to muscle capacity for movement against gravity) (Noreau and Vachon, 1998; Kirshblum, 2002; Jonsson *et al.*, 2000; Dobkin, 2003; Marino *et al.*, 2003, 2004; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Graves *et al.*, 2006; Ho *et al.*, 2007; Fawcett *et al.*, 2007; Steeves *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). Lastly, AIS-E indicates intact sensorimotor functions. The ASIA Impairment Scale is based on motor and sensory assessement and subscoring. The *ASIA Motor Score* is calculated by summing up the score (0-5 of the standard 5 grade-neurological scale for muscle strength) of each of 20 muscles, for a maximum of 100. The ASIA Motor Score is in fact the sum of two subscores the *Upper Extremity Motor Score* (UEMS; C5-T1) and a *Lower Extremity Motor Score* (LEMS; L2-S1) in each of which 10 key muscles are evaluated (5 on each side, 0-5 for each muscle) for a maximum of 50 (Ditunno *et al.*, 1992; Ditunno *et al.*, 2001; van Tuijl *et al.*, 2002; Biering-Sørensen *et al.*, 2006; Fawcett *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). As is evident, thoracic level motor assessement is lacking in the ASIA examination (Fawcett *et al.*, 2007; Steeves *et al.*, 2007). *Motor level* is defined as the most caudal spinal level that corresponds to a key muscle which scores ≥ 3 given that the key muscle of the segment above scores 5. The *ASIA Sensory Score* is calculated by the summation of a qualitative (0-2) score for light touch and pinprick sensation at each of 28 dermatomes on each side of the body, for a maximum of 112 for each sensory modality. *Sensory level* is defined as the most caudal spinal level that corresponds to a normal sensory score for both sensory modalities (Biering-Sørensen *et al.*, 2006; Fawcett *et al.*, 2007; Ho *et al.*, 2007; Steeves *et al.*, 2007). *Quantitative sensory testing* (QST) techniques can provide finer discrimination of injury level and density, both for the early assessement of degree of impairment, as an adjunct to the less sensitive ASIA sensory score, and for the prospective monitoring of SCI patients, but are tedious and not yet part of mainstream clinical praxis (Davey *et al.*, 2001; Hayes *et al.*, 2002; Biering-Sørensen *et al.*, 2006; Nicotra and Ellaway, 2006; Savic *et al.*, 2006, 2007). A useful alternative to QST is the *electrical potential threshold* test which measures the sensory threshold for each dermatome (Davey *et al.*, 2001; Biering-Sørensen *et al.*, 2006; Savic *et al.*, 2006, 2007). Neurological evaluation of SCI can be complemented by electrophysiological assessment. Electromyographic (EMG) recordings, as well as *somatosensory-evoked potentials* (SSEP) and *motor-evoked potentials* (MEP) provide objective quantitative data about spinal conductivity, residual

function, peripheral plasticity and possibly remyelination (Thompson *et al.*, 1987; Clarke *et al.*, 1994; Marino *et al.*, 1994; Curt *et al.*, 1997; Thomas *et al.*, 1997a; Thomas *et al.*, 1997b; Curt *et al.*, 1998; Curt *et al.*, 1999; Kirshblum *et al.*, 2001; Biering-Sørensen *et al.*, 2006; Fawcett *et al.*, 2007). *Discomplete* SCI is a term used by some electrophysiologists to describe a clinically complete SCI with electrophysiological findings compatible with anatomically incomplete SCI (Sherwood et al., 1992). Finally, a useful term is that of the *zone of partial preservation* (ZPP), which refers to the 1-2 spinal cord segments, situated right below the uppermost common motor and sensory level, that retain some sensorimotor function (Steeves *et al.*, 2007). In SCI it is within the ZPP that spontaneous sensorimotor recovery is primarily observed, even during the first days after injury, to a degree that may warrant a reclassification of neurological level of injury (Fawcett *et al.*, 2007). Recovery within the ZPP is believed to depend on both CNS and/or peripheral plasticity, while recovery beyond the ZPP theoretically would necessitate some degree of axonal regeneration in the CNS (Fawcett *et al.*, 2007). Given the possible presence of cognitive impairment of the patient, because of brain trauma (25% of SCI patients have an associated head injury), alcohol and recreational drug effects, sedation or lack of cooperation, ASIA evaluation at injury time and at 24 hours are inherently unreliable (Tator *et al.*, 1993b). Moreover, *spinal shock* is a transient state of flaccid paralysis that affects the neurotomes below injury level, with reduced or absent reflexes and paralytic bladder (Bach-Y-Rita *et al.*, 1993). Although an ASIA assessment must be performed at injury time and a few more times during the first days postinjury, it has been shown that the critical assessment point is 72 hours postinjury (Brown *et al.*, 1991; Burns *et al.*, 2001; Burns *et al.*, 2003). Several studies have concluded that the 72-hour examination has the most accurate prognostic value, especially in motor complete SCI (AIS-A and B), while the 24-hour and 48-hour examinations were less reliable (Blaustein *et al.*, 1991; Brown *et al.*, 1991; Burns *et al.*, 2001; Burns *et al.*, 2003; Coleman *et al.*, 2004; Biering-Sørensen *et al.*, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b).

According to some estimates c. 50% of cervical and c. 75% of thoracic SCI are graded AIS-A, while c. 35% of cervical and c. 65% of lumbar SCI are classified as AIS-D (Kirshblum 2002; Jonsson *et al.*, 2000; Dobkin, 2003; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Graves *et al.*, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). Mortality is highest for complete cervical injuries and all individuals with AIS-A above C3 are ventilator dependent while many of those with AIS-A immediately below C3 will depend on temporary ventilation support (Hughes, 1974; Bohlman, 1979; Ikata *et al.*, 1989; Bohlman and Ducker, 1992; Tator *et al.*, 1993b; Katoh and el Masry, 1995; Jonsson *et al.*, 2000; National Spinal Cord Injury Database, 2001; Kirshblum, 2002; McDonald and Sadowsky, 2002; Dobkin, 2003; National Spinal Cord Injury Statistical Center, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Johnson *et al.*, 2006; Kwon *et al.*, 2006; McCall *et al.*, 2006; Miyanji *et al.*, 2006; Wyndaele and Wyndaele, 2006; Fawcett *et al.*, 2007; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). However, for those that survive a complete lesion the greatest neurologic recovery occurs in the more rostral injuries, with the likelihood of conversion from AIS-A to AIS-B and AIS-D being double for quadriplegics than paraplegics (Gresham *et al.*, 1986; Ikata *et al.*, 1989;

Marino *et al.*, 1991; Katoh and el Masry, 1995; Waters *et al.*, 1993; Waters *et al.*, 1994; Waters, 1998; Sekhon *et al.*, 2001; McDonald and Sadowsky, 2002; Saillant *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Fawcett *et al.*, 2007; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). In general, the probability of spontaneous recovery correlates well with severity of SCI and the AIS classification, with motor and sensory recovery following roughly the same time course (Waters, 1998).

Spontaneous recovery is least probable for those patients with sensorimotor complete SCI, i.e. AIS-A (about 10% converting to each, AIS-B and AIS-C), more probable for sensory incomplete, i.e. AIS-B (up to 40% for conversion to each AIS-C and AIS-D) and very probable for sensorimotor incomplete SCI, i.e. AIS-C (between 60-80% convert to AIS-D) or AIS-D (up to 95% improve further, although very few AIS-D convert to AIS-E, i.e. normality) (Brown *et al.*, 1991; Waters *et al.*, 1993, 1994, 1995; Waters, 1998; Marino *et al.*, 1999; Burns and Ditunno, 2001; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Fawcett *et al.*, 2007; Ho *et al.*, 2007; Jackson *et al.*, 2008; van den Berg *et al.*, 2010a, 2010b). As for the time frame of this recovery, for up to 60% of patients it is apparent by 2 months postinjury, and for up to 80% of patients by 3 months postinjury (Ditunno *et al.*, 1992; Waters, 1998; Ditunno *et al.*, 2001; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Fawcett *et al.*, 2007; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). The chronic state is by consensus attained after 6 months of functional plateau have elapsed, i.e. at least 1 year postinjury since recovery rates significantly decrease or abate after 6 months postinjury (Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Fawcett *et al.*, 2007; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). However that does not exclude further improvement beyond the 12-months limit. A long-term prospective study demonstrated that 5,6% of patients graded as AIS-A at 1 year improved enough to convert to AIS-B or AIS-C (1%) by 5 years postinjury (Stauffer, 1984; Waters *et al.*, 1993, 1994, 1995; Waters, 1998; Kirschblum *et al.*, 2004; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). Some types of SCI, however, are known to have higher rates of overall sensorimototor recovery, such as *Brown-Séquard*, *cervicomedullary*, *central cord* or *cauda equina* syndromes (Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). *Central cord syndrome* results from incomplete cervical injury with hemorrhagic damage of the midcervical spinal gray matter and medial laminas of the lateral corticospinal tract and is characterized by a greater distal-than-proximal motor deficit in the upper limbs and lesser motor deficit in the lower limbs with walking ability recovered in most cases (Schneider *et al.*, 1954, 1958; Quencer *et al.*, 1992; Levi *et al.*, 1996; Alexeeva *et al.*, 1997). Finally, *transient* SCI syndromes are a rare exception to the usually bleak SCI prognoses. They are mostly encountered in young athletes and are characterized by significant but transient sensorimotor deficit, often in the setting of an underlying cervical stenosis or spondylosis (Brown *et al.*, 2001; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). The mildest transient syndromes are *stingers* and the *burning hand syndrome*, that refer to unilateral

and bilateral upper extremity paresthesias/dysesthesias respectively, secondary to spinal root or brachial plexus traction injury (Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Jagannathan *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007). A more serious transient self-limiting SCI syndrome is *cervical cord neurapraxia* that may occur after hyperextension or hyperflexion injury of the cervical spine and by definition lasts from afew minutes to 48 hours (Torg *et al.*, 1986, 1997; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Jagannathan *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; van den Berg *et al.*, 2010a, 2010b). The term *neurapraxia* refers to transient conduction block of an injured peripheral nerve without axonal degeneration (*neurotmesis* o*r axonotmesis*), so it would seem out of context when employed in the setting of SCI. This term has been borrowed to denominate the neurologic phenomenon of transient, sensorimotor impairment (monoparesis, hemiparesis, paraparesis or even tetraparesis) without radiologic findings or sequelæ, that typically resolves within 15 minutes (Boockvar *et al.*, 2001; Torg *et al.*, 1997; Perks, 2005).

In more practical terms, less than 5% of the victims presenting with complete sensorimotor impairment below the level of injury (AIS-A) will regain the ability to walk and most of them with assisting devices, whereas this proportion rises considerably for those that retain even modest sensorimotor capacity at the time of admission for inpatient rehabilitation (Hughes, 1974; Ikata *et al.*, 1989; Waters *et al.*, 1993, 1994, 1995; Waters, 1998; Marino *et al.*, 1999; Ditunno *et al.*, 2001; Kirshblum, 2002; McDonald and Sadowsky, 2002; Dobkin, 2003; Kirschblum *et al.*, 2004; National Spinal Cord Injury Statistical Center, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Fawcett *et al.*, 2007; Jackson *et al.*, 2008; van den Berg *et al.*, 2010a, 2010b). The *discomplete syndrome* hypothesis has been put forth to explain the aforementioned recovery of ambulation in AIS-A (Sherwood et al. 1992; Dimitijevic et al., 1992). Although the ASIA motor score is considered more reliable than the ASIA sensory score as a prognosticating tool, preservation of *sacral pinprick sensation* (S4-S5) has been shown to be a useful prognostic factor of not only motor recovery in general but ambulation in particular, in patients with non-AIS-A SCI (Marino and Graves, 2004; Oleson *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Jackson *et al.*, 2008). While sacral pinprick sparing in AIS-B patients at the critical evaluation time-point of 3 days postinjury was associated, but not with statistical significance, with higher percentage of ambulation by 26 or 52 weeks, sacral pinprick sparing at 4 weeks postinjury could predict ambulation at 12 months after SCI to a statistically significant degree (Crozier *et al.*, 1991; Waters *et al.*, 1993, 1994, 1995; Katoh and el Masry, 1995; Marino *et al.*, 1999; Oleson *et al.*, 2005; Biering-Sørensen *et al.*, 2006; DeVivo *et al.*, 2006; Wyndaele and Wyndaele, 2006; Ho *et al.*, 2007; Jackson *et al.*, 2008; Jabbour *et al.*, 2008; van den Berg *et al.*, 2010a, 2010b). In one study, about 54% of AIS-B patients with sacral sparing 3-7 days postinjury improved in motor function to convert to AIS-C/D, while of the AIS-C/D patients with sacral sparing at 3 days postinjury up to 86% would eventually regain ambulation. In fact, if also a lower limb (L2-S1) maintained pinprick sensation in >50% of its dermatomes then ambulation became even more probable (Waters *et al.*, 1993, 1994, 1995; Marino *et al.*, 1999; Oleson *et al.*, 2005; Jackson *et al.*, 2008). Another

prognostic factor is *age* at the time of injury. Among patients with central cord syndrome 91% of those younger than 50 regain ambulation, while only 41% of those older than 50 at the time of injury regain ambulation (Waters *et al.*, 1995; Marino *et al.*, 1999; Rekate *et al.*, 1999; Lovasic, 1999; Villanueva, 2000; Brown *et al.*, 2001; National Spinal Cord Injury Statistical Center, 2005; Jagannathan *et al.*, 2006; Jackson *et al.*, 2008; Jabbour *et al.*, 2008; van den Berg *et al.*, 2010a, 2010b).

Unfortunately, SCI not only leads to permanent neurological sequelæ and functional impairment but also other health complications that are in fact the prinicipal reasons for rehospitalization of a patient with stabilized sensorimotor deficit. Life with SCI is marred by deep vein thrombosis, pressure sores, recurrent urinary tract infections (UTI), bowel dysfunction, autonomic dysreflexia, spasticity, osteoporosis with falls and fractures, cardiorespiratory complications, posttraumatic myelopathy and syringomyelia, chronic neuropathic pain, impotence, infertility, depression and even the metabolic syndrome among other ailments (Eljamel *et al.*, 1994; Yarkony *et al.*, 1994; Young, 1994; Catz *et al.*, 1997; Christensen *et al.*, 1997; Nielsen *et al.*, 1999; Sköld *et al.*, 1999; Westgren *et al.*, 1999; McDonald and Sadowsky, 2002; Dijkers *et al.*, 2003; Itzkovich *et al.*, 2003; Chiodo *et al.*, 2005; Cotton *et al.*, 2005; Cruz-Almeida *et al.*, 2005; Rintala *et al.*, 2005; Kogos *et al.*, 2005; Lammertse *et al.*, 2005; Manns *et al.*, 2005; Nash *et al.*, 2005; Bach *et al.*, 2006; Barat *et al.*, 2006; Biering-Sorensen *et al.*, 2006; Brotherton *et al.*, 2007; Claydon *et al.*, 2006; de Groat *et al.*, 2006; Girard *et al.*, 2006; Hanley *et al.*, 2006; Lechner *et al.*, 2006; Paker *et al.*, 2006; Patki *et al.*, 2006, 2007; Raichle *et al.*, 2006; Salomon *et al.*, 2006; Stroud *et al.*, 2006; Taricco *et al.*, 2006; Vall *et al.*, 2006; Widerstrom-Noga *et al.*, 2006; Zinck *et al.*, 2006; van den Berg *et al.*, 2010a, 2010b). To give a number sense, in one large study, rehospitalization after the acute phase (which can reach 10%) was the result of spasticity (25%), need for further rehabilitation (21.4%), pressure sores (17.9%), UTI (16.1%), spinal surgery (8.9%), urinary system surgery (5.4%) and finally pain (5.4%) (Paker *et al.*, 2006).

The psychological, family-, work- and social-life repercussions and ramifications of SCI, such as sexual impotence, invalidity, career set-backs, underemployment or unemployment, weigh heavily upon patients and relatives (Gresham *et al.*, 1986; Marino *et al.*, 1991; Berkowitz *et al.*, 1998; Dijkers *et al.*, 2003; Fisher *et al.*, 2005; Noreau *et al.*, 2005; Post and Noreau, 2005; Raj *et al.*, 2006; Craig *et al.*, 2009). Depression and other psychiatric morbidity are more common in SCI patients, but were also found to be overepresented and associated to poor leisure satisfaction in members of their family or even their unrelated care givers (Boekamp *et al.*, 1996; Berkowitz *et al.*, 1998; Post and Noreau, 2005; Raj *et al.*, 2006; Stroud *et al.*, 2006). Thus, social, emotional and moral support, for patient and family, is imperative not only during the acute and subacute phases after SCI but also later on (DeSanto-Madeya, 2006). This support should aim at reviving in the patient a sense of hope and joy for life, and should be on offer in the clinical setting of rehabilitation by specially trained personnel but also in the social and family context (Noreau *et al.*, 2005; DeLisa, 2005; Lohne and Severinsson, 2006; DeSanto-Madeya, 2006; Damiano and DeJong, 2009). With increasing life expectancy after SCI, social participation becomes the next major goal of

rehabilitation after improving the ADL score (Activities of Daily Living), and also a means to an end, a self-goal for both patient and society (Catz *et al.*, 1997; Berkowitz *et al.*, 1998; Dijkers *et al.*, 2003; Itzkovich *et al.*, 2003; Noreau *et al.*, 2005; Damiano and DeJong, 2009). Despite their disability individuals with SCI receive their highest daily satisfaction within the social domain and it has been shown that involvement in social sports and physical recreation greatly improves their quality of life (Berkowitz *et al.*, 1998; Anderson, 2004; Nash, 2004; Fisher *et al.*, 2005; Tasiemski *et al.*, 2005; Post and Noreau, 2005).

Improving quality of life (QoL), therefore, so severely affected on the physical, mental and social levels, becomes the primary concern for patients and the major consideration and goal for rehabilitation workers. After the catastrophic experience of SCI and the partial or total incapacitation it entails, even small functional gains can make a big difference in terms of QoL for the patient (Kakulas, 1999; Dijkers *et al.*, 2003; Dobkin, 2003; Anderson, 2004; Nash, 2004). Though SCI victims tend to hierarchize QoL improvements depending on their individual functional impairment, breathing, bladder and bowel voiding, hand use and sexual function are always at the top of their wish-lists (Dijkers *et al.*, 2003; Fisher *et al.*, 2005). These tendencies have been studied more thoroughly to obtain a 'patient's view' on QoL after SCI in order to prioritize rehabilitation subgoals (Dijkers *et al.*, 2003; Anderson, 2004; Nash, 2004; Fisher *et al.*, 2005). It was found, not unexpectedly, that the most determining factors for the QoL of paraplegics and quadriplegics differed significantly (Anderson, 2004; Fisher *et al.*, 2005). Hand or arm function ranked higher among quadriplegics whereas recovery of sexual function ranked highest among paraplegics followed by recovery of bladder/bowel control (Anderson, 2004; Fisher *et al.*, 2005). What is important, regaining these abilities outranked elimination of chronic pain, spasticity or autonomic dysreflexia, which means that patients would rather suffer somatically and have small increments in function, than simply avoid the suffering. During childhood, children with SCI rated their QoL higher than their parents did, without statistical difference between quadriplegic and paraplegic children (Rekate *et al.*, 1999; Anderson *et al.*, 2006; Oladeji *et al.*, 2007; Damiano and DeJong, 2009).

Today, in developed countries, life expectancy for SCI victims that survive the injury and its complications, is increasingly approaching that of individuals without SCI (Devivo *et al.*, 1999; Kirshblum, 2002; McDonald and Sadowsky, 2002; National Spinal Cord Injury Statistical Center, 2005; Garshick *et al.*, 2005; Saillant *et al.*, 2005). However, what goes for longevity cannot also be said for QoL since most SCI survivors will live with significant physical disability and mental burden (Sekhon *et al.*, 2001; McDonald and Sadowsky, 2002; National Spinal Cord Injury Statistical Center, 2005; Saillant *et al.*, 2005; Craig *et al.*, 2009). Anyone who has worked in an emergency or admission hospital unit for SCI patients has certainly witnessed the devastation of these, often young, people (Lohne and Severinsson, 2006). By the time they enter a rehabilitation facility, the new reality has barely begun to sink in as they embark on a sisyphean task of readjusting to their new lives (DeLisa, 2005; Capoor and Stein, 2005; DeSanto-Madeya, 2006; Raj *et al.*, 2006; Furlan and Fehlings, 2006; Craig *et al.*, 2009).

When one happens to meet the same patients months or years later it is often remarkable to observe the astounding change towards a more optimistic view of life (Noreau *et al.*, 2005; DeLisa, 2005; DeSanto-Madeya, 2006; Raj *et al.*, 2006; Wee, 2006; Craig *et al.*, 2009). 'Starting-over' after SCI is ultimately more an uphill battle of the mind than the body. Patients have to fight back at their self-pity, regrets and recurrent depression at the same time they endure bodily sufferings and face the ills of social injustice, isolation or rejection. Not everyone will manage well at this prerogative but the truth is that most will, reclaiming a new state of normalcy and life satisfaction, ultimately enjoying productive and rewarding lives. The same is true for many individuals with pediatric-onset SCI, who manage to achieve stability of independent living, employment, and life satisfaction as adults (Anderson *et al.*, 2006; Oladeji *et al.*, 2007).

GENERAL BACKGROUND:
PATHOPHYSIOLOGICAL MECHANISMS IN SCI

The spinal cord is a vital but fragile organ in vertebrate species. Its elongated anatomical structure makes it vulnerable to injury along its entire length, which is why evolutionary providence has had the cord cushioned by the cerebrospinal fluid, enveloped by the meningeal coverings and extradural fat and enclosed in the flexible but sturdy vertebral column. Despite this formidable barrier, high-energy impact, missiles or penetration with sharp instruments can lead to injury of the spinal cord. The whole construction is rather efficient though since only a small percentage of spinal injuries are accompanied by cord damage. Traditionally, SCI is viewed as the consequence of a chronologically two-phase process, comprising primary and secondary injury (Tator, 1991, 1992, 1995, 1996; Kakulas, 1999, 2004; Profyris *et al.*, 2004).

PRIMARY INJURY, CLINICAL SYNDROMES AND SPINAL SHOCK

The initial physical trauma on the cord tissue results in *primary injury* and encompasses the damage caused by both the initial *impact* and the subsequent *compression*. The spinal cord may be subjected to a combination of forces (flexion, hyperextension, rotation, traction, shear or compression) with resultant trauma of graded severity (Schwab and Bartholdi, 1996; Coleman and Geisler, 2004; Kakulas, 1999, 2004; Profyris *et al.*, 2004; Choo *et al.*, 2008). *High-speed impact* SCI may result in ligamentous injury, vertebral luxation, atlantoaxial dissociation/dislocation, spinal fracture with fragment dislocation, burst fracture or intervertebral disc herniation, with or without cord compression (Schwab and Bartholdi, 1996; Coleman and Geisler, 2004; Kakulas, 1999, 2004; Profyris *et al.*, 2004; Choo *et al.*, 2008). The severity of the primary injury is proportionate to the amount of energy transfered to the cord tissue and inversely proportionate to the duration (Δt) of this energy transfer (Schwab and Bartholdi, 1996; Coleman and Geisler, 2004). Therefore, in high-velocity missile injuries such as by gun-shot or shrapnel the spinal cord may be completely transected without actually being touched by the missile whose energy is dissipated fats over a large area and leads to a *blast effect* that damages the cord indirectly. On the other hand, in stab-wound injuries, for a SCI to occur the blade would have to enter the spinal canal, puncture the meninges and penetrate the cord parenchyma, which is less likely to happen since the anatomical structure of the vertebral column usually drives the offending blade to one side or the other of the column. However, SCI can even result from low-energy insults such as minimal trauma by fall, violence or other mishaps, especially in predisposed elder individuals, with risk factors such as degenerative spondylosis, stenosis of the spinal canal, hypertrophied ligamentum flavum or atlantoaxial instability as in rheumatoid arthritis (Schwab and Bartholdi, 1996; Coleman and Geisler, 2004; Kakulas, 1999, 2004; Profyris *et al.*, 2004; Choo *et al.*, 2008). Finally, SCI of purely ischæmic nature (spinal infarct), can accompany direct trauma to the the radicular feeding arteries of the spinal cord after abdominal or chest

penetrating wounds (Schwab and Bartholdi, 1996; Kakulas, 1999, 2004; Profyris *et al.*, 2004).

Because of the differences in the transverse area of the spinal cord along its length, the free (intradural and extradural) *space available for the cord* (SAC) inside the spinal canal after a fracture depends on the injured region of the spine (Tator, 1995, 1996; Kakulas, 1999, 2004; Profyris *et al.*, 2004). This is one reason why different fracture patterns or levels of injury carry different prognoses. Thus, at the level of the foramen magnum and atlantoaxial unit (C1-2) the SAC is large relative to the regional cross-sectional area of the spinal cord and so most fractures of the occipital condyles or C1 and C2 do not result in SCI, with the exception of *atlantoaxial dissociation/dislocation*, usually after a ligamentous injury in which axial traction is applied to the cord often with catastrophic results (Coleman and Geisler, 2004; Kakulas, 1999, 2004; Profyris *et al.*, 2004; Choo *et al.*, 2008). In mild cases only bulbar symptoms and some long tract symptoms may occur, but often atlantoaxial junction injuries are fatal. The SAC gradually decreases as one reaches the lower cervical zone (cervical enlargement) and anterior vertebral dislocations or fracture dislocations at this region lead more often to SCI, while laminar fractures which can actually increase the SAC or spinous process fractures are less likely to cause SCI. The SAC reaches its minimum in the thoracic zone with an increased risk of SCI after vertebral or fracture dislocation. At the transition zone between thoracic and lumbar regions the SAC increases again in order to accommodate the lumbosacral enlargement. The conus medullaris commonly found at the upper lumbar level (L1-2) is susceptible to injuries that range from mild to complete. Finally, the cauda equina in the lumbosacral zone has a safety margin of SAC and is therefore less vulnerable to injury by compression. Thus, burst fractures or fracture dislocations of the lower lumbar zone with as much as 75% canal compromise, may spare the cauda equina and not result in neurological symptoms (Coleman and Geisler, 2004; Kakulas, 1999, 2004; Profyris *et al.*, 2004; Choo *et al.*, 2008). Consequently, various incomplete SCI syndromes have been characterized as a result of the different level or pattern of injury. Although most of them were characterized by Schneider in the 50's, for some of them the exact anatomical basis is still controversial. The major incomplete SCI syndromes encountered in a trauma center are the *cervicomedullary* syndrome, the *central cord* syndrome, the *anterior cord* syndrome, the *posterior cord* syndrome, the *Brown-Séquard* syndrome, the *conus medullaris* syndrome and the *cauda equina* syndrome. The transient SCI syndromes and the SCIWORA or SCIWORET syndromes mentioned earlier are rare.

The primary injury on the cord parenchyma *per se* is roughly classified into concussion, contusion, laceration or maceration. *Concussion* (or *commotion*) signifies a transient functional deficit in the absence of anatomical damage demonstrable with the available neuroimaging techniques (Schwab and Bartholdi, 1996; Coleman and Geisler, 2004; Kakulas, 1999, 2004; Profyris *et al.*, 2004). Diffuse axonal strain or damage, resulting from instantaneous compression, shear or stretch forces, most probably lies behind this type of mild injury (Freeman and Wright, 1953; Schwab and Bartholdi, 1996; Choo *et al.*, 2008). *Contusion* is always accompanied by anatomical damage because of cord compression with or without laceration of the meninges and cord tissue, e.g. after traffic

or diving accidents (Freeman and Wright, 1953; Schwab and Bartholdi, 1996; Kakulas, 1999, 2004; Profyris *et al*., 2004 Choo *et al*., 2008). *Laceration* or even *transection* of the cord can occur after marked fracture dislocation or foreign body penetration, e.g. in stabbing or gunshot injuries, but it must be stressed that a serious degree of contusion is always present even in these injuries (Schwab and Bartholdi, 1996; Kakulas, 1999, 2004; Profyris *et al.*, 2004Choo *et al*., 2008). The term *maceration* can be used when laceration and severe contusion occur simultaneously, while Cajal uses the term *trituration* (Cajal, 1928). The majority of human spinal injuries involve cord compression with or without laceration and naturally this is the type of primary injury that scientists strive to replicate in animal models. The endresult of a typical compression injury of the human spinal cord is the destruction to a certain degree of dorsal and ventral horn gray matter over one or more levels, the partial or complete disconnection of ascending and descending white matter tracts and the avulsion or tearing of dorsal and ventral roots at the level of the lesion (Kakulas, 1999, 2004; Profyris *et al.*, 2004).

At the microscopic level the mechanical forces implicated in primary injury translate into neuronal damage or axotomy, damage to neuroglial cells, vascular damage on smal-calibre vessels and indirect damage by ischaemia. Capillary or venule shearing leads to haemorrhagic pools (*petechiæ*) predominantly in the highly vascularized gray matter (Tator, 1991, 1992, 1995, 1996; Kakulas, 1999, 2004; Profyris *et al.*, 2004). The softer consistency of the grey matter, attributable in part to its higher vascularity and lower membrane content compared with white matter, leads to an uneven dissipation of impact energy with greater tissue displacement rostrocaudally along the central grey matter column (Blight, 1988; Tator, 1991, 1992, 1995, 1996; Kakulas, 1999, 2004; Profyris *et al.*, 2004). These biomechanical properties of primary injury may explain the expansion of central cord haemorrhage and necrosis during secondary injury. White matter is directly damaged during primary injury too. Contusion of the spinal cord leads to stretching and distortion of fiber bundles and ultimately *diffuse axonal injury*. The special biomechanics of contusive injury result in the relative sparing of peripheral (subpial) axons under the epicenter of impact as compared to the central white matter surrounding the gray matter, which is more severely damaged (Young, 2002). Obviously, this is not true whenever laceration is involved, whereby the peripheral white matter is damaged first. The myelination status of axons is relevant to their durability after SCI. Thus, after acute contusive SCI , large myelinated fibres suffer more than unmyelinated ones, because the nodes of Ranvier bear the brunt of the stretching forces with resultant perinodal microtubule disruption and axonal disintegration (Blight, 1988; Maxwell, 1996; Gruner *et al.*, 1996). However, under chronic compressive injury, small unmyelinated fibres are more vulnerable, while large myelinated fibres are relatively spared (Blight and Decrescito, 1986).

Spinal shock is the clinical syndrome characterised by flaccid paralysis with areflexia, somatosensory anæsthesia, loss of sympathetic autonomic function and bladder/ bowel paralysis after major SCI to the cervical or upper thoracic spinal cord (Atkinson and Atkinson, 1996). The syndrome is transient with variable duration but, as a rule, the more severe and the higher the lesion the greater its gravity and duration. In general, sensory

loss resolves within 1 h after injury and any residual sensorimotor deficit beyond the first postinjury hours should be attributed to primary injury of the cord. In the best case scenario of the transient SCI syndromes encountered in athletes, sensorimotor abnormalities resolve within minutes from injury. On the other hand the reflex loss and dysautonomia may last from days to months depending on the severity and level of lesion (Bach-Y-Rita, 1993; Atkinson and Atkinson, 1996). Indeed, autonomic dysregulation which results in systemic hypotension, cutaneous hyperæmia, and bradycardia due to unopposed vagotonia can be life-threatening, particularly during the first week after injury. While the spinal shock syndrome results from a general conduction block, the exact pathophysiological mechanism behind it remains unclear (Bach-Y-Rita, 1993; Atkinson and Atkinson, 1996). Hypoperfusion, alterations of ionic homeostasis and neurotransmitter effects have all been proposed. The occurrence of spinal shock can confound the neurological assessement as long as it lasts. Moreover, in the polytraumatic patient the neurogenic circulatory shock secondary to spinal shock may compound or be confused for hypovolemic shock due to internal hemorrhage. It is important to distinguish between the two because their treatments differ. Thus, hypovolemic shock is accompanied by hypotension and tachycardia while neurogenic shock by hypotension and bradycardia because the vagotonia inhibits the normal physiological response of heart rate acceleration to increase cardiac output. While hypovolemic shock responds to volume repletion, neurogenic shock responds better to sympathomimetics than to volume replacement alone (Bach-Y-Rita, 1993; Schwab and Bartholdi, 1996).

SECONDARY INJURY

The term *secondary injury* or *secondary degeneration* is nowadays used to encompass all the cellular and subcellular pathophysiological events that progressively exacerbate the damage, neuronal or non-neuronal, already effected by primary injury (Tator and Fehlings, 1991; Tator, 1991, 1992, 1995, 1996; Kakulas, 1999, 2004; Profyris *et al.*, 2004). The temporospatial distinction between primary and secondary injury is artificial, since secondary injury essentially begins right after the primary insult (Schwab and Bartholdi 1996; Zhang *et al.*, 1997; Tator and Fehlings, 1991; Schwartz and Fehlings, 2002; Blight, 1992; Tator, 1992; Schnell *et al.*, 1999; Bilgen *et al.*, 2000; Olson, 2002). Before the term 'secondary degeneration' had acquired its current meaning, Cajal used it to refer to the process of 'Wallerian-type degeneration' of long fiber tracts after a CNS lesion (Cajal, 1928). Cajal recognized two types of axonal degeneration after lesions in the CNS. A relatively slow one that appeared late and was the CNS equivalent to *Wallerian degeneration* of the PNS, which Cajal called *secondary degeneration*, and affected the distal stump (Cajal, 1928). And an extremely rapid axonal degeneration process, first described by Schiefferdecker, that was the immediate result of the traumatic insult on the axons and extended to a small but variable distance from the "lips of the wound" in the *distal and proximal* stumps (Cajal, 1928), and corresponded to what we mean today by the term *acute axonal degeneration* (AAD) (Kerschensteiner *et al.*, 2005; Misgeld, 2005; Misgeld *et al.*, 2007).

The concept of a secondary injury mechanism complicating acute SCI was postulated by Allen in 1911 when he found that removal of posttraumatic hematomyelia in dogs after acute contusion SCI led to improvement in neurologic function (Allen, 1911). To explain these findings Allen speculated the generation of a 'biochemical factor' in the hemorrhagic necrotic tissue with putative pathogenic effects on healthy cord tissue (Allen, 1914). Since that time, and especially during the last 20 years secondary injury after SCI has been studied extensively in animal models at the histological and biochemical levels and lately also with the use of microarrays. A full understanding of secondary injury is of utmost importance for a rational approach to neuroprotection but also neuroregeneration (Reier *et al.*, 1988; Blight, 1988, 1992; Schwab and Bartholdi 1996; Tator, 1991, 1992, 1995, 1996; Kakulas, 1999, 2004; Olson, 2002; Profyris *et al.*, 2004). Secondary injury after SCI is not invariant in the different species that have been studied (Zhang *et al.*, 1996; Guth *et al.*, 1999; Steward *et al.*, 1999; Hausmann *et al.*, 2003; Sroga *et al.* 2003; Profyris *et al.*, 2004; Byrnes *et al.*, 2010). Thus, certain aspects of glial scar formation and inflammatory reactions after SCI differ remarkably between rats and mice and between different strains of the same species (Zhang *et al.*, 1996; Guth *et al.*, 1999; Steward *et al.*, 1999; Hausmann *et al.*, 2003; Sroga *et al.* 2003). However, as shown by clinicopathological studies of human SCI, when it comes to the basic pathophysiologic mechanisms of secondary injury one can safely extrapolate from experimental data to the human condition. We will here focus on these basic mechanisms of secondary injury after a typical contusion injury.

Vascular changes, ischæmia, hæmorrhage, infarction, œdema and reperfusion injury

SCI in the acute stage leads to changes into both systemic and local circulation with the former further aggravating the latter (Griffiths, 1973; Sandler and Tator, 1976a, 1976b, 1976c, 1976d; Green *et al.*, 1981; Hayashi *et al.*, 1983a, 1983b, 1983c; Tator and Fehlings, 1991; Tator, 1992; Tator, 1992, 1995, 1996, 1998; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002; Choo *et al.*, 2008). In the hyperacute stage of experimental SCI, i.e. immediately after injury, spinal cord blood flow (SCBF) at the lesion site is greatly reduced and this condition aggravates over the first hours to the point of veritable ischæmia (Griffiths, 1973; Sandler and Tator, 1976a, 1976b, 1976c, 1976d; Ducker *et al.*, 1978a, 1978b, 1978c; Němecek, *et al.*, 1978; Rivlin and Tator, 1978; Dolan *et al.*, 1980a, 1980b; Dolan and Tator, 1980, 1982; Anderson *et al.*, 1982; Hayashi *et al.*, 1983a, 1983b, 1983c; Wallace and Tator, 1986; Fehlings and Tator, 1989; Holtz *et al.*, 1989; Tator and Fehlings, 1991; Tator, 1992, 1995, 1996; Ross and Tator, 1993; Anthes *et al.*, 1996; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002). Hypoperfusion and ischæmia at and around the lesion site may persist for over 24 hours as shown in rat and monkey models (Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b, 1973c; Griffiths, 1973; Sandler and Tator, 1976a, 1976b, 1976c, 1976d; Ducker *et al.*, 1978a, 1978b, 1978c; Němecek, *et al.*, 1978; Rivlin and Tator, 1978; Dolan and Tator, 1980, 1982; Dolan *et al.*, 1980a, 1980b; Anderson *et al.*, 1982; Hayashi *et al.*, 1983a, 1983b, 1983c; Tator,1992, 1995, 1996; Ross and Tator, 1993; Anthes *et al.*, 1996; Tator

and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002). Because of the richer vascularity of the grey matter, under normal conditions grey/white matter blood flow is maintained at a 3:1 ratio approximately (Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b, 1973c; Griffiths, 1973; Ducker *et al.*, 1978a, 1978b, 1978c; Němecek, *et al.*, 1978; Hayashi *et al.*, 1983a, 1983b, 1983c; Dolan and Tator, 1980, 1982; Dolan *et al.*, 1980a, 1980b; Anderson *et al.*, 1982; Banik *et al.*, 1985; Wallace and Tator, 1986; Koyanagi *et al.*, 1993a, 1993b, 1993c; Tator, 1992, 1995, 1996; Ross and Tator, 1993; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002). *In vivo* studies have confirmed that grey matter and its immediately adjacent white matter are particularly susceptible to posttraumatic hypoperfusion and hypoxia compared to peripheral white matter, whereas in the latter both hyperæmia and ischæmia have been reported (Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b, 1973c; Griffiths, 1973; Ducker *et al.*, 1978a, 1978b, 1978c; Němecek, *et al.*, 1978; Rivlin and Tator, 1978; Dolan and Tator, 1980, 1982; Anderson *et al.*, 1982; Hayashi *et al.*, 1983a, 1983b, 1983c; Banik *et al.*, 1985; Wallace and Tator, 1986; Holtz *et al.*, 1989; Tator, 1992, 1995, 1996; Koyanagi *et al.*, 1993a, 1993b, 1993c; Ross and Tator, 1993; Anthes *et al.*, 1996; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002). Thus, blood flow in the white matter decreases within 5 minutes of SCI but begins to normalize within 15 minutes and once normalized within the first hour postinjury it remains so during the next 24 hours. On the contrary, as shown by microangiography and fluorescent tracer studies, in the already hæmorrhagic (from primary injury) grey matter, blood flow severely diminishes within 1 hour after injury and remains low for the next 24 hours (Fried *et al.*, 1971; Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b, 1973c; Griffiths, 1973; Sandler and Tator, 1976a, 1976b, 1976c, 1976d; Ducker *et al.*, 1978a, 1978b, 1978c; Němecek, *et al.*, 1978; Rivlin and Tator, 1978; Lohse *et al.*, 1979; Senter *et al.*, 1978, 1979; Senter and Venes *et al.*, 1978, 1979; Dolan and Tator, 1980, 1982; Anderson *et al.*, 1982; Hayashi *et al.*, 1983a, 1983b, 1983c; Holtz *et al.*, 1989; Tator and Fehlings, 1991; Tator, 1992, 1995, 1996; Koyanagi *et al.*, 1993a, 1993b, 1993c; Ross and Tator, 1993; Anthes *et al.*, 1996; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002b).

Hypoperfusion is the result of many factors operating in a vicious circle. First, ischæmia is certainly promoted by the multiple *hæmorrhages* and *ecchymoses* that occur in the central region of the spinal cord because of the primary injury (Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b; Ducker *et al.*, 1978a, 1978b, 1978c; Dolan and Tator, 1980, 1982; Dolan *et al.*, 1980a, 1980b; Green *et al.*, 1981; Anderson *et al.*, 1982; Wallace and Tator, 1986; Tator, 1992, 1995, 1996; Koyanagi *et al.*, 1993a, 1993b, 1993c; Ross and Tator, 1993; Anthes *et al.*, 1996; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002; Choo *et al.*, 2008). The primary injury leads to direct mechanical disruption of the arterioles, capillaries and venules. Microangiographic studies of the effects of the primary injury on spinal cord blood flow in humans and experimental animals with SCI show that, although the large arteries remain patent, the local microcirculation is severely compromised in smaller vessels (mainly capillaries and venules) (Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b; Griffiths, 1973; Ducker *et al.*, 1978a, 1978b, 1978c; Green *et al.*, 1981; Anderson *et al.*, 1982; Banik *et al.*, 1985; Wallace and Tator, 1986; Tator, 1992; Koyanagi *et al.*, 1993a, 1993b, 1993c; Ross and Tator, 1993; Dumont *et al.*, 2001a, 2001b, 2002; Young,

2002). This was also shown by histopathological studies that detected extravasated albumin and fibronectin in and around the lesion area after spinal cord compression injury (Dohrmann and Allen, 1975; Green *et al.*, 1981; Hsu *et al.*, 1985; Farooque *et al.*, 1992; Koyanagi *et al.*, 1993, 1997; Dumont *et al.*, 2001a, 2001b, 2002). In contusion SCI, posttraumatic hæmorrhage begins within a few minutes after primary injury (Banik *et al.*, 1985; Tator, 1995, 1996). The hæmorrhagic front at the site of trauma begins to expand as early as 2 hours post injury with the appearance of numerous petechial haemorrhages mainly in the grey matter (Ducker *et al.*, 1978a, 1978b, 1978c; Wallace and Tator, 1986; Tator and Fehlings, 1991; Tator, 1992, 1995, 1996, 1998; Ross and Tator, 1993; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002). Hæmorrhage progresses during the first day after injury with enlargement and confluence of the hæmorrhagic fronts (Griffiths, 1973; Tator, 1992, 1995, 1996, 1998; Ross and Tator, 1993; Koyanagi and Tator, 1997; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002). Hæmorrhagic pools are still visible at 3 days but by the 8th day they have been resorbed and the lesion site is filled with tissue debris (Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b, 1973c; Ducker *et al.*, 1978a, 1978b, 1978c; Anderson *et al.*, 1982; Banik *et al.*, 1985; Beattie *et al.*, 2000, 2002; Dumont *et al.*, 2001a, 2001b, 2002). Second, neurogenic shock and loss of vascular autoregulation exacerbate hypoperfusion and ischæmia (Lohse *et al.*, 1979; Senter *et al.*, 1978, 1979; Senter and Venes *et al.*, 1978, 1979; Young, *et al.*, 1980; 1982; Anderson *et al.*, 1982; Tator and Fehlings 1991; Tator, 1992, 1995, 1996, 1998; Koyanagi *et al.*, 1993a, 1993b, 1993c; Koyanagi and Tator, 1997; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002). Neurogenic shock, that lasts several days after primary injury leads to systemic hypotension which worsens blood flow in the lesion zone (Guha *et al.*, 1985. 1987a, 1987b, 1989a, 1989b; Guha and Tator, 1988; Atkinson and Atkinson, 1996; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002; Choo *et al.*, 2008). Moreover, alterations in local vascular autoregulation, with vasoconstriction, vasospasm and reduced microcirculatory flow are also believed to occur, secondary to the local secretion of vasoactive substances suchs as thromboxanes, leucotrienes, kinins, kallikreins, serotonin, endogenous opioids and *platelet activating factor* (PAF) to name a few (Tator and Fehlings, 1991, Olsson *et al.*, 1992; Tator, 1992, 1995, 1996, 1998; Young, 2002). Vasospasm could also be the result of mechanical damage of arteries and arterioles and serve as a reflex mechanism to prevent bleeding, as is known to occur in wounds in general (Tator and Fehlings, 1991; Tator, 1992, 1995, 1996, 1998; Koyanagi *et al.*, 1993). Thirdly, microthromboses may exacerbate local ischæmia and lead to microinfarcts (Němecek, *et al.*, 1978; Green *et al.*, 1981; de la Torre, 1981; Anderson *et al.*, 1982; Tator, 1992, 1995, 1996; Young, 2002). Microthromboses could be favored by damage in the vessel walls and endothelium and of course by local mediators that have proven effects on the coagulation pathway such as thromboxanes, leucotrienes, kinins and PAF (Tator and Fehlings, 1991, Olsson *et al.*, 1992; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002). Finally, infarction may be the cumulative result of all the abovementioned mechanisms of ischæmia, i.e. local hypotension, bleeding and vasospasm (de la Torre, 1981; Koyanagi *et al.*, 1993; Tator, 1992, 1995, 1996, 1998; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002; Choo *et al.*, 2008).

Infarction leads to necrosis of the directly affected tissue and within 6 hours the formation

of a *penumbra* and œdema around the zone of necrosis (Lemke *et al.*, 1987; Lemke and Faden, 1990; Tator, 1992, 1995, 1996, 1998; Tator and Koyanagi, 1997; Guth *et al.*, 1999; Choo *et al.*, 2008). In experimental animals with SCI, swelling initially involves the central portion of the cord and later spreads in a centrifugal fashion into white matter (Green and Wagner, 1973; Green *et al.*, 1981; Lemke *et al.*, 1987; Lemke and Faden, 1990). Œdema is maximal in the first days after SCI injury affecting predominantly the white matter (Wagner *et al.*, 1971; Green and Wagner, 1973; Lemke and Faden, 1990; Tator, 1992, 1995, 1996, 1998, 1999; Guth *et al.*, 1999; Dumont *et al.*, 2001a, 2001b, 2002). Because of the swelling, grey and white matter become softer and anatomical distinction between the two is lost at the vicinity of injury (Tator, 1992, 1995, 1996, 1998; Dumont *et al.*, 2001a, 2001b, 2002). *Vasogenic œdema* can also result from BBB disruption, endothelial damage or the actions of vasoactive amines on the vessel wall not directly related to infarction (Wells *et al.*, 1978; Anderson *et al.*, 1982; Lemke *et al.*, 1987; Lemke and Faden, 1990; Tator and Fehlings, 1991; Farooque *et al.*, 1992; Olsson *et al.*, 1992; Tator, 1992, 1995, 1996, 1998; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002; Choo *et al.*, 2008). Alterations in endothelial cell function with increased vascular permeability and extracellular œdema of the vasogenic type have been well documented in experimental SCI, e.g. by immunohistochemical detection of plasma proteins, such as albumin or fibronectin, that have escaped into the CNS parenchyma through the leaky microvessels (Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b, 1973c; Griffiths and Miller, 1974; Lewin *et al.*, 1974; Dohrmann and Allen., 1975; Beggs *et al.*, 1975; Wells, 1978; Wells *et al.*, 1978; Stewart and Wagner, 1979; Green *et al.*, 1981; Anderson *et al.*, 1982; Hsu *et al.*, 1985; Iizuka *et al.*, 1987; Lemke *et al.*, 1987; Lemke and Faden, 1990; Farooque *et al.*, 1992; Tator, 1992, 1995, 1996, 1998; Ross and Tator, 1993; Tator and Koyanagi, 1997; Dumont *et al.*, 2001a, 2001b, 2002). Œdema results in further tissue compression with venule collapse once the interstitial pressure exceeds the venous blood pressure. Thus, œdema causes secondary hypoperfusion and ischæmia and a vicious-circle effect (Lewin *et al.*, 1974; Lemke *et al.*, 1987; Lemke and Faden, 1990; Farooque *et al.*, 1992; Tator, 1992, 1995, 1996, 1998, 1999; Tator and Koyanagi, 1997; Young, 2002; Choo *et al.*, 2008). Eventually, microcirculation and tissue perfusion is restored in the first few days after SCI but reperfusion exacerbates tissue damage and secondary injury (Anderson *et al.*, 1982). The pathophysiological mechanism is believed to be similar to the *reperfusion injury* that occurs after myocardial or cerebral infarction and is attributed to a dramatic rise in *reactive oxygen intermediates* (ROIs) with catastrophic results for the postischæmic tissue (Tator, 1995, 1996, 1998; Tator and Koyanagi, 1997; Tator and Fehlings, 1999; Carlson *et al.*, 1997, 1998; Guth *et al.*, 1999; Dumont *et al.*, 2001a, 2001b, 2002; Choo *et al.*, 2008).

Metabolic changes, acidosis, free redicals and ionic disturbances

One of the earliest metabolic events after SCI is a linear decrease in oxygen tension at the injury site during the first hours, with local *hypoxia* lasting for several hours during which the metabolic rate is depressed (Ducker *et al.*, 1978a, 1978b, 1978c; Anderson *et al.*,

1980a, 1980b, 1982; Green *et al.*, 1981; Rawe *et al.*, 1981; Stokes *et al.*, 1981; Hayashi *et al.*, 1983a, 1983b, 1983c; Tator, 1992, 1995, 1996, 1998; Sala *et al.* 1999; Dumont *et al.*, 2001a, 2001b, 2002; Choo *et al.*, 2008). In the first 4 hours after trauma metabolism shifts from the high-energy yield aerobic to less efficient anaerobic pathways (Anderson *et al.*, 1980a, 1980b; Rawe *et al.*, 1981; Sala *et al.* 1999). During that period not only is oxidative phosphorylation shut-down but glycolysis skips altogether pyruvate generation and the citric acid cycle due to lack of renewable electron carriers, e.g. *nicotinamide adenine dinucleotide* (NAD^+) (Zhang *et al.*, 1993c). Glucose conversion to lactate is therefore increased in both gray and white matter (Anderson *et al.*, 1980a, 1980b, 1982; Rawe *et al.*, 1981; Hasse *et al.* 2000; Dumont *et al.*, 2001a, 2001b, 2002). Thus, in the first postinjury hours glucose is rapidly depleted, and so are the few energy reserves in the form of high energy phosphates, such as ATP, which together result in lactic acid accumulation and acidosis and exact their toll on cell viability with massive tissue necrosis (Anderson *et al.*, 1980a, 1980b, 1982; Rawe *et al.*, 1981; Sala *et al.* 1999; Hasse *et al.* 2000; Dumont *et al.*, 2001a, 2001b, 2002). Between 4 and 24 hours postinjury circulation and tissue oxygenation improve and oxidative phosphorylation resumes but necrosis continues to propagate (Anderson *et al.*, 1980a, 1980b, 1982; Walker *et al.*, 1979). One reason for that is *reperfusion injury* which exacerbates tissue damage within the cord in the first few days postinjury. Ischæmia results in metabolic changes and enzymatic changes that render subsequent reperfusion hazardous. During the initial ischæmic phase the endothelial *xanthine dehydrogenase* undergoes limited proteolysis and is transformed into xanthine oxidase which transfers electrons to molecular oxygen. Reexposure of endothelial cells to O_2 during the reperfusion phase leads to the formation of *reactive oxygen intermediates or species* (ROI) by the action of *xanthine oxidase* (Guth *et al.*, 1999). This is of course only one way of ROI generation after SCI as we will see later on (Dumont *et al.*, 2001a, 2001b, 2002).

Studies on whole spinal cord segments from monkeys, cats and rats that have undergone experimental spinal cord injury have verified the presence of local acidosis and increased lactate concentration in the lesion site (Feldman *et al.*, 1971a, 1971b; Locke *et al.*, 1971; Paulson *et al.*, 1971; Braughler *et al.*, 1983; Tator, 1992, 1995, 1996, 1998; Zhang *et al.*, 1993c; Sala *et al.* 1999; Hasse *et al.* 2000; Dumont *et al.*, 2001a, 2001b, 2002; Choo *et al.*, 2008). *Acidosis* is the fall in pH caused by the tissue accumulation of inorganic and organic acids. Tissue ischæmia or hypoxæmia result in cellular energy generation under anaerobic conditions and lactate overproduction. However, while lactate accumulation is necessary for acidosis to occur it is *not always* sufficient. Lactate is also overproduced during muscle exercise but the intracellular and extracellular buffering systems manage to maintain homeostasis. In the case of ischæmia or infarction, and likewise after SCI, there is a net loss in ATP that is depleted because of an energetically inefficient anaerobic glycolysis with secondary lactate accumulation. Whenever ATP is hydrolysed, a hydrogen ion is released. A surplus of ATP-derived hydrogen ions and lactate lead to a net shift towards lactic acid generation, which is primarily responsible, in combination with a failing tissue buffering capacity, for the decrease in pH and the intracellular and extracellular acidosis (Tator, 1992, 1995, 1996, 1998; Siesjö, 1993; Siesjö *et al.*, 1993). Acidosis has many serious repercussions for the cord tissue that escaped primary injury.

It seriously affects the optimal conditions for enzymatic and chemical reactions in the protoplasm or extracellular fluid. It also perturbs the function of many ion channels and so transmembrane ion exchange and equilibrium. Finally, acidosis favors the generation of free radicals by various synergistic or independent mechanisms. Some of them involve iron, whose release from extravasated hæmoglobin, transferrin and ferritin stores is facilitated by acidosis. Indeed, the hemorrhagic pools that form after SCI are a rich source of haemoglobin from which iron can be liberated and then participate in free radical formation and lipid peroxidation reactions.

Free radicals are molecules with unpaired electrons in their outer orbitals and therefore a high chemical reactivity (Demopoulos *et al.*, 1980). They exist everywhere in nature. In the lower atmosphere the most common naturally occuring free radical is molecular dioxygen [·O-O·], which is actually a diradical. In biological systems free radicals are natural byproducts of metabolism (especially oxygen metabolism in mitochondria where up to 2% of electrons are thought to end up in free radicals) and can participate in vital processes such as the bacteriotoxic action of neutrophil granulocytes or cell signalling (*redox signaling*) by which platelets and leucocytes are recruited to a wound sites. It has even been suggested that the small amounts of free radicals generated physiologically in mitochondria may actually increase the cellular defense capacity against exogenous radicals, a phenomenon called *mitohormesis* (*hormesis*: a dose-response phenomenon characterized by positive effects at low doses of a chemical agent or toxin as opposed to large doses). However, due to their unpaired valence shell electrons free radicals may also be cytotoxic mediators of oxidative stress through chain reactions that result in their geometric proliferation and the disintegration of lipid bilayers, proteins and nucleic acids (Del Maestro, 1980). In biological systems free radicals can be micromolecules that are oxygen based (e.g. superoxide [$\cdot O_2^-$], hydroxyl radicals [OH·], singlet oxygen [·O] etc.) collectively designated as ROI or nitrogen based (e.g. nitric oxide radical [·NO], peroxynitrite [ONO_2^-] etc.) designated as RNIs, or they can be macromolecules with oxygen or nitrogen carrying groups (e.g. carboxyl groups on fatty acids, amide groups on proteins, etc.). Under normal conditions, free radicals are contained by task-specific enzymatic systems (e.g. superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and other peroxiredoxins) and endogenous antioxidants (e.g. ascorbic acid, tocopherol, glutathione, polyphenol antioxidants, possibly even uric acid, bilirubin) that act as free radical scavengers. Another protective mechanism is the tight regulation of reactive iron ions that are potent catalysts of free radical formation and lipid peroxidation. For example, the superoxide free radical produced in mitochondria is converted into hydrogen peroxide by superoxide dismutase {$2[\cdot O_2^-] \rightarrow H_2O_2$}. Hydrogen peroxide is broken down to water and molecular oxygen by *catalase*, a peroxisomal enzyme specialized for the task {$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$}. Hydrogen peroxide can be converted into highly toxic hydroxyl radicals via a spontaneous chemical reaction that uses Fe^{2+} as a catalyst, the *Fenton reaction* {$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + [OH\cdot] + [OH^-]$}. Hydrogen peroxide degradation is also effected by *glutathione peroxidase* that uses *selenium* as the cofactor that transfers reactive electrons from the hydrogen peroxide to glutathion, a small detoxifier protein that flip-flops between a reduced and an oxidized state thanks to its ($H^+ + e^-$) donating cysteine *thiol group* and the enzyme *glutathione*

reductase {2GSH + H_2O_2→GSSG + $2H_2O$}. Glutathion exists in cells in relatively high concentrations, especially so in liver cells and in normal conditions less than 10% is in its disulfidic form, while the remaining 90% is in its reducing form and in reserve to fend off the challenge of oxidative stress. Finally, hydrogen peroxide is also done away with by other *peroxiredoxins* within mitochondria, cytosol and nucleus. RNIs are less common free radicals produced mainly in neutrophils and macrophages along with ROI when they undergo *oxidative burst* under special conditions and *inducible nitric oxide synthase* (iNOS) plays a central role by delivering nitric oxide in this cascade (Satake *et al.*, 2000a; Xu *et al.*, 2001; Pearse *et al.*, 2003). *Peroxynitrite* (or *peroxonitrite*) is derived *in vivo* from the reaction of the ROI superoxide with the RNI nitric oxide radical [$\cdot O_2^- + \cdot NO \rightarrow ONO_2^-$] (Liu *et al.*, 2000; Satake *et al.*, 2000a). Peroxynitrite, although not a RNI *sensu stricto*, is nevertheless a powerful oxidant and nitrating agent that damages proteins and nucleic acids (Liu *et al.*, 2000; Satake *et al.*, 2000a). Another dangerous chemical but not a *bona fide* free radical, that is produced by neutrophils through the action of *myeloperoxidase* is *hypochlorous acid* [HOCl] that in its oxidized form, *hypochlorite* [$^-$OCl], can be highly cytotoxic, which is why sodium hypochlorite is used as a bactericidal antiseptic. In the setting of SCI, hypochlorite is scavenged by *taurine*, a sulphur-containing aminoacid that makes up about 0.1% of total body weight (Petegnief *et al.*, 1995; Gupta, 2006; Gupta *et al.*, 2006).

When the cellular antioxidant defences begin to fail, free radicals proliferate and wreak havoc in the cell, disrupting many fundamental cellular structures and functions and inducing cell death (Demopoulos *et al.*, 1980). First, free radicals, and especially ROI, cause widespread damage of the phospholipid and cholesterol components of biological membranes (plasma and organellar lipid bylayers) through a chain reaction of lipid peroxidation (Demopoulos *et al.*, 1980; Aust *et al.*, 1985; Thomas and Aust, 1985; Thomas *et al.*, 1985; Braughler and Hall, 1989; Tator, 1992, 1995, 1996, 1998; Dumont *et al.*, 2001a, 2001b, 2002). One way they do so is by attacking the double-bonds of polyunsaturated fatty acids, yielding peroxides that lead to lipid peroxidation in a self-propagating fashion (Anderson *et al.*, 1985a, 1985b, 1985c; Demediuk *et al.*, 1985a, 1985b, 1987; Horrocks *et al.*, 1985). Second, free radicals can damage proteins in many ways, e.g. by oxidation of protein backbones and amino acid side chains, causing protein fragmentation or protein–protein cross-linkage (Demopoulos *et al.*, 1980; Anderson *et al.*, 1985a, 1985b, 1985c; Demediuk *et al.*, 1985a, 1985b, 1987; Horrocks *et al.*, 1985; Hall and Braughler, 1993). They can also inactivate many enzymes either by direct oxidtion or by oxidation of their co-factors (Demopoulos *et al.*, 1980; Anderson *et al.*, 1985a, 1985b, 1985c; Demediuk *et al.*, 1985a, 1985b, 1987; Horrocks *et al.*, 1985). Finally, free radicals can damage nucleic acids, both nuclear and mitochondrial. One way they achieve this is by reacting with thymine in DNA to produce single strand breaks (Demopoulos *et al.*, 1980; Anderson *et al.*, 1985a, 1985b, 1985c; Demediuk *et al.*, 1985a, 1985b; Horrocks *et al.*, 1985; Braughler *et al.*, 1987 ; Hall and Braughler, 1993).

Free radicals are implicated in the secondary injury after SCI since their formation is either favored by many posttraumatic pathophysiological processes as explained above (e.g. primary injury, hæmorrhage, ischæmia, hypoperfusion, acidosis, tissue reperfusion,

tissue necrosis and inflammatory cell invasion) or itself exacerbates some of these processes (e.g. hypoperfusion, acidosis, tissue necrosis and inflammatory cell invasion) (Demopoulos *et al.*, 1980; Demediuk *et al.*, 1985a, 1985b, 1987; Horrocks *et al.*, 1985; Hall *et al.*, 1987; Tator, 1992, 1995, 1996, 1998; Hall and Braughler, 1993). To give a few examples of the complex positive-feedback mechanisms that operate after SCI and involve free radicals: 1) Free radicals cause necrotic and apoptotic cell death by their detrimental actions on biological membranes and cellular proteins. But the massive cellular necrosis that occurs after SCI also promotes free radical generation through various mechanisms, both directly (e.g. the spill-out of toxic free radicals from lysosomal content, the release of sequestrated mitochondrial iron or endosomal calcium) and indirectly (e.g. by exacerbating tissue œdema, acidosis, electrolyte disturbances etc.) (Demediuk *et al.*, 1985a, 1985b, 1987; Horrocks *et al.*, 1985; Braughler *et al.*, 1987; Tator, 1992, 1995, 1996, 1998; Hall and Braughler, 1993; Carlson *et al.*, 1997, 1998; Lewén *et al.*, 2000). 2) Another source of ROIs and RNIs postinjury are the inflammatory cells that invade and persist in the the lesion area for many days (especially neutrophils and macrophages) (Tator, 1992, 1995, 1996, 1998, 1999; Carlson *et al.*, 1998; Satake *et al.*, 2000a; Guzik *et al.*, 2003). But free radicals are also known to mediate recruitement of leucocytes after tissue injury. 3) Proinflammatory cytokines, such as *tumor necrosis factor-alpha* (TNFα), that are being secreted by neutrophils and macrophages and exist in abundance at the injury site in the acute stage of SCI, are also known to induce ROI and RNI formation (Tator, 1992, 1995, 1996, 1998; Satake *et al.*, 2000a; Kamata *et al.*, 2005). TNFα is also a known proapoptotic agent. This effect is achieved both through the activation of membrane-bound *death receptors* and via free radicals. For example, TNFα-induced ROIs cause the overactivation of JNK (*Jun N-terminal Kinases*) by inhibiting JNK-phosphatases through oxidation of their catalytic cysteine thiol group into sulphenic acid. In turn, net JNK activation leads to apoptotic cell death by *cytochrome c* release and *caspase-3* cleavage, but also necrotic cell death (Kamata *et al.*, 2005). Cell necrosis as mentioned above causes further free radical release. 4) Free radicals overwhelm and consume the antioxidant reserves of the ailing cells. Antioxidant deficit leads in turn to a higher oxidative stress for the cell and formation of more free radicals through uncontrolled chain reactions (Anderson *et al.*, 1985; Braughler *et al.*, 1987; Saunders *et al.*, 1987a, 1987b; Tator, 1992, 1995, 1996, 1998; Hall and Braughler, 1993).

There is consistent evidence for the early generation of ROIs and lipid peroxidation in CNS injury or ischæmia in general (Demopoulos *et al.*, 1980; Hall and Braughler, 1993). Animal studies of contusive or compressive SCI have shown that free radicals take action already in the hyperacute stage after injury. Lipid peroxidation was in fact shown to begin already within the first 5 minutes after an acute SCI (Demopoulos *et al.*, 1980; Demediuk *et al.*, 1985; Braughler *et al.*, 1987; Hall and Braughler, 1993). This is in accordance with other data that corroborate the importance of many synergistic pathophysiological mechanisms in ROI formation and free radical mediated secondary injury. Thus, it is known that spinal cord blood flow decreases within 5 minutes of SCI and that polyunsaturated fatty acids are being released as early as 5-15 minutes after CNS injury following phospholipid hydrolysis by Ca^{2+}-dependent phospholipases (Fried *et al.*, 1971; Dohrmann, 1971, 1972a, 1972b, 1973a, 1973b, 1973c; Griffiths, 1973; Ducker *et al.*,

1978a, 1978b, 1978c; Němecek, *et al.*, 1978; Demopoulos *et al.*, 1980; Hayashi *et al.*, 1983a, 1983b, 1983c; Banik *et al.*, 1985; Demediuk *et al.*, 1985; Wallace and Tator, 1986; Braughler *et al.*, 1987; Janssen and Hansebout, 1989; Tator, 1992, 1995, 1996, 1998; Hall and Braughler, 1993; Koyanagi *et al.*, 1993a, 1993b, 1993c; Tator and Koyanagi, 1997; Tator and Fehlings, 1997; Dumont *et al.*, 2001a, 2001b, 2002; Young, 2002). Free fatty acids and lipid peroxidation peak again 4 hours later and remain high for at least 24 hours which temporally coincides with reactivation of the respiratory chain with oxidative phosphorylation in mitochondria (the main cellular locus of ROI generation) and reperfusion injury (Demopoulos *et al.*, 1980; Demediuk *et al.*, 1985; Faden *et al.*, 1987; Hall and Braughler, 1993). Thus, during the first 24 hours after SCI, accumulation of phospholipid degradation products, cholesterol and their oxidation products (e.g. malonyldialdehyde) shows a biphasic pattern that reflects its diverse ætiology and is accompanied by a decrease in tissue antioxidants (Anderson et al., 1985; Hall and Braughler, 1993). Membrane lipid degradation both fuels and is exacerbated by lipid peroxidation with irreversible consequences for the cells (Hall and Braughler, 1986, 1989, 1993; Braughler *et al.*, 1987; Saunders *et al.*, 1987; Braughler and Hall, 1989, 1992). Also during the first days after SCI, hæmorrhage is a constant source of iron that has a booming effect on free radical levels in the already ailing cells (Hall and Braughler, 1986, 1989, 1993; Braughler *et al.*, 1987; Saunders *et al.*, 1987; Braughler and Hall, 1989, 1992; Rehncrona *et al.*, 1989). It is obvious therefore why levels of free radicals in the lesion site follow a time-course that fits well with secondary injury related cell loss (Hall and Braughler, 1986, 1989, 1993; Braughler *et al.*, 1987; Saunders *et al.*, 1987; Braughler and Hall, 1989, 1992; Tator, 1992, 1995, 1996, 1998, 1999; Hamada *et al.*, 1996; Streit *et al.*, 1998; Liu *et al.*, 2000; Satake *et al.*, 2000a; Dumont *et al.*, 2001a, 2001b, 2002; Xu *et al.*, 2001; Chatzipanteli *et al.*, 2002; Pearse *et al.*, 2003).

We have hinted at the deleterious role played by calcium in acute SCI, however ionic disturbances after CNS trauma do not concern just calcium but all major electrolytes. Animal studies of contusion or compression SCI have shown that although $[Ca^{2+}]_e$ falls rapidly after injury, total tissue $[Ca^{2+}]$ increases. Despite the confounding effect of cell necrosis and tissue œdema on $[Ca^{2+}]_e$, this indicates that Ca^{2+} is translocating into the cells (Balentine and Spector, 1977; Young *et al.*, 1982; Stokes *et al.*, 1983; Hall and Braughler, 1986, 1989, 1993; Braughler *et al.*, 1987; Saunders *et al.*, 1987; Braughler and Hall, 1989, 1992; Tator, 1992, 1995, 1996, 1998, 1999; Dumont *et al.*, 2001a, 2001b, 2002; Sayer, 2002a; Berliocchi and Bano, 2005). It has been verified that $[Ca^{2+}]_i$ after SCI increases and that calcium is sequestered preferentially in certain intracellular compartments, e.g. in the axoplasm (Balentine and Spector, 1977; Young *et al.*, 1982; Stokes *et al.*, 1983; Stys and LoPachin, 1996, 1998; Stys *et al.*, 1998; LoPachin *et al.*, 1999). Total tissue calcium levels are significantly elevated at 45 min, maximize at 8 hours postinjury and remain elevated for at least 1 week thereafter while intraaxonal calcium levels rise as early as 30 min after injury (Balentine and Spector, 1977; Balentine, 1978; Hapel *et al.*, 1981; Stokes *et al.*, 1983; Braughler and Hall, 1989, 1992; Hall and Braughler, 1986, 1989, 1993; Moriya *et al.*, 1994; Stys and LoPachin, 1996, 1998; Stys *et al.*, 1998; Berliocchi and Bano, 2005). However, Ca^{2+}-mediated actions, such as phospholipid hydrolysis by phospholipases are known to occur as early

as 5 minutes postinjury. The net increase in $[Ca^{2+}]_i$ can be explained by aberrant calcium influx through voltage-gated Ca^{2+} channels, overactivation of the NMDA receptor channel by excitatory amino acids or failure of Ca^{2+}-ATPase mediated Ca^{2+} extrusion and reverse Na^+/Ca^{2+} exchange due to ATP depletion, Na^+/K^+ ATPase pump failure and subsequent increase in $[Na^+]_i$. Release from intracellular stores could also partly explain the axoplasmic accumulation of calcium. Intracellular calcium overload affects a score of Ca^{2+}-regulated enzymes (phospholipases, calpain, protein kinase C, CaMkinaseII, endonucleases etc.) with deleterious effects on many cellular structures and functions (Stokes *et al.*, 1983; Siesjö *et al.*, 1988; Stys and LoPachin, 1996, 1998; Stys *et al.*, 1998; Doble, 1999; Dumont *et al.*, 2001a, 2001b, 2002; Sayer, 2002a; Berliocchi and Bano, 2005). Activation of phospholipase C and phospholipase A_2 results in the breakdown of lipid bilayer phospholipids and arachidonate production among several other byproducts with second messenger functions (Hall and Braughler, 1986, 1989, 1993; Rasmussen, 1986a, 1986b; Braughler *et al.*, 1987; Saunders *et al.*, 1987; Braughler and Hall, 1989, 1992; Berliocchi and Bano, 2005). Phospholipid metabolism boosts lipid peroxidation as mentioned above. Arachidonate metabolism yields thromboxanes and leucotrienes which have vasoregulatory and proinflammatory actions (Hall and Braughler, 1986, 1989, 1993; Braughler *et al.*, 1987; Saunders *et al.*, 1987; Demediuk and Faden, 1988; Braughler and Hall, 1989, 1992; Xu *et al.*, 1991). Calpain is a Ca^{2+}-dependent protease which, when overactivated, targets cytoskeletal or other critical proteins (Saido *et al.*, 1994; McIntosh *et al.*, 1997, 1998). High $[Ca^{2+}]_i$ activates protein kinase C and CaMkinaseII (Ca^{2+}/calmodulin-dependent protein kinase II) which are two central proteins in many signaling pathways which result in the transcription of many stress-related genes (e.g. immediate early genes) or lead to neuronal apoptosis (Herdegen *et al.*, 1999; Berliocchi and Bano, 2005). Intracellular calcium excess also perturbs the storage and release of neurotransmitters at synaptic junctions. It also disturbs mitochondrial function affecting energy production and leading to free radical formation (Berliocchi and Bano, 2005). Finally, Ca^{2+} overload may cause failure of many ion channels both by direct and many indirect actions disturbing sodium and potassium fluxes with negative effects on neuronal excitation and overall cellular homeostasis (e.g. swelling) (Schwab and Bartholdi, 1996; Stys and LoPachin, 1996, 1998; Stys *et al.*, 1998; Berliocchi and Bano, 2005).

Of all the biologically important ions calcium has the more harmful consequences in SCI but postinjury $[Na^+]_i$ surge has dire consequences too (Fried, 1965). *Sodium* influx is mediated through several different mechanisms. First, ATP depletion disrupts the function of key active transporters such as the Na^+/K^+ ATPase, and Na^+/Ca^{2+} exchangers, as explained above (Fried, 1965). Second, glutamate binding on AMPA and NMDA receptors leads to a cycle of membrane depolarisation, activation of voltage-gated Na^+ channels, sodium entry and further depolarisation. Third, the chronicity of this response releases NMDA receptors from their magnesium block with resultant overactivation by glutamate and additional Na^+ entry. Finally, this net sodium influx is accompanied by Cl^- for reasons of electric charge equilibrium. Because of osmotic forces water floods in causing intracellular oedema, failure of many vital cellular functions, including ion transport, and in a positive feedback fashion, further $[Na^+]_i$ increase (Fried, 1965;

Wrathall *et al.*, 1994; Teng and Wrathall, 1997; Faden *et al.*, 1988; Agrawal and Fehlings, 1996, 1997; Li *et al.*, 1999; Rosenberg *et al.*, 1999; Doble, 1999; Dumont *et al.*, 2001a, 2001b, 2002). This is how sodium is believed to mediate white matter damage and participate in glutamate mediated excitotoxicity (Fried, 1965; Wrathall *et al.*, 1994; Teng and Wrathall, 1997; Faden *et al.*, 1988; Agrawal and Fehlings, 1996, 1997; Li *et al.*, 1999; Rosenberg *et al.*, 1999; Doble, 1999). As for *potassium*, in the acute stage after contusion SCI, $[K^+]_e$ increases but then gradually falls to very low levels in the damaged tissue (Fried, 1965; Eidelberg, 1975; Stokes, *et al.*, 1983; Young and Koreh, 1986). The immediate rise in $[K^+]_e$ results in partial depolarization in both gray and white matter and conduction block while the subacute loss of potassium could be the result of loss of cellular membrane integrity and cell death (Lewin *et al.*, 1974; Eidelberg, 1975; Stokes, *et al.*, 1983; Young and Koreh, 1986; Anderson *et al.*, 1991). High extracellular potassium levels are known to contribute, together with glutamate, to neuronal excitotoxicity in the setting of ischæmia. One delineated pathway for this effect involves chloride ions and activation of NKCC1 (*$Na^+/K^+/2Cl^-$ cotransporter*) through serine-threonine phosphorylation (Su *et al.*, 2002; Schomberg *et al.*, 2001). *Chloride* is the primary permeant anion in mammals and $[Cl^-]_i$ homeostasis is critical for many basic neurophysiological processes, such as cell volume regulation and efficient GABA (*γ-aminobutyric acid*) neurotransmission. Chloride ion fluxes and $[Cl^-]_i$ are tightly regulated by *cation-chloride cotransporters* (CCCs), which include the inwardly directed NKCC1 and several outwardly directed *K^+/Cl^- cotransporters* (Delpire *et al.*, 2000; Payne *et al.*, 2003; Gamba *et al.*, 2005). In the setting of traumatic CNS injury or ischæmia a pathologic increase of NKCC1 activity leads to a rise in $[Cl^-]_i$ that in turn triggers neuronal hyperexcitability to glutamate and excitotoxicity (Beck *et al.*, 2003; Yan *et al.*, 2003; Chen and Sun, 2005; Chen *et al.*, 2005).

Cell death: necrosis, excitotoxicity, apoptosis

In neuropathology two different modes of cell death, mechanistically and morphologically, have been described, necrosis and apoptosis (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Bargmann, 1991; Cohen, 1993a, 1993b, 1993c; Majno and Joris, 1995; Bredesen, 1995; Bär, 1996). *Necrosis* is the inevitable demise of the cell caused by the irreversible failure of the many vital and homeostatic cellular functions that are overwhelmed by an external insult such as heat, anoxia, inflammation or trauma (Bargmann, 1991; Kristian and Siesjö, 1996; Zhang *et al.*, 1997). Necrosis is a strictly pathological process that results from a haphazard multiorganellar failure that leads to a *point of no return* for the cell, while it does not exact any energy requirements and does not depend on *de novo* gene transcription or protein synthesis (Bargmann, 1991). Necrosis is therefore viewed as an accidental 'passive' process, hence the denomination *Accidental Cell Death*, that was proposed to encompass all forms of necrosis irrespective of injurious stimulus. *Apoptosis* (αποπιπτω: Gr. for "to wither and fall") on the other hand is a well-orchestrated and energy-demanding form of cell death that is the end result of stereotypical and preprogrammed molecular final common pathways triggered by endogenous or exogenous cytopathic stimuli (Kerr *et al.*, 1972; Wyllie *et al.*, 1980;

Bargmann, 1991; Cohen, 1993a, 1993b, 1993c; Majno and Joris, 1995). Thus, apoptosis is a tightly regulated 'active' process that has been likened to a carefully executed 'cellular suicide', hence its alternative denomination *Programmed Cell Death* (PCD) (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Bargmann, 1991; Majno and Joris, 1995). Apoptosis is a physiological process of paramount importance for living organisms that operates under physiologic conditions, e.g. *in utero* during embryonic development as the result of lack of trophic stimuli for the doomed cells, but can also occur in pathologic conditions alone or in parallel with necrosis (Lockshin and Williams, 1964; Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Oppenheim and Haverkamp, 1988; Williams and Herrup, 1988; Oppenheim, 1989; Oppenheim *et al.*, 1989; Bargmann, 1991; Raff *et al.*, 1993; ; Majno and Joris, 1995; Steller, 1995). In general, severe and/or abrupt injury to the cell leads to necrosis, whereas apoptosis is the result of less severe but insidious damage that eventually becomes irreparable. *Excitotoxic* cell death results from the neurotoxic actions of the excitatory neurotransmitter glutamate on different receptors on CNS neurons, especially under ischæmic conditions, and is in fact a variant of necrosis (Bargmann, 1991).

Morphologically, the two main forms of cell death could not be more different (Bargmann, 1991; Majno and Joris, 1995). During necrosis the cell and many of its organelles, including the nucleus (*karyolysis*), essentially swell up and burst, spilling their content, including many noxious lysosomal enzymes, in the extracellular space, and triggering a cytotoxic chain reaction and a robust inflammatory reaction that clears up the cellular debris but also compounds tissue damage (Bargmann, 1991; Majno and Joris, 1995). This is especially true in ischæmia-induced cell death in the CNS, so the term ***oncosis*** was proposed in 1910 by von Recklinghausen to describe the form of necrosis that today is better known as *excitotoxic* cell death, a term that alludes to mechanism and not morphology of cell death (Majno and Joris, 1995). As a rule, the insult that causes necrosis is so overwhelming that necrotic cell death occurs *en masse*, with large swaths of tissue being liquefied (Bargmann, 1991; Majno and Joris, 1995). On the contrary, during apoptosis the cell shrinks, chromatin aggregates and the whole nuclear content condenses (*nuclear pyknosis*) and disintegrates (*karyorrhexis*), with ensuing parceling and fragmentation of the cell and uptake by neighboring cells with phagocytic and pinocytic capacitiy, i.e. mostly tissue macrophages, without discharge of any intracellular material in the extracellular space and in the absence of local inflammatory response (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Bargmann, 1991; Savill *et al.*, 1993; Cohen *et al.*, 1993; Majno and Joris, 1995; Thompson *et al.*, 1995).

The molecular workings of ***necrosis*** vary considerably since necrosis is not a stereotypical process. Many different injurious stimuli will lead to cell necrosis through varying subcellular cascades but what is common is the irreversibility of its outcome, which is invariably the lysis of the cell. Heat, anoxia, inflammation, and trauma will not trigger the exact same subcellular pathways but they will all cause cellular demise by a necrotic process, instantly or not. In the case of SCI, necrosis will be the result of the pathophysiological processes described so far, i.e. direct trauma, hypoxia, tissue œdema, reperfusion injury, free radical generation, neuroinflammation (Bargmann, 1991; Tator *et al.*, 1991, 1992, 1995, 1996, 1998; Zhang *et al.*, 1997; Guth *et al.*, 1999; Carlson *et al.*,

1998; Hall *et al.*, 1993; Halliwell *et al.*, 1992; Braughler *et al.*, 1983 Braughler *et al.*, 1982; Hall *et al.*, 1988; Demopoulos *et al.*, 1982; Hall *et al.*, 1984; Young *et al.*, 1982; Braughler *et al.*, 1984; Hall, 1992; Braughler *et al.*, 1987; Lewén *et al.*, 2000; Satake *et al.*, 2000; Anderson *et al.*, 1985; Saunders *et al.*, 1987a, 1987b; Hall *et al.*, 1987; Schwab and Bartholdi, 1996; Janssen and Hansebout, 1989; Demediuk *et al.*, 1985; Demopoulos *et al.*, 1980; Faden *et al.*, 1987; Del Maestro, 1980; Braughler and Hall, 1989; Dumont *et al.*, 2001a, 2001b, 2002;). The subcellular metabolic, chemical and structural perturbations that lead to cell lysis have already been detailed. These secondary injury mechanisms initiate a centripetal and rostro-caudal necrotic wave that by 8 h postinjury will be irreversible and will sweep as far as two myelotomes above and below the lesion center (Tator, 1995). The fusiform cystic degeneration of the cord parenchyma at the lesion site after contusion or transection injury is entirely the result of extensive tissue necrosis and liquefaction. Similar cystic lesions with complete disappearance of brain parenchyma can be seen after cerebral infarcts where ætiology is purely ischæmic. Necrosis is the principal mechanism of cell death after CNS trauma in general and SCI in particular ocurring immediately after the primary injury. Necrosis accounts for almost the totality of secondary injury-related tissue loss in SCI and is directly proportional to severity of primary injury (Tator *et al.*, 1995, 1996; Zhang *et al.*, 1996, 1997).

Excitoxicity is a specific form of necrotic cell death triggered by the neurotoxic effects of glutamergic overactivation (Bargmann, 1991; Dumont *et al.*, 2001a, 2001b, 2002). *Glutamate* is the primary excitatory neurotransmitter in the CNS while *aspartate* is also an excitatory amino acid of the CNS. They are stored in presynaptic vesicles and are released into the synaptic cleft during neural transmission whereupon they activate ionotropic NMDA (*N-methyl-D-aspartate*) and nonNMDA, i.e. AMPA (*DL-alpha-amino-3-hydroxy-5-methylisoxazol-propionic acid*), kainate and metabotropic (mGluR) receptors. Under normal conditions the extracellular fluid content in glutamate is very low because immediately after neurotransmission glutamate is taken up by active transport into neurons and astrocytes. Neurons and astrocytes cooperate closely in glutamate recycling so that glutamergic neurons are timely replenished with their neurotransmitter. The sequence of molecular events in excitotoxic cell death has been elucidated to some extent. Under pathological conditions causing excitoxicity, e.g. after CNS traumatic or ischæmic injury or both, glutamate binds initially to neuronal AMPA receptors. This leads to neuronal membrane depolarisation and subsequent voltage-dependent sodium channel activation. Sodium influx through these channels causes further membrane depolarisation and increase in $[Na^+]_i$. These conditions favor the release of NMDA receptors from their magnesium block and their activation by free extracellular glutamate resulting in additional sodium and chloride influx and increase in $[Na^+]_i$ and $[Cl^-]_i$. A parallel excitotoxic pathway involves calcium. The glutamate receptor-mediated chronic membrane depolarisation unleashes a massive increase in $[Ca^{2+}]_i$ calcium via: 1) influx through voltage-dependent calcium channels, 2) reverse operation of the membrane Na^+/Ca^{2+} exchanger forced by the upswing of $[Na^+]_i$ and 3) calcium mobilisation from intracellular stores. Among the serious consequences of the simultaneous surge in intracellular $[Na^+]_i$, $[Cl^-]_i$, and $[Ca^{2+}]_i$ are a) the perturbation of the electrolyte and osmotic homeostasis of the cell with secondary influx of water, cell swelling and lysis and b) the

activation of calcium-dependent cascades and enzymes (proteases, kinases and phospholipases) that lead to cell death by necrosis (Bargmann, 1991; Choi, 1992, Doble, 1999). The role of NMDA and AMPA receptors in secondary injury in CNS trauma has been established by studies showing that specific blockers were able to reduce the extent of histological changes (Faden *et al.*, 1988, 1992; Gomez-Pinilla *et al.*, 1989; Sun and Faden, 1994; Wrathall *et al.*, 1994).

In the acute stage of SCI, excitotoxicity becomes inevitable by the combination of neurotrauma and ischæmia (Tator, 1991, 1995, 1996). In experimental models it has been shown that extracellular concentrations of both glutamate and aspartate rise immediately after contusion SCI, peak a few minutes later and continue to remain at toxic levels as a result of: a) the massive release of intracellular glutamate by cell lysis, b) the exocytosis of glutamate-containing synaptic vesicles triggered by increased neuronal $[Ca^{2+}]_i$ and c) the failing reuptake by neurons and astrocytes (Panter *et al.*, 1990). The abundance of extracellular glutamate and aspartate in the hypoxic postinjury milieu overstimulates ionotropic receptors, including kainate receptors, and trigger a wave of excitotoxic cell death that sweeps through both grey and white matter (Faden *et al.*, 1988; Panter *et al.*, 1990; Liu *et al.*, 1991, 1993; Choi, 1992, 1996; Liu and McAdoo, 1993a, 1993b; Wrathall *et al.*, 1994; Doble, 1999; Teng and Wrathall, 1997;). Activation of voltage-gated Na+ channels and AMPA or kainate receptors plays an important role in direct damage to white matter structures (Agrawal and Fehlings, 1996, 1997; Li *et al.*, 1999; Rosenberg *et al.*, 1999; Dumont *et al.*, 2001a, 2001b, 2002). In SCI, excitotoxic cell death affects also glial cells, and most importantly oligodendrocytes who happen to be the most vulnerable (McDonald *et al.*, 1998). The susceptibility of glial cells to excitotoxicity is partly due to their lack of NMDA receptors (Steinhauser and Gallo, 1996). This means that AMPA and kainate receptors are activated on glial cells during excitotoxicity. In mature oligodendrocytes these two receptors are more permeable to calcium, compared to their isoreceptors on neurons. Mature oligodendrocytes have also a poor capacity to buffer calcium. The combination of these factors facilitates the excitotoxic cell death of oligodendorocytes after SCI (Mattson *et al.*, 1991; Puchalski *et al.*, 1994; Matute *et al.*, 2001).

Oligodendrocytes are also targets of apoptosis after SCI. ***Apoptosis*** is a form of PCD that can result from multiple complex molecular cascades which activate one of the two main apoptotic pathways, the *extrinsic* and the *intrinsic*. In SCI apoptosis is a less important mediator of secondary injury and it seems to take place in two spatiotemporally distinct postinjury phases (Crowe *et al.*, 1997; Beattie *et al.*, 2000, 2002; Satake *et al.*, 2000a; Dumont *et al.*, 2001a, 2001b, 2002). As early as 6 hours postinjury it occurs together with necrosis around the lesion center affecting multiple cell types (neurons, oligodendrocytes, astrocytes, microglia, infiltrating inflammatory cells). By 1 week this initial indiscriminate apoptotic wave subsides but at that time a new apoptotic wave begins to spread away from the injury and as far as 4 myelotomes on each side, involving oligodendrocytes mainly and leading to long-term and persistent demyelination (Crowe et al., 1997; Satake *et al.*, 2000a; Dumont *et al.*, 2001a, 2001b, 2002).

DELAYED CYSTIC CAVITATION

Already at 21 days postinjury cystic degeneration of the spinal cord is under way and by 14 weeks cavitations coalesce into large fluid-filled cysts, which are sometimes septated and are surrounded by glial scar tissue originating mainly from astroglia and to a lesser extent from the PNS (Wakefield and Eidelberg, 1975; Cohen *et al.*, 1985; Zhang *et al.*, 1997; Schwartz *et al.*, 1999; Beattie *et al.*, 2002; Dumont *et al.*, 2001a, 2001b, 2002; Radojicic *et al.*, 2005). In 30% of rats with a compression SCI injury the cystic regions finally expand for considerable distances rostrocaudally to the lesion site (Cohen *et al.*, 1985; Tator, 1995; Guizar-Sahagún *et al.*, 1994a, 1994b; Zhang *et al.*, 1997; Schwartz *et al.*, 1999; Thompson *et al.*, 2001; Wirth *et al.*, 2001). *Progressive post-traumatic cystic myelopathy* (PPCM) has been is found by MRI in up to 50% of patients with SCI in different series of patients, but is clinically manifest in less than 10% (Barkovich *et al.*, 1987; Milhorat *et al.*, 1992; Falcone *et al.*, 1994; Quencer, 1998; Åkesson *et al.*, 1998). *Progressive posttraumatic myelomalacic myelopathy* (PPMM) may clinically mimic PPCM but MRI as well as intraoperative sonography can confirm the absence of a confluent cyst (Hackney *et al.*, 1986; Backe *et al.*, 1991; Shimada and Tokioka, 1999; Barkovich *et al.*, 1987; Milhorat *et al.*, 1992; Falcone *et al.*, 1994; Quencer, 1998; Åkesson *et al.*, 1998). Intradural arachnoid cysts are other associated posttraumatic lesions, and may appear to compress the spinal cord and nerve roots (Barkovich *et al.*, 1987; Milhorat *et al.*, 1992; Falcone *et al.*, 1994; Quencer, 1998; Åkesson *et al.*, 1998; Dumont *et al.*, 2001a, 2001b, 2002). Seemingly isolated central intramedullary microcysts can be detected by MRI in SCI patients and may represent areas of cystic myelomalacia (Hackney *et al.*, 1986; Backe *et al.*, 1991; Shimada and Tokioka, 1999; Barkovich *et al.*, 1987). Blockade in the CSF circulation altering the direction of the flow into communicating or *cul de sac* cisternæ with increasing pressure and ascending expansion of the cavity affecting the less resistant grey matter could be one hydrodynamic ætiology of posttraumatic syringomyelia or syringobulbia. Cord tethering by arachnoidea scarring has also been speculated (Guizar-Sahagún *et al.*, 1994a, 1994b; Barkovich *et al.*, 1987). Indeed, in traumatic transverse cord lesions, arachnoiditis or tumors, the cavities usually ascend from the site of injury, although in upper cervical lesions downward cavitation is also encountered (Barkovich *et al.*, 1987; Milhorat *et al.*, 1992; Falcone *et al.*, 1994; Quencer, 1998). A combination of factors such as venous obstruction, exudation of protein, ischemia and œdema have been implicated in the formation of the cavity which is always surrounded by gliosis (Barkovich *et al.*, 1987; Milhorat *et al.*, 1992; Falcone *et al.*, 1994; Quencer, 1998; Schwartz *et al.*, 1999). A new hypothesis on the pathogenesis of syringomyelia and cyst formation based on MRI studies of CSF dynamics has been proposed (Greitz, 2004; 2006; Rushbridge *et al.*, 2006; Josephson *et al.*, 2001) and tested experimentally in our lab in a CSF flow obstruction by constriction rat model (Josephson *et al.*, 2001). According to the hypothesis, descending and ascending CSF pressure waves, arriving at points of spinal canal posttraumatic obstructions, are transmitted centripetally into the cord where they generate the tension that leads to generation and propagation of cysts rostral and caudal to the constriction point. These studies also demonstrated that cysts can develop anywhere in the cord parenchyma, rather then just in connection with the central canal, that cysts are always preceeded by œdema but not *vice versa*, and that

the cyst fluid is typically extracellular fluid and not CSF. Indeed, the pathophysiological mechanisms that underlie the generation of intraspinal cysts and syringomyelia after SCI are believed to be responsible for the limited success of neural tissue transplantation therapies in patients with posttraumatic syringomyelia (Falci *et al.*, 1997; Thompson *et al.*, 2001; Wirth *et al.*, 2001).

GENERAL BACKGROUND:
CLINICAL MANAGEMENT OF SCI

ACUTE STAGE

Management of SCI begins in the field, and initial mismanagement can result in aggravation of a spinal column injury into SCI or an incomplete SCI into a complete one, though this risk used to be higher in the past. Patients with SCI are often multitrauma cases (80% of SCI are polytraumatic and 20% of SCI patients have another life-threatening injury, e.g. traumatic brain injury, hemopneumothorax, abdominal organ damage) and standard practices of trauma care must therefore always be followed. A guiding principle is that all multitrauma patients should be treated as if they had SCI until proven otherwise and all patients with SCI should be treated as polytraumatic cases until proven otherwise (Jurkovich, 2009). Moreover, every spinal injury must be initially managed as potentially unstable (e.g. burst fractures) where every move can increase the extent of the injury, hence the use of cervical collars. For example, polytraumatic cases needing intubation may have unstable cervical spine fractures rendering intubation a very risky procedure (Jurkovich, 2009). Although the indications for nasotracheal and endotracheal intubation in patients with SCI still remain unclear, the ability to speak is a rough indicator of adequate air movement through the airway. Also during extrication and mobilization, both before and after placement on a backboard, the spine should be kept at as neutral a position as possible. However, in older adults with kyphotic deformities and particularly those with ankylosing spondylitis the spine should not be forced into an anatomically neutral alignment, because that carries the risk of subluxation at the level of the fracture, which could endanger further neurological impairment. For the same preventive reasons, if the patient, especially the cervically injured, constitutes a danger to himself because of agitation or uncooperativeness due to stress, head injury or intoxication, he should be sedated or chemically paralyzed and then intubated. However, because emergency intubation carries its own risk and because sedation or chemical paralysis abolishes the possibility for serial neurological examination this measure is only a last resort. Indeed, even after arrival to the trauma unit, until a spinal fracture has been ruled as stable either by excluding cord injury or after surgical intervention, the spine should remain in as neutral position as possible by the use of a cervical collar, a halo or traction tongs, because it is believed that micromotion of the injured swollen cord causes further neural tissue damage (Jurkovich, 2009).

Polytraumatic or not, SCI patients are initially treated in the critical care unit, where ventilatory support is available and where their clinical condition can be closely monitored until it is stabilized. The purpose is to avoid hypoventilation, hypotension and hypoxia that are serious acute complications in spinal cord injury and must be addressed promptly and adequately (Jurkovich, 2009). The clinical goal in the hyperacute period is for patients to remain well ventilated with a P_{O_2} and P_{CO_2} in the normal range and mean arterial pressure above 85 mm Hg. Hypoventilation, hypotension and hypoxia may be the direct result of SCI because of paralysis of respiratory muscles and interruption of

vasoregulatory autonomic pathways. Injuries above C3 result in diaphragmatic paralysis rendering the patients *ventilator-dependent*. Cervical injuries below C3 as a rule do not lead to permanent dependence on ventilator but *temporary ventilatory support* may be necessary initially. However, hypoventilation, hypotension and tissue hypoxia can also be attributed to other factors in polytraumatic SCI. Polytrauma patients can present with hypoventilation caused by pulmonary restriction syndrome secondary to pneumo/hæmothorax, chest wall injury, or abdominal compartment syndrome with resultant abdominal distention or airway obstruction syndrome secondary to prevertebral soft tissue hematoma or oedema, despite neurologically intact mucles of respiration. Moreover, altered mental status secondary to traumatic brain injury or intoxication may complicate respiratory function. They may also suffer from hypotension caused by occult internal hæmorrhage, cardiac tamponade or pneumothorax and may develop *systemic inflammatory response syndrome* (SIRS) or *adult respiratory distress syndrome* (ARDS) further compromising tissue perfusion and oxygenation. For patients whose hypotension and bradycardia do not respond to standard volume resuscitation, autonomic dysfunction and *neurogenic shock* should be suspected and the use of *vasopressors* is imperative. This because prolonged hypoperfusion and hypoxia may have a devastating effect on a traumatized spinal cord that already experiences decreased perfusion pressure due to oedema (Senter and Venes, 1978, 1979; Dolan *et al.*, 1980a, 1980b; Dolan and Tator, 1980, 1982; Tator, 1991, 1992, 1995, 1996, 1998; Tator and Fehlings, 1999; King *et al.*, 2000).

Computer assisted tomography (CAT scan or CT) is the most widely used imaging modality for acute SCI, followed by *magnetic resonance imaging* (MRI). Plain X-ray films are also very useful as a rapid and easy method of assessment of the spinal skeleton in the acute setting, while *conventional tomography* still remains the most sensitive technique to visualize horizontally oriented vertebral fractures, particularly of the odontoid. *Myelography*, which relies on the injection of contrast material intrathecally prior to plain X-ray or CT imaging, can still substitute for MRI when the question of spinal canal compromise or disc herniation arises. CT myelography also remains the gold standard in the evaluation of CSF leakage in nerve root avulsion. However, MRI has revolutionized spinal cord imaging because it can demonstrate an occult fracture of the thoracolumbar spine missed on plain films, as well as identify ligamentous and disc injuries not seen on CT. However, MRI does not provide adequate anatomical detail of bony structures and vertically oriented fractures are best seen on CT. CT is also most sensitive in defining vertebral body alignment and bony encroachment on the spinal canal (Zhang *et al.*, 1993a). Moreover, MRI is not useful in SCI cases where metal fragments may lead to artifacts, and the technique remains cumbersome and often will necessitate deep sedation or even chemical paralysis of the agitated or claustrophobic patient. Several parameters can be varied in MRI in order to alter the imaging outcome according to the indication or question at hand. The most widely used sequences in the acute SCI setting are *T1-weighted* images that offer the greatest anatomical detail while *T2-weighted* images are the most sensitive for the assessment of tissue œdema and extent of cord injury. The extent of injury on T2 is not a good indicator of prognosis while the presence of hæmatoma is a more sensitive predictor of long-term disability. However, the

appearance of hæmatoma on T2 is very much dependent on the interval between injury and imaging. *Gadolinium-enhanced T1*, *FLAIR* (fluid attenuation inversion recovery), *T2** (*gradient-echo*), and *diffusion-weighted* MRI sequences are not routinely used in the acute management of acute SCI.

An old question in acute SCI management is whether and if so, when to resort to surgery. The nature of the traumatic insult, the gravity of spinal column damage and their repercussions on spinal column stability and spinal cord tissue integrity should determine whether or not to undertake surgery with removal of bone, disc or ligament tissue that impinge on the already œdematous cord. This is not an easy clinical decision, because precise guidelines for surgical treatment of SCI are not established. Moreover, polytraumatic SCI patients may not tolerate general anesthæsia and surgery, and associated co-morbidities such as sepsis, pulmonary embolism, deep vein thrombosis or skin wounds can thus delay acute surgical intervention. Early nonsurgical decompressive manipulations can also be remarkably beneficial. Thus realignment and prevention of dislocation after cervical spinal injury with halo or tongs, even as late as 8 hours postinjury can result in significant pain reduction and improvement of neurological function. However, closed reduction in the thoracolumbar region can be contraindicated, because of the risk to nearby great vessels (Silber and Vaccaro, 2001; Fehlings and Perrin, 2005; Kishan *et al.* 2005; Rabinowitz *et al.*, 2008; Schinkel, 2008; Fehlings, 2009; Fehlings and Arvin, 2009; Jurkovich, 2009; Rahimi-Movaghar *et al.*, 2009; Tederko *et al.*, 2009).

Surgical management of acute SCI has two goals, *decompression* and *spine stabilization*. Between 40-70% of SCI patients undergo spine surgery within one week from trauma. However, despite important advances in spinal surgical techniques and tools the indications remain controversial. In fact the cornerstone of SCI clinical practice is to avoid acute surgery whenever possible. Even the practice of acute laminectomy that had been advocated by some specialists as a way to improve spinal cord perfusion pressure in the acute setting has been abandoned. Progressive *neurological deterioration* in the setting of an *incomplete* (non ASIA-A) injury with *ongoing compression* and *unstable spinal fracture* are the main indications for acute surgery. In the absence of ongoing compression and neurological deterioration, acute surgery is generally contraindicated, while the situation is more complex in patients with ongoing compression but fixed (stable) incomplete injury, especially of the thoracolumbar cord. The presence of an intradural foreign body (e.g. after penetrating spinal cord injury that can result in osteomyelitis, epidural abscess and chemical or infectious meningitis), CSF leak or depressed bone fragments, evidence of intervertebral disc herniation and irreducible dislocation or inability to maintain alignment are also considered indications for acute surgical intervention. Often painful ligamentous injuries that heal poorly may require surgical fixation. Complete SCI (ASIA-A) will seldom require surgery, unless if one of the above indications is present (Silber and Vaccaro, 2001; Fehlings and Perrin, 2005; Kishan *et al.* 2005; Rabinowitz *et al.*, 2008; Schinkel, 2008; Fehlings, 2009; Fehlings and Arvin, 2009; Jurkovich, 2009; Rahimi-Movaghar *et al.*, 2009; Tederko *et al.*, 2009).

The first hours after injury are critical for cord decompression lest neural tissue damage becomes irreversible. In experimental animals decompression up to 6-8 hours postinjury enhances recovery but the *window-of-opportunity* has not been clearly defined in man (Dolan *et al.*, 1980a, 1980b). Most centers favor decompression and if possible even stabilization within 48-72 h of injury, especially for patients with cervical injuries and central cord syndrome or for patients with anterior cord syndrome, who in case of reducible instability are immediately placed in traction and operated within 48 h, while in case of irreducible malalignment or acute disc herniation undergo emergency surgery. Nevertheless, some studies have shown that delayed decompression surgery between 24-72 hours after SCI does not yield satisfactory results and thus it is seldom practiced beyond 72 hours postinjury. Rapid intervention is also known to be determinant of the outcome of cauda equina syndrome, partial or complete. Elective *fixation surgery* (*spinal fusion* and *bracing*) of unstable injuries within 1 week postinjury is often practiced to allow for early mobilization. However, in certain cases, such as in central cord syndrome, stabilization surgery can be delayed until 6-12 weeks after injury when the patient has reached a plateau phase of recovery (Vaccaro *et al.*, 1997; Silber and Vaccaro, 2001; Ball and Sekhon, 2006; Schinkel, 2008; Tederko *et al.*, 2009).

The question of the effect of surgical intervention in acute SCI on long-term outcome has not been adequately answered yet. It has mostly been addressed by retrospective observational studies. Very few *multicenter randomized controlled comparative* studies have been published concerning operative versus nonoperative approaches or the timing of surgery. A general conclusion of most studies is that surgical intervention, especially if undertaken after 24 hours postinjury, may shorten recovery and speed mobilization of the patient but probably does not result in significant improvement of long-term outcome or quality of life (Vaccaro *et al.*, 1997).

Surgical treatment of *chronic spinal injury* becomes an option in a small percentage of patients suffering from delayed posttraumatic syndromes such as *syringomyelia* or *arachnoiditis*. Surgical approaches against syringomyelia are dural augmentation, lysis of subarachnoid adhesions, cyst fenestration and shunting (syringosubarachnoid, syringopleural or syringoperitoneal). Some reports exist of patients that have been treated with fetal nervous tissue implantation inside the syrinx. Results of all these approaches are mixed at best, with arrest in syrinx progression being considered a relative success (Falci *et al.*, 1997, 2009). However, failure rates as high ar 50% are to be expected in the first two years after operative treatment of syringomyelia. Arachnoiditis, which can be the direct result of SCI but also a rare complication of intraoperative durotomy during surgical decompression, and may lead to tethering of healthy spinal cord or nerve roots, has a poor prognosis despite the possibility of untethering operations. Attempts to repair human SCI at the chronic stage have been reported sporadically and have been generally met with scepticism by the scientific community and with good reason.

Administration of *methylprednisolone* (MP), a synthetic glucocorticoid and potent immunosuppressive, was the first pharmacological treatment for acute SCI to be introduced in many trauma centers in the 1990s (Baptiste and Fehlings, 2006). This

breakthrough occurred in the wake of the multicenter randomized clinical NASCIS (National Acute Spinal Cord Injury Study) trials, whereby MP was shown to prevent extensive tissue destruction and to enhance neurological recovery when administered within 8 h after trauma (Bracken *et al.*, 1990a, 1990b, 1992, 1993). On the basis of the results of the NASCIS I and II, MP is currently administered in a 24-hour regimen as follows:

1. intravenous bolus of *methylprednisolone sodium succinate* (30mg/kg) over 15 minutes, and after a pause of 45 minutes,
2. continuous drip (perfusion of 5,4 mg/kg/h) for 23 hours.

The NASCIS III trial compared a 24-hour with a 48-hour MP protocol and concluded that when MP treatment was started in the window of 3-8 hours postinjury the 48-hour regimen was superior in preserving motor function, albeit without significant functional benefit (Bracken *et al.*, 1997). Yet many centers in the US prolong the MP administration at a perfusion rate of 5.4 mg/kg/h until 48 hours instead of 24 hours, if treatment is initiated between 3-8 hours postinjury. However, treatment with high-dose MP is not free of complications since it is associated with higher frequency of gastric bleeding and wound infection. Indeed, side effects were greater in those patients receiving the 48-hour infusion regimen in the NASCIS III trial (Bracken *et al.*, 1998). Moreover, corticosteroid use is contraindicated in cases of concurrent infection or risk of infection, e.g. gunshot wounds, penetrating injury with intradural foreign body, CSF leak or bowel injury, which renders many patients with acute SCI ineligible for MP treatment. In these cases antibiotic therapy is mandatory. Nevertheless, even the efficacy of MP has been called into question by many clinicians and experimental scientists (Nesathurai *et al.*, 1998).

The proponents of MP believe it to be *neuroprotective* in a variety of ways (Anderson *et al.*, 1982; Bracken *et al.*, 1990a, 1990b; Bracken *et al.*, 1992a, 1992b; Bracken *et al.*, 1993; Constantini and Young, 1994; Ducker and Zeidman, 1994; Young *et al.*, 1994; Gerndt *et al.*, 1997). MP has been shown experimentally to promote neurological recovery in animal models of SCI and to attenuate, directly or indirectly, several detrimental postinjury pathophysiological processes or biochemical cascades, such as posttraumatic ischæmia, tissue œdema, disturbance of aerobic metabolism and resultant lactic acidosis, inflammatory reactions, TNFα production by monocytes/macrophages, nitric oxide production, free radical generation, lipid peroxidation and cellular membrane desintegration, degradation of neurofilaments, intracellular calcium accumulation, excitotoxicity, apoptosis and more (Anderson *et al.*, 1982, 1985, 1988; Braughler *et al.*, 1982, 1983, 1984; Demopoulos *et al.*, 1982; Young *et al.*, 1982, 1996; Hall *et al.*, 1984, 1988, 1992; Holtz *et al.*, 1990, 1992; ; Radi *et al.*, 1991; Liu *et al.*, 1993; Ducker and Zeidman, 1994; Farooque *et al.*, 1994, 1995, 1996a, 1996b, 1997a, 1997b; Bartholdi and Schwab, 1995; Hall, 1996; Gerndt *et al.*, 1997; Franchimont *et al.*, 1999; Xu *et al.*, 1998; 2001; Fu and Saporta, 2005; Li *et al.*, 2000). On the clinical front, the NASCIS trials provided evidence that treatment with MP in high doses within 8 hours of injury resulted in modest but significant improvement and thus MP was finally introduced as an empirical treatment of acute SCI in many centers (Ducker and Zeidman, 1994; Bracken *et*

al., 1997a, 1997b; Bracken *et al.*, 1990a, 1990b). However, its efficacy has been constantly called into question (Ducker and Zeidman, 1994; George *et al.*, 1995; Rabchevsky *et al.*, 1999; Coleman *et al.*, 2000; Hurlbert *et al.*, 2000; Qian *et al.*, 2000; Short, 2000; Short *et al.*, 2000; Sipski and Pearse *et al.*, 2006). Several groups have reported a lack of long-terms effects of MP on functional recovery after SCI in clinical trials, while in animal studies where a failure in hindlimb funcional recovery was reported, stereological analysis showed at best a marginal effect on lesion volume and no effect on the amount of sparing of grey and white matter (George *et al.*, 1995; Gerndt *et al.*, 1997; Nesathurai *et al.*, 1998; Hurlbert *et al.*, 2000; Faden *et al.*, 1984; Ross and Tator, 1993; Ross *et al.*, 1993; Haghighi *et al.*, 1998; Rabchevsky *et al.*, 1999; Coleman *et al.*, 2000; Short, 2000; Short *et al.*, 2000; Qian *et al.*, 2000; Baptiste and Fehlings, 2006). In conclusion, MP still remains a controversial *empirical* treatment that despite its widespread use has not reached the status of *standard* treatment since it lacks both FDA (Federal Drug Administration) approval and endorsement by the AANS/CNS (American Association of Neurological Surgeons/Congress of Neurological Surgeons) guideline committee (Ducker and Zeidman, 1994; George *et al.*, 1995; Nesathurai *et al.*, 1998; Hurlbert *et al.*, 2000; Short, 2000; Short *et al.*, 2000; Baptiste and Fehlings, 2006; Sipski and Pearse *et al.*, 2006). In fact the AANS/CNS has rated all NASCIS publications only as class C evidence because of faults in study design, data presentation, data interpretation and data analysis, and as a result its use has been suspended in many trauma centers (Coleman *et al.*, 2000; Short, 2000; Short *et al.*, 2000; Baptiste and Fehlings, 2006; Sipski and Pearse *et al.*, 2006).

A few other experimental drugs have been tested clinically, but none achieved the *primary endpoints* of the trials (naloxone, GM1, tirilazad). Naloxone has been studied in animal models in the 1980s where it was shown to reverse spinal shock and improve SCBF after SCI, although these results were not unequivocal. Small clinical trials did not support the early promise of naloxone and other opiate antagonists. Experimental studies had also shown that the monosialoganglioside GM1 could reduce excitotoxic neuronal death, enhance the survival effect of growth factors and promote axonal sprouting (Constantini and Young, 1994; Young *et al.*, 1994; Skaper and Leon, 1992). Extended delivery of GM1 in a small trial yielded promising results (Geisler *et al.*, 1991, 1992, 2001). A new family of 21-aminosteroids was designed that would be free of some of the unwanted side-effects of glucocorticoids while retaining some of their positive properties (Hall *et al.*, 1988). The prototype 21-aminosteroid, *tirilazad*, has been shown *in vitro* to act as a free radical scavenger and to inhibit iron-catalyzed lipid peroxidation in rat brain tissue homogenates among others. It was also shown *in vivo* that tirilazad, like MP, reduced the posttraumatic decrease in SCBF and improved recovery of hind limb motor function after experimental SCI in the rat (Anderson *et al.*, 1988; Hall *et al.*, 1988; Holtz *et al.*, 1992; Farooque *et al.*, 1994, 1996). However, it was later shown in animal studies that tirilazad lacked the attenuating effect of MP on glutamate excitotoxicity, arginine and NO production, lactic acidosis and tissue œdema (Braughler *et al.*, 1987; Farooque *et al.*, 1994, 1996). Unfortunately, the NASCIS III clinical trial did not demonstrate any positive effect for tirilazad (Bracken *et al.*, 1997). More neuroprotective agents have been tested in animal models of SCI that either never made it to clinical trials or gave

disappointing results in small scale clinical trials (e.g. modulators of noradrenaline receptors such as clonidin, excitatory aminoacid NMDA and AMPA-kainate antagonists such as gacyclidine and MK-801, voltage-gated Na+ channel blockade, hypothermia, etc.). An alternative approach, which does not have a neuroprotective mode of action, is offered by *4-aminopyridine* (Halter *et al.*, 2000). This is a K+ channel blocker and aims at improving the conduction of action potentials along demyelinated but otherwise spared axons (Haghighi *et al.*, 1998; Halter *et al.*, 2000). Demyelination of axons, which may occur in SCI even at some distance from the lesion site because of oligodendroglial losses, leads to the exposure of the large population of internodal potassium channels normally insulated by healthy myelin, and to failure of saltatory conduction. Blocking the shunting function of potassium channels by 4-aminopyridine, is believed to improve conduction of denuded axons (Halter *et al.*, 2000). Small scale trials have shown indications of a modest improvement in muscle strength and less muscle fatigue at repetitive movement tasks (Haghighi *et al.*, 1998; Halter *et al.*, 2000; Blight *et al.*, 2002). Due to its mechanism of action *fampyridine* is undergoing clinical trials not only for SCI but also multiple sclerosis (Baptiste and Fehlings, 2006).

Since the early 1980's advances in the field of neuroregeneration have led to the formulation of rational hypotheses about how to abrogate or reverse some of the secondary injury processes in order to increase neuroprotection and enhance tissue repair (Richardson *et al.*, 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982; Schnell *et al.*, 1994; Bregman *et al.*, 1995; Cheng *et al.*, 1996; Kalderon and Fuks, 1996a; Kalderon and Fuks, 1996b; Davies *et al.*, 1997; Li *et al.*, 1997; Ramon-Cueto *et al.*, 1998, 2000; Chen *et al.*, 2000; Merkler *et al.*, 2001; Blight and Zimber, 2001; Teng *et al.*, 2002; GrandPré *et al.*, 2002; Bradbury *et al.*, 2002; Lee *et al.*, 2002; Silver, 2002; Neumann *et al.*, 2002; Fraidakis *et al.*, 2004). Some of these hypotheses have provided the basis for potential treatments. Although few of these hold promise to reach the clinic and improve the prognosis of SCI, the progress during the last 25 years has been astonishing. A therapeutic approach that is not neuroprotective *a priori* is the usage of neutralizing humanized antibodies against Nogo-A, the primary myelin inhibitor to axonal regeneration after CNS injury. Experiments in animal models of SCI during the past 20 years have shown that infusions of various different anti-Nogo antibodies, promote axonal regeneration and long distance axon growth (up to 1 cm), neuroplasticity and, most importantly, functional recovery (Schnell *et al.*, 1990; Schnell and Schwab, 1990; Chen *et al.*, 2000; Merkler *et al.*, 2001). The reproducibility of these results in many rodent and primate experiments led to a phase I clinical trial of neutralizing anti-Nogo humanized antibodies as a treatment for human SCI (Buchli and Schwab, 2005; Buchli *et al.*, 2007).

Cell or tissue transplantation approaches are powerful alternatives to single drug approaches because they offer the potential for neuroprotection, neuronal regeneration and neuroplasticity altogether. A variety of cells or tissues, autologous, homogeneic or allogeneic, have been experimentally implanted in the lesioned spinal cord with variable results. In reality, the experimental results in SCI animal models though they have verified some of the guiding principles of transplanation approaches they have not

produced dramatic effects. The problem is that cell replacement therapies in general, do not circumvent some of the major obstacles of axonal regeneration in spinal cord injury, i.e. the formation of glial scar and the inhibitory effect of white matter. *Olfactory ensheathing cells* (OECs) may be the best candidate for a clinical autoimplantation treatment of SCI. Preclinical evidence has been relatively robust and small-scale clinical trials have also been conducted although with very modest results.

SUBACUTE AND CHRONIC STAGES

After the early phase measures, SCI patients must be followed-up closely to avoid deterioration of their condition and prevent numerous possible complications. For cervical injuries not initially intubated *vital capacity* should be monitored at least thrice daily for the first few days and if it declines steadily or rapidly intubation should be performed. The ætiology of respiratory system malfunction in cervical and high thoracic injuries is manyfold. First, the expiratory flow rates are diminished (because of the interruption of the neural pathways to the expiratory musculature) and thus inadequate for an efficient coughing reflex. Second, while sympathetic innervation to the airways and lung may be seriously compromised (because of the interruption of the sympathetic pathway or damage to the intermediolateral column at cervical and upper thoracic levels) parasympathetic tonus via the vagus is unopposed. This leads to poor airway dilatation and hypersecretion from the mucosa with resultant airway obstruction which in combination with inefficient coughing aggravates the already compromised respiratory function of the patient. Treatment with bronchodilators, mucolytic agents in conjunction with chest physiotherapy and assisted coughing can alleviate the respiratory symptoms.
As an advanced measure, phrenic nerve stimulation may be used when there is damage to respiratory pathways above the origin of the phrenic nerve (C3).

Deep venous thrombosis (DVT) and *pulmonary embolism* (PE) are other frequent and feared acute complications of SCI. They occur often in individuals with complete cervical or thoracic SCI because of failure of the muscle pump that aids venous flow in the lower limbs. The prevention of PE and DVT usually begins on admission with the use of pneumatic devices and elastic stockings. Prophylactic anticoagulation treatment is also imperative for a certain period after trauma in the absence of contraindication. For patients with complete injuries and poor prognosis prophylactic inferior vena cava filter may become an option.

Autonomic dysreflexia is a frequent and potentially life-threatening complication affecting SCI patients with midthoracic (T1-T5) or higher level lesions and subsequent interruption of the sympathetic pathways that course towards the sympathetic spinal center. Autonomic dysreflexia may appear soon after injury and occur for days or weeks or reoccur after years, although its incidence peaks during the first week after injury. The autonomic imbalance with peripheral parasympathetic dominance caused by the lesion leads to precipitous changes in blood pressure, heart rate and temperature usually triggered by pain, adbominal distention, fecal impaction, voiding but also benign

manœuvers such as repositioning. Thus, patients are susceptible to a vicious circle of *autonomic storms* consisting of hypertension above injury level, chills, diaphoresis, mydriasis, headache, pallor and piloerection and simultaneous reflex bradycardia with transient 3rd degree heart block, or even asystole. Compensatory vagus outflow can be so severe as to be the cause of death, therefore depending on the gravity of the vagal symptomatology, vagolytics such as atropine should always be at hand to reverse the condition, but also antihypertensive agents to reduce central hypertension and its repercussions. If the episodes become life threatening pacemaker implantation should be considered. Most important though is prevention of the attacks by adequate bladder and bowel care. Autonomic dysfunction may also lead to *orthostatic hypotension* at later stages when the patient is mobilized during rehabilitation.

Urogenital and bowel functions are severely impaired in complete SCI and a cause of much social and medical trouble for the patient. Complications of bladder and sphincter dysfunction were until 10 years ago the second leading cause of death for SCI patients (Frankel *et al.*, 1998). SCI results in *neurogenic (paralytic) bladder* that, depending on whether the lesion is central (suprasacral) or peripheral (sacral parasympathetic center at S2-S4 or cauda equina), will either recover a voiding reflex (suprasacral SCI) or develop *arreflexia*. Voiding will be affected in both cases, with urinary retention (more so in the arreflexic bladder and during the spinal shock phase) and dysuria. If the lesion is also above the sacral center of somatic innervation of the external (striated) sphincter (Onuf's nucleus at S1-S4) *detrusor-spincter dyssynergia* (DSD) is inevitable. DSD is a very serious pathology because it can lead to destruction of the supravesicular apparatus due to the high-pressure voiding that it entails, leading progressively to bladder distention, bladder diverticulae, vesicoureteral reflux, hydronephrosis and eventually renal failure. Urinary retention and DSD predispose also for bladder stones, urinary tract infections (UTIs), pyelonephritis and urosepsis. The goal of treatment is to alleviate obstructive symptoms caused by DSD and control retention symptoms. In this last respect intermittent self-catheterization for the patient with sufficient upper limb use can have satisfactory results. The pharmacological means of urologists are not very efficient however, and consist mainly of antimuscarinic, antiadrenergic and spasmolytic drugs to address the bladder hypperreflexia, sphincter obstructivity and DSD. When conservative treatment fails several surgical alternatives exist to improve symptomatology and prevent renal damage. As a last option, *functional electric stimulation* (FES) allows the SCI patient to regain voluntary control of his bladder and bowel function. SCI leads to paralytic bowel that similarly to the bladder may more or less regain a reflex function and the anal sphincter may suffer similar dysregulation as the urethral sphincter, depending on level of injury. The end result is dyschezia and constipation. The FDA-approved VOCARE system is an implantable FES device that activates the anterior sacral nerve roots (S2-S4) containing the parasympathetic fibers to initiate coordinated contractions of detrusor/urethral sphincter and large bowel/anal sphincter. However, this FES system necessitates dorsal rhizotomy (S2-S5) in order to improve bladder compliance and prevent DSD with resultant loss of sacral sensation and reflex erection. Thus, VOCARE is primarily used in ASIA-A patients who do not have sacral sensory function and cannot have erections (Creasey *et al.*, 2001). The neurogenic erectile dysfunction in men with

SCI can be addressed with technical (vacuum devices, implantable prostheses) and pharmacologic means (sildenafil *per os*, or injection of vasoactive agents into the corpora cavernosa, e.g. phentolamine mesylate, papaverine or prostaglandin E_1). Some of these medications, especially sildenafil, may also have a potential in treating sexual dysfunction of women with SCI.

Spasticity is another life-long complication of SCI. The ætiopathogenesis of *spastic muscle hypertonia* (or simply *spasticity*) in SCI, is the interruption of the long descending supraspinal pathways, that exert a modulatory effect on segmental spinal circuits in order to constantly regulate muscle tone in static or kinetic conditions, not much different than the case of spasticity that settles in after sroke in patients that have not commenced early and rigorous physiotherapeutic regimen and can lead to contractions with limb deformation (Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; Taricco *et al.*, 2006). Postapoplectic spasticity is the result of spinal cord denervation from upper motor neurons, secondary to the demise of pyramidal and extrapyramidal cortical neurons at the cortical level or damage to their axons at subcortical levels (Sköld *et al.*, 1999; Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; Taricco *et al.*, 2006). In SCI however, for obvious neuroanatomical reasons, in addition to corticospinal pathways, other s*upraspinal* (such as rubrospinal, vestibulospinal, reticulospinal, raphespinal, cœrulospinal) and *propriospinal* pathways are interrupted, which are also involved in muscle tone control for locomotor, motor coordination or postural purposes (Sköld *et al.*, 1999; Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; Taricco *et al.*, 2006). Spasticity is all the more severe if most or all of these pathways are interrupted, and this is the reason why spasticity after SCI is a more complicated problem than spasticity after stroke (Sköld *et al.*, 1999; Kirshblum, 2002; Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; Taricco *et al.*, 2006). For example, SCI patients not only suffer from permanent tonic spasticity of certain muscles (as the one seen after stroke), but can also experience painful spastic crises, which are triggered by proprioceptive (active or passive limb movements) or exteroceptive (cutaneous stimulation) sensory input fed into overactive, damaged, or aberrant neoplastic monosegmental or polysegmental reflex loops that are no longer under supraspinal and/or proriospinal control (Sköld *et al.*, 1999; Kirshblum, 2002; Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; ; Taricco *et al.*, 2006). The patient is overcome by a wave of painful spasms that sweeps whole muscle groups until it subsides after seconds or minutes (Sköld *et al.*, 1999; Pandyan *et al.*, 1999; Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; Taricco *et al.*, 2006). Scales to evaluate spasticity that rely on a subjective, semiquantitative measure of resistance to passive movement (Ashworth and modified Ashworth scales) are in use, but are limited by interrater variability and essentially fail to comprehensively assess the 'spastic syndrome' in its entirety (Bohannon and Smith, 1987; Pandyan *et al.*, 1999; Sköld *et al.*, 1999; Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; Taricco *et al.*, 2006). Botulinum toxin has limited therapeutic potential in spasticity associated with SCI. Antispastic medications, though not equally efficacious (baclofen, dantrolene, tizanidine, diazepam, and to a lesser extent gabapentine, clonidine, amytal), do not extinguish the problem. Intrathecal administration of baclofen by a special indwelling pump device may thus become the last resort (Sköld et al., 1999; Biering-Sørensen *et al.*, 2006; Lechner *et al.*, 2006; Taricco *et al.*, 2006).

In the acute and subacute phase after SCI, pain syndromes unrelated to spasms are also known to occur, and in the chronic SCI pain syndromes affect about 25% of individuals with SCI. These pain syndromes are mostly of the *neuropathic* type and their exact pathogenetic mechanisms are being vigorously investigated. Neuropathic pain can be the result of injuries of both the PNS and CNS, however the pathophysiological mechanisms always involve a central mechanism. In SCI, aberrant axonal regrowth, plasticity or circuitry reorganization, and disturbed local neuromodulation at spinal level can only partly explain this phenomenon. However, pain thresholds are not only modified at spinal level but also at more proximal pain centers such as the thalamus, while the firing properties of nociceptive pathways seem to be involved as well. Different medications have shown an effect reflecting the different pathogenic mechanisms of neuropathic pain, such as opiates, antidepressants (tricyclics, SSRIs, NSRIs, etc.) and of course various antiepileptic drugs or anesthetic agents.

Neurorehabilitation after SCI aims at maximizing the functional autonomy of the patient and if possible ambulation, by exploiting the residual functional and plastic capacity of the nervous system of the injured individual to the fullest. In *spinalized* animals, i.e. fully transected at the spinal cord level excluding supraspinal input, locomotor improvement below the level of transection never occurs spontaneously. Sherrington noticed that *spinalized* animals could regain complex alternating patterns of hindlimb movements after a period of retraining and first postulated the existence of a *spinal pattern generator* (Sherrington, 1910). Spinal and supraspinal *central pattern generators* (CPG) have now been well-characterized in both higher vertebrates e.g. the cat, the rat etc., and lower vertebrates, e.g. the lamprey (Grillner *et al.*, 1979; Cazalets *et al.*, 1995; de Leon *et al.*, 1998; Edgerton *et al.*, 2004; Bouyer *et al.*, 2005; Grillner and Dubuc, 1988; Rossignol and Dubuc, 1994; Dimitrijevic et al., 1998; Hultborn *et al.*, 1998; Pinter and Dimitrijevic, 1999). It thus seems that spinal pattern generators have evolved during phylogenesis into an important common element of the hardwiring of the invertebrate and vertebrate CNS that organizes locomotion (Grillner and Dubuc, 1988; Rossignol and Dubuc, 1994; Dimitrijevic et al., 1998; Hultborn *et al.*, 1998; Pinter and Dimitrijevic, 1999; Grillner *et al.*, 1979; Edgerton *et al.*, 2004). In four-legged animals, spinal pattern generators are found in both cervical and lumbar enlargements while in man a lumbar central pattern generator is dominant (Grillner *et al.*, 1979; Edgerton *et al.*, 2004). Afferents to such pattern generators are derived from both supraspinal centers and peripheral organs of locomotion (Edgerton *et al.*, 2004). Brain stem nuclei connect to lumbar spinal motoneurons via reticulospinal, raphespinal and vestibulospinal projections (Jordan *et al.*, 1992; Peterson *et al.*, 1979). Animal studies have shown that monoaminergic systems originating in the brainstem, such as serotoninergic (raphespinal) and noradrenergic (cœrulospinal from locus cœruleus and subcœruleus) are especially important (Bowker *et al.*, 1982; Holstege and Kuypers, 1982; Holstege *et al.*, 1987; Westlund *et al.*, 1982). Functional improvement in completely spinalized animals is attributed to reactivation of oscillating motor programmes generated by the CPG and plasticity of the CPG neuronal circuitry below the lesion level as the result of *peripheral sensory input* from the limbs during training (Edgerton *et al.*, 2004, 2006; Engesser-Cesar *et al.*, 2005). Indeed,

functional recovery of the hindlimbs in spinalized animals achieved after gravity-assisted treadmill training could be improved by FES or pharmacologic intervention, e.g. intrathecal administration of serotonin or α-adrenergic agonists such as clonidine (Rossignol *et al.*, 2000; Rossignol *et al.*, 1999; Barbeau *et al.*, 1999; Chau *et al.*, 1998; de Leon *et al.*, 1998; Ichiyama *et al.*, 2005). In rats with contusion SCI the expected spontaneous recovery could be further improved by training or simply by environmental enrichment according to some reports while ther has been other syudies dipsuting that (Lankhorst *et al.*, 2001;Van Meeteren *et al.*, 2003; Hutchinson *et al.*, 2004; Moon *et al.*, 2006; Erschbamer et al., 2007).

On the basis of the paradigm of retrained spinalized animals it was hoped that vigorous sessions of repetitive movement tasks by body weight-supported treadmill training would reactivate the central pattern generators in human patients with SCI (Barbeau *et al.*, 1999; Curt *et al.*, 2004; Edgerton *et al.*, 2004, 2006; Bouyer *et al.*, 2005; Damiano and DeJong, 2009). Augmentation of treadmill training with FES has also been investigated while tendon transfer operations aim at increasing the amount of functionally useful muscle. Multicenter clinical trials evaluating the importance of each of these neurorehabilitative measures have been undertaken (Edgerton *et al.*, 2004; Barbeau *et al.*, 2006; Wolpaw, 2006; Damiano and DeJong, 2009). In fact, a locomotor activity pattern has been induced in incomplete and to a small extent even complete paraplegic patients that underwent a regular training protocol (Edgerton *et al.*, 2006; Barbeau *et al.*, 2006). Physical therapy alone has poor results on ambulation capacity in patients with recent severe SCI (ASIA A and B) (Edgerton *et al.*, 2004; Field-Fote *et al.*, 2005; Dobkin *et al.*, 2006; Damiano and DeJong, 2009). However, physical therapy and treadmill locomotor training with body weight support begun soon after the acute phase of SCI enhanced the ability of neurologically complete SCI patients to stand/step on a treadmill (Edgerton *et al.*, 2004, 2006; Field-Fote *et al.*, 2005; Dobkin *et al.*, 2006; Wolpaw, 2006; Damiano and DeJong, 2009). The results were more encouraging for patients, especially young, with motor incomplete SCI who regained some ambulatory capacity when retrained conventionally or on a treadmill with weight support (Edgerton *et al.*, 2004; Field-Fote *et al.*, 2005; Dobkin *et al.*, 2006; Wolpaw, 2006; Damiano and DeJong, 2009). Conventional overground locomotion training or gravity-assisted treadmill training were modestly beneficial even for patients with chronic incomplete SCI (Herman *et al.*, 2002; Wernig, 2005; Edgerton *et al.*, 2004; Field-Fote *et al.*, 2005; Dobkin *et al.*, 2006; Wolpaw, 2006; Damiano and DeJong, 2009). Moreover, FES of the dorsal surface of the lumbar cord region of patients with complete SCI could induce not only rythmic electromyographic activity in leg muscles but also step-like movements, while the combination of treadmill training and epidural FES was superior to treadmill training alone in improving overground or treadmill stepping/walking quality in patients with incomplete SCI (Wolpaw, 2006). Lower limb spasticity can be of functional use to the paraplegic during locomotor training by enabling antigravity support of the body during the stance phase of gait (Barbeau *et al.*, 2006). On the other hand, uncontrolled muscle hypertonia or spasms can parasitize the swing phase of gait or interfere with the swing/stance pattern, and therefore spasticity control by pharmacological means or better yet by FES at specific points in the step cycle could be beneficial (Fung *et al.*, 1994). The muscles suffering

central paresis in paraplegics are believed to undergo plastic changes in the peripheral innervation that regulates muscle tonus at rest but also during the functional movements of gait training (Dietz *et al.*, 1981; Berger *et al.*, 1984). Residual muscle strength and peripheral plasticity are of obvious benefit in gait training, however alterations in spinal output seem to be also responsible for the results obtained by gait training (Dietz *et al.*, 1981; Berger *et al.*, 1984). Indeed, promotion of central plasticity is presumably a very important mechanism behind functional recovery of SCI patients after neurorehabilitation therapy. In complete SCI plasticity, only spinal circuit plasticity and especially at the CPG level is operative in any functional recovery of the paralysed segments of the body (Raineteau *et al.*, 1999, 2001, 2002; Z'Graggen *al.*, 2000; Bareyre *et al.*, 2004; Raineteau, 2008). After incomplete SCI, there is the possibility for supralesional neuroplasticity and formation of spinal circuits that bypass the lesion by sprouting of lesioned fibers, or collateral formation by lesioned or unlesioned fibers which finally synapse onto spared long tracts (Raineteau *et al.*, 1999, 2001, 2002; Z'Graggen *al.*, 2000; Raineteau, 2008). Neuroplasticity after both complete and incomplete SCI entails functional rearrangements along the entire neuraxis, at cortical (sensorimotor cortex) and subcortical levels and in experimental animals with partial spinal lesions it has been shown that the loss of supraspinal input can be compensated for by extensive neuroplasticity of the corticospinal tract (CST) and other descending systems (rubrospinal, vestibulospinal, reticulospinal) at brain stem level with collateral formation and midline cross-over of new collaterals (Raineteau *et al.*, 1999, 2001, 2002; Z'Graggen *al.*, 2000; Raineteau, 2008).

In conclusion, although gravity-assisted treadmill locomotor training may not enable people with severe SCI (ASIA A or B) to become ambulatory it may help individuals with motor incomplete injuries (ASIA-C and D) to regain substantial overground locomotion, while the combination of centrally and peripherally induced stepping, by FES and treadmill/overground training respectively, has a synergistic beneficial effect (Barbeau *et al.*, 1999; Raineteau *et al.*, 1999, 2001, 2002; Z'Graggen *al.*, 2000; Baptiste and Fehlings, 2006; Raineteau, 2008; Damiano and DeJong, 2009).

GENERAL BACKGROUND:
NEUROIMMUNOLOGICAL PERSPECTIVES

The nervous and immune systems interact intricately under physiological and pathological conditions, e.g. autoimmune, infectious, neoplastic or paraneoplastic disorders (Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995; Steinman, 2004; Allan and Rothwell, 2001, 2003; Bradley *et al.*, 2003; Bauer *et al.*, 2001; Becher *et al.*, 2000). The role of the immune system in ischæmic and mechanical injury of the nervous system, such as stroke, traumatic brain injury and spinal cord injury has therefore become the object of intensive research and the therapeutic potential of immunomodulation is increasingly investigated but also hotly debated (Jones *et al.*, 2005a; Popovich and Jones, 2003; David and Ousman, 2002; Schwartz, 2001; Ghirnikar *et al.*, 1998; Dirnagl *et al.* 1999; Bethea *et al.*, 2002; Dietrich *et al.*, 2004; Crutcher *et al.*, 2006). Lastly, even the neuroimmunological aspects of diseases traditionally classified as neurodegenerative such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis have not escaped this trend (Haga *et al.*, 1993; Itagaki *et al.*, 1988; Togo *et al.*, 2002; Przedborski *et al.*, 2003; Ringheim and Conant, 2004; Orr *et al.*, 2002; McGeer *et al.*, 1988; McGeer and McGeer, 2000, 2002; Wyss-Coray and Mucke, 2000; Vila, 2001; Engelhardt *et al.*, 1993; Kawamata *et al.*, 1992; Vila *et al.*, 2001; Dietrich *et al.*, 2004). Nevertheless, the nature, scope and teleology of the interaction between the two most elaborate systems of the human body are still largely unknown to us.

THE IMMUNE SYSTEM: AN INTRODUCTION

In vertebrates, the immune system has two components, one subserving *innate* immunity and the other *acquired* (or *adaptive*) immunity (see Table). Each is capable of *cellular* and *humoral* effector responses. This double dichotomy serves only an academic purpose since, in reality, innate and acquired immunity form a functional continuum, with the innate response always preceding the acquired response and the latter always ensuing the former to a certain degree. The innate immune response is fast and efficient but has limited specificity, while an astonishing diversity and ultraspecificity are the hallmarks of the acquired immune response, expressed by the existence of millions of different clones of immune cells that can produce antibodies to virtually any antigen encountered, hence the use of the term *adaptive immunity*.

As regards the *cellular arm* of the immune system, the *innate* immunological cellular response is the primary, omnipresent cellular reaction to noxious agents and a *sine qua non* of tissue inflammation, operating with a few cell types of low specificity for pathogens (see Table). Among the leucocytes, *neutrophil polymorphonuclear granulocytes* constitute the first line of defence, followed by monocytes with their enhanced phagocytic and proinflammatory potential. On the other hand the *acquired* immunological cellular response, that is somewhat delayed compared to the innate response, is secondary but very specific, and with its enormous variety of T cell and B

cell clones shields the organism with a *ne plus ultra* adaptive immunological reaction to *non-self antigens*. The immunogenicity of foreign antigens is mainly determined by their molecular composition and is mediated by their presentation to B and T cell receptors. *Antigen presentation* is the central event in the initiation of an adaptive immune response and the mode of antigen presentation is determinant of the type of response. Antigen presentation is effected by specialized *antigen-presenting cells* (APCs) (see Table). The antigen is internalized and digested inside the organelles of the APC, and *peptidic* or *glycopeptidic* fragments of it (up to 25 amino acids long) with their salient molecular motifs (*epitopes*) are then displayed on the surface of the APC, snugly fit onto a *major histocompatibility complex* (MHC) class II molecular scaffold, for recognition by the T helper *T cell receptors* (TCR) specific for the given epitopes. Which antigenic peptides and epitopes of the same antigen are displayed on the surface of the APCs of each individual of a vertebrate species is largely dependent on the MHC class II genotype of that individual, since MHC genes are highly *polymorphic*. MHC class II molecules are exclusively expressed on APCs and comprised of two polymorphic chains. They bind peptides derived from *extracellular proteins*, e.g. those of a bacterium phagocytosed and processed in the endosomal system of an APC and are subsequently recognized by the antigen-specific T cell receptors of $CD4^+$ *T helper* cells (T_H),. On the contrary, MHC class I molecules, comprised of one polymorphic chain and a constant β_2-microglobulin chain, are expressed on the surface of most cell types, are exclusively recognized by the T cell receptors (TCR) of the $CD8^+$ *cytotoxic T lymphocytes* (CTLs), and bind peptides (usually up to 9 amino acids long) derived from *intracellular* proteins, e.g. viral proteins manufactured by the virus-infected cell. MHC class I molecules are also the signature molecules in cellular self vs. non-self recognition (*histocompatibility*) and mediate graft-rejection by CTLs. Interestingly, MHC class I molecules have also been implicated in CNS development, plasticity and synapse regulation (Corriveau et al., 1998; Huh et al., 2000; Boulanger et al., 2001; Boulanger and Shatz, 2004; Goddard et al., 2007).

The genes coding for the so called *classical* and *nonclassical* MHC molecules, which definitely have functions other than the strictly immunological described here, are located on chromosome 6, 20 and 17 in humans, rats and mice respectively (Gunther and Walter, 2000, 2001; Dressel *et al.*, 2001). The MHC coding region of chromosome 6 in humans is the *human leucocyte antigen* (HLA) region, while the rat and mouse chromosomal regions are designated *RT1* and *H2* respectively. The homology between HLA, RT1 and H2 is striking, with each containing four MHC subregions (three for the classical MHC-I, -II, -III molecules and one for the so-called nonclassical MHC molecules) as well as other intercalated genes. Among the genes encoded inside the MHC class III region are those of *Notch 4* and *Heat shock protein 70*, while the nonclassical MHC subregion harbors more than 60 genes with mostly unknown functions. It is known however, that some nonclassical MHC molecules have remarkable structural and functional similarities with MHC class I molecules, e.g. they can interact with β2-microglobulin and so are expressed on cellular surfaces and they are capable of binding to the TCR of CTLs and can thus induce T cell mediated cytotoxicity (Leong *et al.*, 1999; Gunther and Walter, 2000, 2001; Dressel *et al.*, 2001). Among the differences, nonclassical MHC molecules are less polymorphic and exposed at a lower concentration on cellular surfaces compared to

classical MHC-I molecules (Gunther and Walter, 2000, 2001; Dressel *et al.*, 2001). Nonclassical MHC molecules can also interact with cellular receptors on *natural killer* (NK) cells and induce their activation (Braud *et al.*, 1999). In terms of evolution and function the NK cells, involved in both types of immunological responses but more so in the innate response, occupy a position inbetween monocytes and lymphocytes. In fact, immunologists have characterized a particular kind of lymphocyte with both NK and T cell features, designated as *NKT* cell, with important actions in immune tolerance, antitumor immunity and regulation of autoimmune responses (Seaman, 2000; Godfrey *et al.*, 2000; Shi *et al.*, 2001; Brutkiewicz *et al.*, 2002; Sharif and Delovitch, 2001; Seino, 2001; MacDonald, 2002; Sharif *et al.*, 2002; Hammond and Godfrey, 2002; Kronenberg and Gapin, 2002; Bach, 2002; Chan *et al.*, 2003; Wilson and Van Kaer, 2003; Papamichail *et al.*, 2004; Yokoyama, 2004; Seino and Taniguchi, 2004; Kronenberg, 2005; Mercer *et al.*, 2005; Van Kaer and Joyce, 2005; La Cava, 2006; La Cava and Shi, 2006; Bendelac *et al.*, 2007; Van Kaer, 2007; Nowak and Stein-Streilein, 2007; Miyake and Yamamura, 2007; Jukes *et al.*, 2007; Schoenborn and Wilson, 2007; Berzofsky andTerabe, 2008; Swain, 2008).

Binding of a loaded MHC molecule on a TCR is not sufficient for T cell activation. In order for that to occur simultaneous T cell costimulation of T cell *co-receptors* is necessary. These T cell co-receptors are also ligands for their respective receptors on APCs and their binding establishes a bidirectional signaling between T cell and APC. Such T cell co-receptors/ligands are CD28 binding to B7-1 (CD80) or B7-2 (CD86) on APC, and the T cell ligand CD154 (CD40L) binding to CD40 (a member of the TNF receptor superfamily) on APC. Absence of costimulation induces *T cell anergy*. Finally, there are distinct subtypes of $CD4^+$ or $CD8^+$ T cells with strictly regulatory or immunosuppressive roles, designated collectively *T regulatory* cells (T_{REG}) (Shevach *et al.*, 2002; Asseman and von Herrath 2002; Ramsdell, 2003). T_{REG} have emerged as crucial players in the homeostasis of the immune system and in autoimmune diseases (Kohm *et al.*, 2002; Mills, 2004; Bacchetta *et al.*, 2007; Kim *et al.*, 2007; Lange *et al.*, 2007). Their classification is complex and under constant reevaluation but they are currently roughly divided in two principal subsets, the *natural* T_{REG} (nT_{REG}) and the *inducible* T_{REG} (iT_{REG}) cells. The main nT_{REG} cells are 1) the $CD4^+25^+FOXP3^+$, which express the IL2 receptor (CD25) and the transcription factor *forkhead box protein P3*, and 2) the HLA-G expressing nT_{REG} cells that can be either $CD4^+$ or $CD8^+$ (Morgan *et al.*, 2005; Wiendl *et al.*, 2005; Feger *et al.*, 2007; Roncarolo *et al.*, 2008). The iT_{REG} cells include, 1) the T_H3 cells, which are derived from naïve $CD4^+$ or $CD8^+$ precursor cells, and 2) the *type 1* T_{REG} (T_R1) which originate from $CD4^+$ precursor cells (Morgan *et al.*, 2005; Wiendl *et al.*, 2005; Feger *et al.*, 2007; Roncarolo *et al.*, 2008). The iT_{REG} produce immunoregulatory cytokines (such as IL10 and TGFβ) that suppress the activities of CTLs and most T_H, designated as *effector T cells* (T_{EFF}), or they interact directly with APCs and render them tolerogenic, as $CD8^+28^-$ iT_{REG} do (Chang *et al.*, 2002; Morgan *et al.*, 2005; Wiendl *et al.*, 2005; Feger *et al.*, 2007; Roncarolo *et al.*, 2008). The role of B cells will be described in conjunction with that of antibodies.

The *humoral responses* of the immune system are subject to the same gross division in innate (antigen-nonspecific or broadly-specific) and acquired (antigen-specific and ultraspecific). The *innate humoral* response operates with antigen-nonspecific or partly specific molecules, with cytokine/chemokine or other antimicrobial actions, produced by cellular elements of the immune or other systems (liver, spleen, reticuloendothelial), such as the complement system and the cytokine/chemokine systems, the acute phase proteins and the interferons (see Table) (Medzhitov and Janeway, 2000a, 2000b, 2000c). The complement system operates via three known pathways or *cascades*: the *classical* pathway triggered by antigen-antibody complexes; the *alternative* or *properdin cascade* which is triggered by foreign antigens not bound to antibodies; and *the Mannan-binding lectin* pathway, triggered by the binding of an MB lectin to mannose sugars on the surface of a microorganism. The complement components, synthesized in a cascade fashion, have chemotactic, opsonization and bacteriotoxic/cytotoxic actions that result in a potent inflammatory reaction and extermination of the pathogen by a *membrane attack complex* (MAC). The innate humoral response also employs a family of receptors, the *Toll-like receptors* (TLRs), whose role is to recognize certain molecular patterns on the surface molecules of pathogens, termed *pathogen associated molecular patterns* (PAMPs) and trigger an immune response (Medzhitov and Janeway, 2000a, 2000b, 2000c, 2000d). The *Toll* protein was discovered in Drosophila as a morphogen important for establishing the dorsoventral axis during embryogenesis. Homologous receptor proteins were later found to be expressed on the surface of mammalian APCs and were termed TLRs. All members of the TLR family (more than 10 according to the current tally), have cytoplasmic domains similar to the IL1 receptor, called *Toll/IL1R homologous regions* or *TIR domains*. All TIR domains activate *nuclear factor κB* signalling pathways that result in the production of many different cytokines/lymphokines. The extracellular domains of TLRs, with their many *leucine-rich repeat* (LRR) motifs, are believed to act as low-specificty receptors for many different PAMPs on cell wall components of Gram positive or negative bacteria (TLR2 for peptidogycan, lipoteichoic acid, porins etc.; TLR4 for LPS, HSP60 etc.; TLR5 for *flagellin*), for bacterial DNA (TLR9 for CpG DNA) and even viral double-stranded RNA (TLR3) (Takeuchi *et al.*, 1999; Hemmi *et al.*, 2000; Alexopoulou *et al.*, 2001; Ashkar and Rosenthal, 2002; Nguyen *et al.*, 2002). The TLRs obviously function as a primordial version of antigen receptors, that have evolved to counteract the different microorganismal challenges, mostly bacterial, faced by eucaryotic organisms and their role is important for the general immunological defence of the body including the nervous system. Indeed, new findings even suggest an association between the CNS innate immune responses and neurodegeneration in diseases such as AD, PD, ALS and MS, as well as a link between the innate and adaptive immune responses in the CNS mediated by TLRs expressed on microglia (Medzitov and Janeway, 2000a, 2000b, 2000c, 2000d; Nguyen *et al.*, 2002; Laflamme *et al.*, 2001; Lehnardt *et al.*, 2004; Zekki *et al.*, 2002; Bsibsi *et al.*, 2002; Dalpke *et al.*, 2002; Akira *et al.*, 2001; Kim *et al.*, 2007). Moreover, mediators of the innate immune response such as complement components and the TLRs have been shown to participate in the development or synaptic plasticity of the healthy CNS (Stevens *et al.*, 2007).

In the *acquired response*, *immunoglobulins* (antibodies) constitute the effector molecules of an ultraspecific humoral response, produced by specialized *antibody-forming cells* (AFC), the *plasma* cells, which are B cells. Antibody diversity and specificity are generated at the genetic level by complex, random DNA rearrangement mechanisms in the absence of a selective force (*antigen-independent* mechanisms) and are gradually refined and boosted by *antigen-dependent* mechanisms until the stage of B cell clonal selection, propagation and plasma cell maturation. Not suprisingly, the enormous diversity and specificity of T cell receptors is generated in a similar fashion since the two lymphocyte groups are interdependent in a complex network. Indeed, antigen specific T_H cells are essential for B lymphocyte activation and clonal selection (both T_H and B cells being specific for the same antigen) in a perfectly orchestrated process. When a *virgin B cell*, with its trademark antibodies embedded in its plasma membrane and their *complementarity-determining regions* (CDRs) exposed on the extracellular surface, encounters a *competent antigen*, i.e. one that is able to cross-link and aggregate several surface antibodies (with *multivalent antigens* being more effective), the antigen is internalized by the B cell by an antibody-mediated mechanism of endocytosis. The B cell then acts as a genuine APC degrading the antigen and displaying antigenic peptides on its surface MHC class II molecule, to be recognized by T_H cells that carry TCRs specific for the same antigenic epitopes. Those T_H cells specific for the same antigen have in their own right been clonally propagated again by a MHC class II mediated mechanism but with macrophages acting as the APCs. The B-to-T cell binding stimulates the T_H cells to release cytokines that in a *paracrine* fashion lead to B cell proliferation and clonal expansion.

Antibodies, which belong to different immunoglobulin types (IgA, IgD, IgE, IgG, IgM) have many and complex actions. Essential for their activity is their binding to an antigen (through their Fab moiety) and the creation of an antibody-antigen complex which can be mebrane-bound or not. Their actions are direct (through their Fab moiety) or indirect (through their Fc moiety), necessitating the participation of other immune system elements, humoral (complement) or cellular (macrophages, CTLs). They can directly neutralize foreign antigens, or indirectly lead to opsonization and phagocytosis or cytotoxicity of antigen-bearing pathogens or tumor cells.

Both innate and acquired humoral responses rely on a plethora of *cytokines*. The list of these potent intercellular messenger molecules which act as proinflammatory or antiintflammatory agents, proapoptotic or antiapoptotic factors, mitogens or growth factors, with effects that stretch beyond the confines of the immune system is ever expanding. It includes among others the *interleukins* (or *lymphokines*), the *chemokines*, the *tumor necrosis factor* superfamily members and their receptors, the *transforming growth factor* superfamily members, the hæmatopoietic *colony stimulating factors*, the *hæmatopoietic cytokines* (CNTF, LIF, IL6, IL11, CT1 etc.), the *interferons*, the *fibroblast growth factor* family members, various vasoactive or angiogenic factors, and even many neurotrophic growth factors. To these one could add *cell adhesion molecules*, *prostanoids* and many *hormones* (or *neurohormones*) with their important effects on neurons, neuroglia and immune cells (Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995).

Apart from the typical immune cells, neuroglial cells, and most notably microglial cells and astrocytes, are capable of producing many different cytokines and cytokine receptors (Constam *et al.*, 1992; Khoury *et al.*, 1992; Matsumoto *et al.*, 1993; Hopkins and Rothwell, 1995; Gold *et al.*, 1996; Chung *et al.*, 1991; De Simone *et al.*, 1998). This is also true for neurons but to a smaller extent (Bartfai and Schultzberg, 1993). Moreover, molecules heretofore known for their role in the immune system and inflammatory response have now been shown to also act as neuromodulators, neurotransmitters and even play a role in the development and wiring of the nervous system (Choi and Benveniste, 2004; Huh *et al.*, 2000; Corriveau et al., 1998; Boulanger et al., 2001; Goddard et al., 2007; Boulanger and Shatz, 2004; Oliveira *et al.*, 2004; Wirjatijasa *et al.*, 2002; Beattie *et al.*, 2002; Barker *et al.*, 2001; Allan and Rothwell, 2001, 2003; Raivich *et al.*, 1999; Ghirnikar *et al.*, 1998). On the other hand, cells of the immune system and neuroglial cells have been shown to secrete neurotrophic factors (Schwartz *et al.*, 1994; Strauss *et al.*, 1994; Schaar *et al.*, 1993; Ho *et al.*, 1995; Garcia-Estrada *et al.*, 1992; Rudge *et al.*, 1994; Kerschensteiner *et al.*, 1999; Albrecht *et al.*, 2003). There is no stronger proof of the close interaction and co-evolution of the nervous and immune systems than their intricate sharing of cytokine networks, complex ligand-receptor interactions and intercellular recognition mechanisms.

As noted above, as the innate and adaptive responses appear in tandem, so do cellular and humoral immune reactions always operate in concert. The complexity of this cellular-humoral interplay is staggering but a basic understaning has been achieved during the past 20 years. The $CD4^+$ T-helper cells, as their name suggests, play a central role in orchestrating these interactions and in modulating immune responses. Specific subtypes of T_H cells are responsible for the specific type of T cellular immune responses in various conditions by secreting different cytokines (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; Mosmann and Sad, 1996). Thus, T_H are subclassified according to the cytokines they secrete, and are mainly designated as T_H1 (secreting TNFα, TNFβ, IFNγ, IL12, IL18) and T_H2 (IL4, IL5, IL10, IL13), first described by Mosmann and Coffman in 1986, and believed to arise from a pool of 'naïve' T_H0 cells, (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; Bendelac and Schwartz, 1991; Abehsira-Amar *et al.*, 1992; O'Garra A and Arai, 2000; Mosmann and Sad, 1996; Ansel *et al.*, 2003). These refined T cell response programmes are believed to have evolved to meet specific needs by specific actions. For example, the T_H1 response is known to be strongly elicited by viral infections and encapsulated bacteria, while the T_H2 response is elicited by parasitic and helminthic infections (Mosmann and Sad, 1996; Alexander and Bryson, 2005). However, the different T cell subtypes not only release overlapping sets of cytokines. For example, TNFα is also secreted by some T_H2 cells, GM-CSF by both T_H1 and T_H2 cells and IL2 by both T_H0 and T_H1 cells. In the past some immunologists even favored a trichotomy of T_H cells having described a T_H3 (TGFβ secreting) subset which serves a local immunosuppressive or antiinflammatory fucntion in autoimmune conditions, eg. in the human gut in man and in the mouse brain in experimental allergic encephalomyelitis. Another separate T_H subset has been described and characterized recently, designated T_H17 (Afzali *et al.*, 2007; Alber and Kamradt, 2007; Betteli *et al.*, 2006; Korn *et al.*, 2009). T_H17 cells produce IL17, IL17F, IL21 and IL22 and thus induce a massive tissue

reaction thanks to the broad distribution of the IL17 and IL22 receptors, while they communicate with other cells of the immune system via IL21 (Agnello *et al.*, 2003). Proliferation of T_H0 and T_H1 is driven by IL2 and T_H2 propagation is driven by IL4, via autocrine and paracrine loops triggered by appropriate stimulatory and costimulatory signals (Belardelli *et al.*, 1995; Rengarajan *et al.*, 2000; Agnello *et al.*, 2003). T_H17 lineage differentiation and proliferation are both driven by a combination of the actions of TGFβ, IL6, IL21 and IL23 (Agnello *et al.*, 2003; Afzali *et al.*, 2007; Betteli *et al.*, 2006; Korn *et al.*, 2009). Stimulation of T_H cells, as mentioned above, is achieved by interaction between their T cell receptor and a MHC-II molecule, loaded with the antigenic peptide the T cell receptor is supposed to be specific to, on APCs such as macrophages, dendritic cells and even microglia (Aloisi *et al.*, 1999; Aloisi, 2000; O'Garra and Arai, 2000). Although they are all designated numerically as different T_H subclasses, the T_H1, T_H17 and T_H2 are viewed as T_{EFF} and more specifically the former two as *proinflammatory* and the latter as *antiinflammatory*, while T_H3 is classed with the T_{REG} as *immunoregulatory*.

INTERACTIONS BETWEEN THE IMMUNE SYSTEM AND THE CNS

In higher vertebrates the CNS has long been considered to be *immunoprivileged*, due to the paucity of immune system activity within it during physiological, pathological and experimental conditions, in relative contrast to the PNS and other tissues. Indeed, the CNS microenvironment was long believed to be devoid of lymphatic drainage, resident antibody-forming cells and cytotoxic lymphocytes and therefore not equipped to mount an efficient inflammatory or immunological response against pathogens. Intracerebral transplantation studies have provided definitive support to the '*immune-privilege hypothesis*' with allografts surviving longer in CNS than in other tissues (Medawar, 1948; Barker and Billingham, 1977; Björklund *et al.*, 1982; Suter *et al.*, 2003; Aloisi *et al.*, 2001). Clinical experience and experimental evidence of the grave consequences of CNS dissemination by various types of microorganisms support this conclusion. For example, experimental infection of peripheral tissues in mice with the *lymphocytic choriomeningitis virus* (LCMV) causes a massive expansion of virus-specific CTLs (that recognize and kill virus-infected cells expressing MHC class I-viral peptide complexes) that results in viral clearance and host survival, whereas viral inoculation directly into the CNS causes a lethal infection supposedly because of an inadequate counteracting immune response (Joly *et al.*, 1991; Mucke *et al.*, 1992; McGavern *et al.*, 2002;). Another straightforward evidence for a CNS immune privilege was the demonstration that the bacillus Calmette-Guerin if inoculated in the brain parenchyma could escape immune recognition (Matyszak *et al.*, 1997; Matyszak, 1998; Matyszak and Perry, 1998).

Understanding of neuro-immune interactions in health and disease has increased thanks to the animal model of *experimental allergic* (traditionally called 'allergic', although 'autoimmune' is more correct and there has been a shift in usage) *encephalomyelitis* (EAE). EAE is an autoimmune model disease that affects the CNS with extensive demyelination and progressive paralysis and since some EAE variants characteristically course in bouts it has been an attractive model of multiple sclerosis (Ben-Nun *et al.*,

1981; Cohen *et al.*, 1981; Ben-Nun and Cohen, 1982a, 1982b; Storch *et al.*, 1998a, 1998b; Tuohy *et al.*, 1989; Weissert *et al.*, 1998; Swanborg *et al.*, 2001; Griffiths *et al.*, 1999). Experimental encephalomyelitis was first employed by Rivers in 1933 who was investigating *post-vaccination encephalomyelitis* (PVE), that had first been observed in 1885 as a complication of vaccination with Pasteur's anti-rabires vaccine, which consisted of dried rabies virus-infected rabbit spinal cord (Ben-Nun and Cohen, 1981; Ben-Nun *et al.*, 1981a, 1981b, 1981c; Cohen *et al.*, 1981; Ben-Nun and Cohen, 1982a, 1982b; Storch *et al.*, 1998a, 1998b; Tuohy *et al.*, 1989; Weissert *et al.*, 1998; Swanborg *et al.*, 2001; Griffiths *et al.*, 1999; Mackay and Andersson, 2010). Rivers noticed that immunization of monkeys even with normal rabbit CNS extracts, i.e. non-infected rabbit CNS tissue, would sometimes cause demyelinating encephalomyelitis. The term 'allergic' was introduced in 1947 when the adjuvant, that had been developed by Freund in the 40's, was first applied in the experimental encephalomyelitis model to potentiate the induction of disease (Mackay and Andersson, 2010). A similar experimental autoimmune demyelinating condition affecting the PNS, *experimental autoimmune neuritis* (EAN), has been employed to model Guillain-Barré syndrome. Both EAE and EAN, in rats or mice, rely on the induction of autoimmune T cell clones against resident myelin antigens of the CNS (MBP, MOG, PLP) and PNS (P0, P2) respectively, by specific immunization protocols that make use of adjuvants to break self-tolerance (Ben-Nun and Cohen, 1981; Ben-Nun *et al.*, 1981a, 1981b, 1981c; Cohen *et al.*, 1981; Ben-Nun and Cohen, 1982a, 1982b; Hartung *et al.*, 1988; Tuohy *et al.*, 1989; Storch *et al.*, 1998a, 1998b; Weissert *et al.*, 1998; Griffiths *et al.*, 1999; van Der Meché and van Doorn, 2000; Swanborg *et al.*, 2001; Gold *et al.*, 2000; Zou *et al.*, 2000).

The purpose of the immunoprivileged status of the CNS is less obvious, an 'evolutionary trade-off' hypothesis being perhaps the most plausible explanation. Most neurons are postmitotic and terminally differentiated. Since neurons combine into neuronal circuits the demise of neurons that are central to a network or important relays within a circuit would have an influence on the nervous system more profound than just the mere arithmetical subtraction of those neurons. The principal aim of the immune system is to eliminate foreign pathogens and protect the host but when an immune reaction to a pathogen goes unchecked irreversible damage to the CNS is inevitable (Medana *et al.*, 2001c). Thus, in a mouse CNS demyelination model by Theiler virus infection, β2-microglobulin knock-out mice, that did not express functional MHC class I molecules, suffered a milder infection with less axonal pathology and overall CNS damage, compared to wild type mice that could express MHC class I molecules on neurons which therefore succumbed to T cell mediated cytotoxicity (Miller *et al.*, 1995; Rodriguez *et al.*, 1997; Rivera-Quinones *et al.*, 1998; Medana *et al.*, 2001a, 2001b; Neumann *et al.*, 2002). Moreover, an inappropriate *autoimmune* reaction to a CNS self-antigen may have devastating consequences as seen in multiple sclerosis and acute disseminated encephalomyelitis. For example, in MS activated T cells, macrophages and microglia are responsible for demyelination through many different noxious pathways, such as secretion of proinflammatory cytokines, chemokines, intrathecal antimyelin antibody production and myelin destruction and phagocytosis by antibody or complement mediated pathways (Törnqvist *et al.*, 1996; Trapp *et al.*, 1998; Medana *et al.*, 2000,

2001a, 2001b, 2001c; Smith *et al.*, 2001;). Given the numerous cytopathic/toxic molecules produced by neutrophils, macrophages, microglia, plasma or T cells, such as proinflammatory cytokines (TNFα, IL1, IL6, IL12, IL18, IL23 etc.), chemokines (IL8, MIP1α, MIP1β, MCP1, RANTES etc.), nitric oxide, oxygen and nitrogen free radicals and prostanoids (PGD2, PGE2, thromboxane A2, thromboxane B2), evolutionary pressures might explain why the immune system has not been allowed free reign in the nervous system in higher vertebrates (Matyszak *et al.*, 1997; Matyszak, 1998; Carson *et al.*, 1995; Dietrich *et al.*, 2004).

The *immune privilege* of the CNS is accomplished primarily by the blood-brain barrier (BBB), which restricts the entry of lymphocytes, antibodies and complement into the CNS, and secondarily by other mechanisms intrinsic to the brain and spinal cord parenchyma that prevent immune effector cells from gaining access or foothold under normal conditions or restrict and regulate them under pathological conditions (Janzer *et al.*, 1993; Gloor *et al.*, 2001; Bezzi and Volterra, 2001; Bernardes-Silva *et al.*, 2001; Pan and Kastin, 1999; Fitch and Silver, 1997; Törnqvist *et al.*, 1996; Dietrich *et al.*, 2004). Although some lymphatic drainage from the CNS does take place, this occurs at a lesser scale than in other organs (Cserr and Knopf, 1992). Another indicator of the immunoprivileged status of the CNS is the low constitutive expression of MHC molecules on neurons and glial cells (Horwitz *et al.*, 1999; Braud *et al.*, 1999; Collawn and Benveniste, 1999; Corriveau *et al.*, 1998; Linda *et al.*, 1998, 1999; Perry *et al.*, 1998; Phillips *et al.*, 1999; Neumann *et al.*, 1995; Neumann *et al.*, 1998; Neumann *et al.*, 1996; Neumann *et al.*, 1997; Streit *et al.*, 1989; Vass *et al.*, 1990; Xu *et al.*, 1994).

Glial cells are important in neuroimmune interactions (Streit *et al.*, 1999; Haydon, 2001; Hatton *et al.*, 2002; Fields and Stevens-Graham *et al.*, 2002). In fact, considering that glial cells approximate one trillion in number (10 times more than neurons) and occupy half of the CNS mass, it is only safe to assume that they are indispensable in all kinds of neuroimmune cross-talk, including the establishment of the immune privilege, and modern research supports this tenet (Williams and Herrup, 1988; Kettenmann and Ransom, 1995). Thus, apart from the traditional role of astrocytes in establishing the BBB with their perivascular end feet that envelop CNS vessels, it is now known that many antiinflammatory or immunomodulatory factors, such as TGFβ, vasoactive intestinal peptide (VIP), some gangliosides and Fas-ligand (FasL), which inactivate (*anergy*) or induce death of immune cells (*programmed cell death* or *apoptosis*), are produced by neuroglial cells (Unsicker *et al.*, 1991; Becher *et al.*, 1998; 2000; Bechmann *et al.*, 1999; Bechmann *et al.*, 1999; Flügel *et al.*, 2000; Medana *et al.*, 2001b; Benveniste *et al.*, 1998; Irani *et al.*, 1996, 1998; Pender *et al.*, 2001; Vitkovic *et al.*, 2001; Kim *et al.*, 2000; Wolfe *et al.*, 2002). Also, in the EAE model, although T cells enter and expand in the CNS, they are nonetheless under a tighter regulation inside the CNS than elsewhere. This is believed to be achieved at multiple sites and by many mechanisms, some of which involve neuroglia. First, at the BBB level, the interactions of the CNS myelin sensitized $CD4^+$ T cells that traffic to the CNS with the endothelial cells may attenuate their overall responsiveness and degree of activation, possibly inducing a state of *anergy* (Bourdoulous *et al.*, 1995; Prat *et al.*, 2000). Second, once inside the CNS parenchyma

many of the activated $CD4^+$ T cells are either inactivated or undergo apoptosis *en masse* (Bauer *et al.*, 1996; Pender *et al.*, 2001). Indeed, in the CNS of rodents with EAE this effect was found to be due to microglia that inhibited T cell proliferation through the release of nitric oxide or induced T cell apoptosis through Fas-FasL interactions (Juedes and Ruddle, 2001; Bonetti *et al.*, 1997; Carson *et al.*, 1999).

Neuroglial cells dampen immune responses in constant regulatory cross-talk with neurons whether in physiological or pathological conditions (Aloisi, 2001; Aloisi *et al.*, 1999). Neurons express MHC class I molecules and are therefore able to establish 'immunological synapses' with cytotoxic $CD8^+$ T cells. They also produce cytokines including IFNγ, with important neuroimmune effects (Olsson *et al.*, 1989, 1994; Neumann *et al.*, 1997). Finally, neurons can even induce T cell apoptosis both directly and indirectly (Flügel *et al.*, 2000; Medana *et al.*, 2001b). Intact CNS neurons render microglial cells quiescent through contact-mediated or paracrine signals. One illustrative example of a contact-mediated neuron-to-glia immunosuppressive mechanism in the CNS is that of neuronal CD200 (previously named OX2), a surface glycoprotein ligand, that binds to its receptor on microglial cells and deactivates them (Hoek *et al.*, 2000). Thus, in CD200-deficient mice, microglial cells lose their ramified morphology, upregulate the expression of activation markers CD11b and CD45 on their surface and exhibit a more rapid and drastic inflammatory response after facial nerve transection or EAE induction (Hoek *et al.*, 2000). Another neuron-to-glia immunoregulatory mechanism by which neurons maintain microglial dormancy involves an electrical activity-dependent step and a probable paracrine (or even direct) action on microglia. Blocking electrical activity of neurons *in vitro* with tetrodotoxin or NMDA antagonists not only altered neuronal expression of MHC class I molecules, turning them more vulnerable to CTLs, but also resulted in MHC class II upregulation by co-cultured glial cells, the latter paracrine effect partly mediated by IFNγ released by neurons (Neuman *et al.*, 1996, 1997, 2001). Indeed, neuronal electrical activity deregulation could trigger a final common pathway for many heteronymous neuronal stimuli that are capable of immunoregulation. Neurotransmitters (norepinephrine, glutamate, VIP) and neurotrophins (NGF, BDNF, NT3) that act principally on neuronal receptors, have all been shown to downregulate the microglial expression of MHC class II and co-stimulatory molecules critical for antigen presentation, such as CD40 and CD86 (Neumann *et al.*, 2001; Wei *et al.*, 1999). Therefore, functional integrity of neuronal elements and networks exercises constitutive immunoregulation on microglia and other immune cells that is partly responsible for the immune privilege of the CNS. When neurons are injured *in vitro*, even by noninfectious insults, this delicate balance is perturbed and microglia become rapidly activated, probably because of a diminished inhibitory input from neurons. Of course, *in vivo* proinflammatory stimuli also participate in microglia activation (Aloisi *et al.*, 2001, Neumann *et al.*, 2001).

Lastly, glial-glial interactions are also involved in maintaining the immune privilege of the CNS and regulating CNS inflammation after axonal injury (Aldskogius and Kozlova, 1998). Astrocytes, which may account for up to half of all glial cells, are versatile neuroglial cells which are more functionally and even electrochemically knit to neurons

than any other glial cell (Kettenmann and Ransom, 1995; Bezzi and Volterra, 2001). Unlike microglia, astrocytes derive from the ectoderm (Ling and Wong, 1993; Barron, 1995; Kettenmann and Ransom, 1995). Although reactive astrocytes express MHC class II *in vitro*, they are not considered as APC *in vivo*, since they do not express the necessary co-stimulatory molecules to form an immunological synapse with $CD4^+$ T cells (Aloisi *et al.*, 2000). They are nonetheless able to fulfill an immunoregulatory function and have been shown to induce T cell apoptosis and to inhibit microglial activation by releasing TGFβ or interleukin-10, both cytokines with anti-inflammatory and neuroprotective effects in the CNS (Dong and Benveniste, 2001; Benveniste *et al.*, 1998; Flanders *et al.*, 1998; Constam *et al.*, 1992; Khoury *et al.*, 1992; Matsumoto *et al.*, 1993; Gold *et al.*, 1996; Aloisi *et al.*, 2001; Vincent *et al.*, 1997; Makwana *et al.*, 2007). Finally, astroglia are able to produce several growth factors known for their neurotrophic actions or their role in remyelination, such as NGF, GDNF, IGF1 and CNTF (Schwartz and Nishiyama, 1994; Strauss *et al.*, 1994; Schaar *et al.*, 1993; Ho *et al.*, 1995; Garcia-Estrada *et al.*, 1992; Rudge *et al.*, 1994; Albrecht *et al.*, 2003). Therefore, CNS neurons and neuroglia, in health or disease, participate in a constant, sophisticated regulation of all cellular elements of the immune system. This is the essence of the CNS immune privilege, which should not to be interpreted as an altogether absence of immune responses inside the CNS, as will be seen later on.

For one thing, a certain degree of immunosurveillance of vital importance for the vertebrate CNS is suggested by various observations (Wekerle et al., 1986; Shinkart and Benveniste, 1996). Indirect evidence to that is that latent viruses, such as varicella zoster virus, cytomegalovirus and JC virus, that are harmless symbiotes in physiological carriers, produce fatal clinical syndromes when affecting the CNS of immunocompromised patients. Moreover, MHC and co-stimulatory molecules, that are indispensable for immune responses, are strongly upregulated not only in CNS infections but in most adverse CNS conditions, e.g. ischæmia, traumatic injury, neoplasia or primary demyelinating and neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease or Parkinson's disease (An *et al.*, 1996; Dorries *et al.*, 2001; McGeer *et al.*, 1993; O'Keefe *et al.*, 2002; Owens *et al.*, 2002; Piehl and Lidman, 2001; Xu *et al.*, 1994). More directly, various types of hæmatogenous or resident immune effector cells have been shown to gain access to and constantly survey the CNS (Wekerle *et al.*, 1986).

CNS immunosurveillance is mediated primarily by microglia and resident or wandering APCs, i.e. perivascular macrophages, pericytes and dendritic cells respectively, and secondarily by infiltrating T and B lymphocytes (Thomas *et al.*, 1999; Wekerle *et al.*, 1986). Microglia, perivascular macrophages, pericytes, dendritic cells and lymphocytes are all of mesodermal origin. Pericytes and dendritic cells are closely related cells of myeloid monocyte lineage while B and T lymphocytes are of lymphoid lineage (Baron and Gallego, 1972; Ling and Wong, 1993; Barron, 1995; Thomas *et al.*, 1999). Microglia probably comprises different subpopulations with special characteristics. Some investigators even consider pericytes (ED2-positive) as a subclass of microglia (ED2-negative) based on immunophenotypic and histological criteria while others view pericytes as monocyte-derived macrophages with a sentinel role at the BBB interface

(Baron and Gallego, 1972; Thomas *et al.*, 1999). What is certain is that parenchymal microglia, which enter the CNS early in development, have very slow or no turnover via bone-marrow derived cells, while perivascular macrophages are continuously replenished by blood-borne monocytes as has been conclusively demonstrated by experiments employing bone-marrow chimeric animals (Bechmann *et al.*, 2001; Carson *et al.*, 1998 1999; Ling *et al.*, 1993; Hickey *et al.*, 1988; Graeber *et al.*, 1989; Lassman *et al.*, 1993; Matsumoto and Fujiwara, 1987; Lassmann *et al.*, 1993; Ford *et al.*, 1995; Barron, 1995; Thomas *et al.*, 1999).

Perivascular macrophages and dendritic cells are phagocytes that have been identified in the Virchow-Robin (perivascular) spaces, the leptomeninges and the choroid plexus (Fischer *et al.*, 2001; McMenamin *et al.*, 1999; Thomas *et al.*, 1999). They constitutively express MHC class II molecules and a rich diversity of cytokines such as IL1, TNFα, IL6, IL8, IL12, IL23, TGFβ (Kingsley *et al.*, 1994; O'Garra *et al.*, 1998; Flanders *et al.*, 1998; McLellan and Kämpgen, 2000). Their phagocytic potential, MHC profile and critical location are only three of the reasons why they are viewed as *bona fide* APCs of the CNS (Hickey *et al.*, 1999; Thomas *et al.*, 1999; Williams *et al.*, 2001). However, contrary to perivascular macrophages, dendritic cells are 'smart' migrating phagocytes that circulate to and fro lymphoid organs and whose main role is antigen exportation from the CNS transportation and presentation to immune system cells in central (thymus, bone marrow) or peripheral lymphoid organs (spleen, lymph nodes, mucosal lymphoid tissue) but also *in situ* (Fisher and Reichmann, 2001). In comparison, the tissue macrophage is more of a 'dirty-work' phagocyte primarily burdened with a scavenging task but also reserves an APC capacity towards T cells, mainly at a local level. Some immunologists even believe that not all dendritic cells are unconditional APCs but that there exist dendritic cells with immunosuppressive actions, which they have termed as *downregulatory dendritic cells* (McLellan and Kämpgen, 2000; Jin *et al.*, 2004). Dendritic cells are scattered in many non-lymphoid organs and tissues, exercising their all-important scouting action, as do for instance Langerhans cells in the skin. It is unclarified though, if dendritic cells regularly cross the BBB and migrate inside the CNS parenchyma. Among the aforementioned types of bone-marrow derived CNS phagocytes, *in vitro* and *in vivo* studies have unequivocally established perivascular macrophages, pericytes and dendritic cells as competent APCs (Ling *et al.*, 1993; Hickey *et al.*, 1988; Graeber *et al.*, 1989; Lassman *et al.*, 1993; Ford *et al.*, 1995)

Microglial cells, comprise about 10% of all neuroglia, and are sensitive homeostatic sensors of the CNS (Barron *et al.*, 1995, 2003). They also have potent reparative but also cytotoxic functions (Barron *et al.*, 1995, 2003; Kreutzberg et al., 1996). Microglia are sensitive to various exogenous (CNS or PNS injury, infections) and endogenous stimuli (neurotransmitters, neuropeptides, cytokines) and their activation programme is prompt but graded and encompasses proliferation, morphological transformations (hypertrophy and dedifferentiation from a resting ramified into a macrophage-like migratory amœboid morphotype) and many functional changes and complex interactions with neighboring neurons, glia and immune cells (upregulation of MHC molecules, phagocytosis, cytokine-chemokine-cytopathic mediator secretion) (Kreutzberg *et al.*, 1996; Delgado *et al.*, 1998;

Kim *et al.*, 2000; Aloisi *et al.*, 1998; Phillips *et al.*, 1999; Aloisi *et al.*, 2000; Hide *et al.*, 2000). Recent evidence even suggests that microglia primed by certain antiinflammatory cytokines may promote neurogenesis (Vitkovic *et al.*, 2001; Ziv *et al.*, 2006 a, 2006b). Microglia participates in both innate and adaptive immune reactions and plays an important role in immunosurveillance of the nervous system. Microglial cells, like macrophages, express complement receptors (such as CR3 that can bind bacterial wall sugars with its lectin-binding site), TLRs that can bind PAMPs and CD14 that is a receptor for endotoxin (Graeber et al., 1988a; Gehrmann *et al.*, 1995; Kreutzberg *et al.*, 1995; Barron *et al.*, 1995, 2003; Kitchens *et al.*, 2000; Becher *et al.*, 1996; Rivest *et al.*, 2003). Indeed, when microglia reverts to an amœboid morphotype after a CNS insult it is indistinguishable from invading blood-borne macrophages. Thus, microglia, like macrophages, are able to produce various cytokines/chemokines (IL1β, TNFα, IL12, IL18, etc.) and cytokine/chemokine receptors (TNF receptors I and II, IL4R, CD40, IFNγR, M-CSF, GM-CSF, etc.) but also nerve growth factors under certain conditions (Liu *et al.*, 1998; Prinz *et al.*, 1999; Gregersen *et al.*, 2000; Yamada and Yamanaka, 1995; Stalder *et al.*, 1997; O'Keefe *et al.*, 1999; Nguyen and Benveniste, 2000; Dopp *et al.*, 1997; Grewal *et al.*, 1996; Batchelor *et al.*, 1999, 2000, 2002a, 2002b, 2008a). In response to various injurious stimuli microgliacytes have been shown *in vitro* (exposure to proinflammatory cytokine or LPS) and *in vivo* (CNS or PNS injury, or infections) to be capable of expressing both MHC class I and II molecules (Streit *et al.*, 1989; Vass and Lassmann, 1990; Xu and Ling, 1994; Gerritse *et al.*, 1996). In EAE and MS lesions microglia has also been found to express CD40, a receptor of the T cell ligand CD40L (De Simone *et al.*, 1995; Li *et al.*, 1996). Microglia therefore seems to be able to provide both stimulation and co-stimulation to T cells which are both necessary preconditions for an APC function. Although resting microglia may not be competent APCs, activated microglial cells are capable of priming naïve T cells, of inducing T cells to produce various cytokines, and of reactivating quiescent memory T cells (Aloisi *et al.*, 2000, 2001). Whether microglia is able *per se* to induce T cell clonal proliferation has been a matter of controversy for some time, but it is now widely accepted that microglia can function as a fully competent APC *in vivo* under certain pathological conditions (Aloisi *et al.*, 2000; Ford *et al.*, 1996; Carson *et al.*, 1998).

Finally, the importance of astroglia in immunosurveillance seems to be minor compared to that of microglia. CNS lesions of mechanical, ischæmic, neoplastic, autoimmune or infectious ætiology result in astrocyte activation (reactive astrocytosis). Reactive astrocytosis entails hyperplasia, overexpression of intermediate filaments (GFAP, vimentin, nestin), cellular hypertrophy, and migration towards the site of injury (Tetzlaff *et al.*, 1988; Eddleston and Mucke, 1993; Frisén *et al.*, 1995; Ridet *et al.*, 1997). This results in spatial delimitation of the pathological process by a dense glial scar which differs from the mesenchymal collagenous scar that appears in penetrating CNS injuries (Profyris *et al.*, 2004). Astrocytes exhibit phenotypic and functional heterogeneity (Wilkin *et al.*, 1990; Benarroch, 2005). They respond to alterations of neuronal function and firing patterns faster than microglia, but they respond slower to inflammatory stimuli compared to microglia. As mentioned earlier, they express mostly cytokines with immunoregulatory or antiinflammatory actions in order to control CNS damage, but

under appropriate conditions astrocytes can also produce TNFα, IFNγ and IL12 (Constam *et al.*, 1992; Khoury *et al.*, 1992; Matsumoto et al., 1993; Gold et al., 1996; Chung *et al.*, 1991; De Simone *et al.*, 1998; Stalder *et al.*, 1997; Vitkovic *et al.*, 2001). Concerning a physiological T cell activating APC capacity for astrocytes the jury is still out. *In vitro*, upon exposure to IFNγ or endotoxin, they are capable of expressing MHC class II and various co-stimulatory molecules (e.g. CD80, CD86, CD40, ICAM-1 or CD54, VCAM-1) and of processing the endocytosed MHC class II-MBP complex for presentation to T cells. Myelin-specific T cells activated by astrocytes in this manner are encephalitogenic, (i.e. able to induce EAE) in SJL/J mice. Even *in vivo*, in experimental inflammatory conditions such as EAE, astrocytes have been shown to present an ostensibly APC phenotype in terms of MHC expression and co-stimulatiory molecules, at least in some studies. However, there is conflicting evidence, both *in vitro* and *in vivo*, that negates a physiological APC role for astrocytes (Aloisi *et al.*, 1999; Cross and Ku, 2000; Matsumoto *et al.*, 1992; Sun *et al.*, 1997). *In vivo*, astrocytes have been shown to only poorly express MHC class II and the requisite costimulatory molecules while *in vitro*, they could activate naive T cells only after IFNγ pretreatment. Moreover, they have even been shown to suppress endotoxin-induced activation of other APCs (Hailer *et al.*, 1998; Aloisi *et al.*, 2000; Dong and Benveniste, 2001).

Bona fide immune cells such as T and B lymphocytes, and even NK cells, also exist in the intact CNS but in very small numbers in normal conditions (Kil *et al.*, 1999; Owens *et al.*, 1998; Bauer *et al.*, 1995; Hickey *et al.*, 1991, 1988; Bradl *et al.*, 2005). For example, in the intact spinal cord of healthy Lewis rats T cells could be found in the cord parenchyma, preferentially located within the grey matter and with a $CD8^+$ (CTLs) to $CD4^+$ (T_H) preponderance (Bradl *et al.*, 2005). In pathological conditions the participation of immune system cells depends on the pathogen. Thus, in infectious or neoplastic disease of the CNS, B and T cells are numerous and important for host defence, while in autoimmune disorders they exacerbate the clinical outcome. Specifically, in MS or EAE, myelin-sensitized autoreactive T cells (and secondarily plasma cells) are the culprits of the demyelinating process. In EAE and MS, $CD4^+$ and $CD8^+$ T cells, together with macrophages, are responsible for oligodendrocytotoxicity through secretion of T_H1 proinflammatory cytokines, cytotoxic mediators or direct injury to the oligodendrocytes (Gœbels *et al.*, 2000; Meinl *et al.*, 2002; Steinman *et al.*, 2000, 2001a, 2001b, 2001c, 2001d, 2004; Zamvil *et al.*, 1985a, 1985b, 1990; Traugott and Reine, 1983; Bradl *et al.*, 2005). In EAE and MS, myelin-sensitized, autoagressive T cells can penentrate the BBB and some evidence suggests that even CNS myelin autoantigens can be transported by cervical lymphatics to cervical lymph nodes (Wekerle *et al.*, 1987; Hickey *et al.*, 1991; Cserr and Knopf, 1992; Cserr *et al.*, 1992). T cell penetration of the BBB in EAE and MS does not necessarily require a barrier breach but occurs by *diapedesis* inbetween the capillary endothelial cells or the apposing astrocytic podocytes. Such interactions between T cells and endothelium involve *cell adhesion molecules*, *integrins* and other surface molecules. Of course, immune cell and monocyte invasion in the CNS is easier and massive in sites of active CNS inflammation where capillary endothelium becomes leaky or damaged by inflammatory mediators. Primed T cells are not only more capable of penetrating the BBB than naïve T cells but they are also more prone to accumulate at

sites of active demyelination and myelinated axon injury, as also do other inflammatory cells (Konno *et al.*, 1990; Maehlen *et al.*, 1989a, 1989b; Piehl and Lidman, 2001). For all these reasons, the CNS is more accessible to the immune system in the setting of EAE. In fact, it has been clearly shown that in animals with EAE even pure peripheral nerve injury by axotomy of the facial nerve leads to a more robust inflammatory and immune response at the site of nucleus facialis, compared to cranial nerve injury in the absence of EAE (Maehlen *et al.*, 1989a, 1989b; Barron *et al.*, 2004).

T cells are found in the CNS even in non-immune hereditary neurodegenerative or myelinodegenerative conditions (Bradl *et al.*, 2005). For example, in Lewis rats with degeneration of white matter axons or hemizygous *proteolipid protein* (PLP)-transgenic Lewis rats with subclincal myelin degeneration and oligodendrocyte cell death, T cells were enriched in the CNS lesions synchronously to degeneration, with a preponderance of $CD4^+$ over $CD8^+$ (Bradl *et al.*, 2005). Moreover, T cells penetrate the CNS and myelin-autoreactive T cells have been found in the CNS even after experimental ischæmic or purely traumatic injury, although to a much smaller extent than in EAE (Hirschberg *et al.*, 1998; Popovich *et al.*, 1996, 1997). Self-reactive T cells have also been detected in patients after stroke or peripheral nerve biopsy (Wang *et al.*, 1992; Olsson *et al.*, 1993). In fact, self-reactive T cells may circulate in the bloodstream of healthy individuals but kept inactivated by peripheral mechanisms of immune tolerance, something that is conceivable given the expression of nervous system antigens in the thymus (Visan *et al.*, 2004; Rose and Mackay, 1998). Immune cells such as macrophages, B cells and T cells have also been shown to be capable of expressing several nerve growth factors such as NGF, BDNF, NT3, NT4 or GDNF and neurotrophic factor receptors (Ehrhard et al 1993a, 1993b; Melamed *et al.*, 1995; Torcia 1996; Batchelor *et al.*, 1999, 2000, 2002a, 2002b, 2008a, 2008b; Besser and Wank, 1999; Kerschensteiner *et al.*, 1999; Moalem *et al.*, 2000). These factors seem to primarily serve a cytokine role related to the function of the immune cells that express them. Thus, NGF was demonstrated to be an antiapoptotic factor for B cells, trkB and BDNF may be involved in T cell development in the thymus, and BDNF was shown to be expressed in MS lesions probably by T cells (Torcia *et al.*, 1996; Maroder *et al.*, 1996; Kerschensteiner *et al.*, 1999; Stadelmann *et al.*, 2002). It was therefore hypothesized by some investigators that the myelin-specific $CD4^+$ T_H cells recruited to sites of axonal or neuronal injury, may actually promote neuronal survival by secreting neurotrophic factors upon specific myelin-antigen recognition and reactivation (Hammarberg *et al.*, 2000; Hohlfeld *et al.*, 2000; Kerschensteiner *et al.*, 1999; Moalem *et al.*, 2000). However, the occurrence of autoreactive T cells in non-autoimmune conditions, like stroke, *traumatic brain injury* (TBI) and SCI, is believed to be more of an epiphenomenon than an orchestrated process of neuroprotection or repair (Popovich *et al.*, 1996; Crutcher *et al.*, 2006).

The proinflammatory actions of macrophages after CNS injury have been known for long (Blight, 1992). In 1990 David *et al.* showed in an *in vitro* system that the CNS white matter nonpermissiveness to axonal regeneration could be mitigated by macrophages (David *et al.*, 1990). Based on this singular finding a group of investigators hypothesized that a possible paucity of postinjury CNS infiltration by macrophages, as compared to

PNS injury, is partly responsible for the failure of axonal regeneration in the CNS and rather counterintuitively suggested for macrophages in particular and inflammation in general, a natural role in neuroprotection and regeneration after CNS injury (Hirschberg and Schwartz, 1995; Schwartz *et al.*, 1995). They then set about to show that implantation of 'activated macrophages', i.e. macrophages preincubated with peripheral nerve tissue, enhanced axonal regeneration in an optic nerve injury rat model, an effect not obtained with naïve macrophages or macrophages preincubated with central myelin. The same macrophage preincubation/implantation protocol was subsequently applied in a complete transection SCI rat model again with results suggesting enhanced CNS regeneration. Myelin-specific T cells implantation or vaccination protocols that served to boost autoimmune responses against CNS antigens were later employed in various CNS injury models always with positive results (Rapalino *et al.*, 1998; Moalem *et al.*, 1999). These researchers generally and loosely proposed that the mechanism behind neurological improvement in all these different experiments was neuroprotection and introduced the notion of 'protective autoimmunity' (Rapalino *et al.*, 1998; Moalem *et al.*, 1999). They then showed that dendritic cells and skin-incubated macrophages had similar neuroprotective effects and went on to tackle disparate disorders such as traumatic brain injury, depression, dementia and glaucoma were amenable to similar vaccination or immunotherapeutic protocols (Hauben *et al.*, 2000a, 2000b, 2003). However, no coherent mechanistic immunobiological explanation for these results has been offered. Moreover, replication of the exact same implantation or vaccination experiments by several other labs gave opposite results, thus contradicting the hypothesis of 'protective autoimmunity', and even the premise of lower macrophage infiltration of the CNS after SCI as compared to macrophage infiltration of PNS has been disputed (Griffin *et al.*, 1993; Popovich *et al.*, 1996, 2006; Rapalino *et al.*, 1998; Batchelor *et al.*, 1999, 2000, 2002a, 2002b, 2008a; Moalem *et al.*, 1999; Leskovar *et al.*, 2000; Jones *et al.*, 2002, 2004; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010; Busch *et al.*, 2009, 2010).

INTERACTIONS BETWEEN IMMUNE SYSTEM AND PNS

To conclude this brief introduction of such a complex subject as neuroimmune interactions, we should make specific mention of the PNS. In broad strokes many of the mechanisms that were presented for the CNS are also at play in the PNS. Thus, the PNS also enjoys a certain degree of immune privilege. Anatomically in the PNS, among the three connective tissue layers inside peripheral nerves, once epineurium is breached, perineurium is the ultimate barrier separating neural and extraneural environment. A finer functional border and the equivalent of the BBB for the PNS is the blood-nerve barrier (BNB) which restricts access of immune cells and soluble mediators. As in specific areas of the CNS (such as the pineal gland, the choroid plexus, the leptomeninges and the circumventricular organs –organum vasculosum of lamina terminalis, the subfornical organ, the median eminence and area postrema) the BBB is more permissive for physiological and homeostatic purposes, so is the BNB anatomically and functionally incomplete at several locations (nerve roots, dorsal root ganglia, nerve terminals). And as the CNS is being constantly patrolled by immune cells so is the PNS, and probably more

so. Lymphocytes, both B and T, can infiltrate the BNB, a capacity related more to their level of activation than to their antigen specificity. In physiological conditions the peripheral nerve tissue is also populated with macrophages, while blood-borne monocytes can easily gain access inside peripheral nerves as has been shown in the EAN model or in Guillain-Barré syndrome. The mechanisms of influx of monocytes, macrophages and lymphocytes during neuritis have been elucidated thanks to the EAN model and seem to be analogous to those operating in EAE and MS. Local macrophages and activated T cells secrete all the necessary chemokines (CXCL10, CCL2, MCP1, MIP1α, MIP1β, RANTES), cytokines (such as IL1, TNFα, IFNγ, IL6, IL12, IL18, IL23) and complement proteins to attract and activate further lymphocytes, leucocytes and APCs and lead to myelin damage (Stübgen, 2008). Infiltrating inflammatory cells secrete in their turn matrix metalloproteinases such as *matrilysin* (MMP7) and *gelatinase* (MMP9) that disrupt the BNB and the connective tissue scaffold of the peripheral nerves and thus prepare the invasion of more cells and macromolecules. Macrophages and occasional dendritic cells play the role of APCs in the PNS and quite possibly Schwann cells as well. As in the CNS or EAE, there exist similar buffering mechanisms that control or downregulate immunological reactions in the PNS and EAN, such as the induction of T cell apoptosis, again with the participation of Schwann cells. However, the neuroimmunological potential of Schwann cells deserves separate mention since it is the only glial element of the PNS but an extraordinarily versatile one.

The Schwann cells are of neuroectodermal origin and their principal physiological role is to protect and insulate the peripheral axons and ensure the saltatory conduction of action potentials along the length of myelinated axons. Their potential as promoters of regeneration after peripheral nerve injury has been hypothesized more than a century ago and during the years it was found that to this end they proliferate to form contiguous *bands of Büngner* within the original basal membrane channels and secrete a host of neurotrophic factors, ECM and axonal guidance molecules. Schwann cells are also believed to act as phagocytes of myelin debris secondary to macrophages that bear the brunt of this task. The immunological exploits of Schwann cells have been elucidated only during the last 30 years (Wekerle, 1986). Schwann cells are thus capable of producing various cytopathic mediators (NO), cytokines (IL1, TNFα, IL6, prostaglandin E_2, thromboxane A_2 etc.), complement proteins (C3, etc.) as well as cytokines (IL1R, IFNγR, TNFR, Fas, FasL, etc.) and complement receptors (CR1, CD49, CD55, CD59, etc.). Upon appropriate cytokine stimulation *in vitro* they are also able to express MHC class I and II and the costimulatory molecules necessary for antigen presentation and there is evidence to suggest that the same happens *in vivo*. Whether these characteristics translate into an APC function *in vivo* is debated, however, Schwann cells could succesfully process and present both P2 and MBP to autoreactive T cells under certain conditions *in vitro*. Further interactions with T cells and macrophages are alluded by their molecular typology. Their expression of Fas (CD95) and other TNF-receptor family members makes them susceptible to apoptotic elimination by FasL- and TNFα-expressing T cells and macrophages, which could explain the demyelination in EAN and the clinical syndrome, *acute inflammatory demyelinating polyradiculoneuropathy* (AIDP) (Griffin and George, 1993; Griffin *et al.*, 1993; Hughes *et al.*, 2006; Stübgen, 2008). On

the other hand, their immunoregulatory potential is supported by their own expression of FasL or NO which could induce T cell apoptosis or deactivation as shown *in vitro*. Finally, it has been shown that the immunological properties of Schwann cells are regulated by their interaction with the axons they are in contact with. It seems therefore that Schwann cells are indeed sensitive sentinels of the PNS environment in ways reminiscent of both microglia and astroglia.

TABLE

	Innate immunity (inflammatory response)	**Acquired immunity (specific immune response)**
Cellular	Polymorphonuclear granulocytes: • Neutrophils • Eosinophils • Basophils Monocytes/macrophages (Mϕ) Resident macrophages • Microgliacytes • Histiocytes Natural killer (NK) cells	T-lymphocytes (CTL, T_H, T_{REG}) B-lymphocytes (memory cells, plasma cells) Antigen-presenting cells-MHC II bearing (APC): • Macrophages (Mϕ) • Dendritic cells • Perivascular Mϕ • Pericytes • Microgliacytes • B cells
Humoral	Acute phase proteins Antimicrobial peptides Complement pathways Cytokine networks Chemokine networks Interferons	Immunoglobulins Cytokine networks Chemokine networks

NEUROIMMUNOLOGICAL ASPECTS OF SCI

In higher vertebrates the nervous system enjoys an '*immune privilege*' because of the paucity of immunological surveillance during physiological but also experimental conditions, eg. intracerebral transplantation studies (Suter *et al.*, 2003; Aloisi *et al.,* 2001). Exactly why this is so still eludes scientists, but they have rightly tried to explain this phenomenon from the evolutionary point of view. Almost all neurons are postmitotic and terminally differentiated, their number begins to decrease early in life and the cell regeneration potential is limited within the CNS. Since neurons combine into neuronal circuits the demise of neurons that are central to a network or important relays within a circuit would have an influence on the nervous sytem more profound than just the mere arithmetical subtraction of those neurons. In tissues that have retained the ability to regenerate, e.g. the skin, inflammation is profitable because it always leads to wound healing and tissue restitution, though severe inflammation always leads to scar tissue. On the contrary, in the CNS, the 'wound healing' process per se (which is not as effective in the soft and delicate architecture of the CNS parenchyma as in more resilient tissues such as skin) can bring more debt than profit. This is the reason why the potent inflammatory or immunological reactions within the CNS, e.g. in neuroinfections such as viral or bacterial meningoencephalitides, and in acute disseminating encephalomyelitis or postinfectious encephalitis, lead to excessive collateral damage that sometimes exceeds and prolongs the harm done by the original pathogen. Evolutionary pressures might therefore explain why the immune system has not been allowed free access in the nervous system and why these two systems are so elaborately separated in higher vertebrates.

However, the immunoprivileged status of the CNS might be an indirect cause of harm. It is believed that autoimmune B and T cell clones against various self-antigens undergo apoptosis or become anergic during development leading to immunological tolerance (Pette *et al.*, 1990; Martin *et al.*, 1992; Kojima *et al.,*, 1997; Goverman, 1997; Harrington *et al.*, 1998). The relatively isolated CNS environment would therefore result in neural antigen sequestration from the tolerogenic machinery and autoimmune clone dysregulation (Pette *et al.,* 1990; Martin *et al.,* 1992). The generation of autoaggressive B and T cell clones targeting antigens specific for the nervous system, especially myelin-related antigens (e.g. *myelin basic protein* or MBP, many different gangliosides, etc.), has been proposed as one pathogenic process in autoimmune/dysimmunitary conditions of the nervous system, such as *acute inflammatory demyelinating polyradiculoneuropathy* (AIDP) (or eponymically Guillain-Barré syndrome) and *chronic inflammatory demyelinating polyradiculoneuropathy* (CIDP) for the PNS and multiple sclerosis (MS) and *acute disseminated encephalomyelitis* (ADEM) for the CNS (Martin *et al.,* 1992; Hughes *et al.*, 2006; McFarland and Martin, 2007). The event triggering the generation of autoreactive lymphocytes is according to one hypothesis a viral or bacterial infection activating *polyspecific* lymphocyte clones, i.e. lymphocytes specific for microbial antigens that also cross-react with CNS autoantigens (Martin *et al.,* 1992; Allen and Brankin, 1993; Miller *et al.*, 1997; Wucherpfennig and Strominger, 1995).

Consequently, the role of *molecular mimicry* between microbial antigens and nervous system autoantigens has been hypothesized in ADEM and established in AIDP, for example in Campylobacter infection-associated AIDP (Griffin and Ho, 1993). It must be stressed though that in most cases of MS no overt infection precedes the onset of the autoimmune disease. Another proposed mechanism is the dysregulation of T_{REG} which seems to be a predisposing factor for autoimmune disease of the nervous system (Reinherz *et al.*, 1980; Viglietta *et al.*, 2004; Yu *et al.*, 2005; Kumar *et al.*, 2006). The potentially autoreactive B and T lymphocytes are believed to be activated upon exposure to the cognate antigens although it is still controversial how they penetrate the BBB/BNB or if they are activated in peripheral lymphoid organs (Andersen, 1993). BBB/BNB dysfunction induced by pathogens, inflammatory cells of the innate immune system (mainly neutrophils and macrophages) or cytokine overproduction, in the setting of a clinical or occult infection is the prevailing hypothesis, while idiopathic microvascular trauma and other predisposing factors cannot be excluded (Eugenin *et al.,* 2006; Karpus and Ransohoff, 1998; Lowenstein and Castro, 2003; Phares et al., 2006; Poser, 1994; Sœrensen *et al.,* 1999; Zirger *et al.,* 2006; Koning *et al.,* 2007). Autoreactive T lymphocytes activation *in situ* in the PNS or CNS via MHC-antigen complex formation leads to macrophage/microglia activation, local inflammation and tissue destruction while autoreactive B lymphocytes are responsible for systemic or intrathecal synthesis of autoantibodies against neuroantigens (Genain *et al.,* 1999; Hafler *et al.,* 2005; Hellings *et al.,* 2002; Hohlfeld *et al.,* 1995; Stinissen *et al.,* 1997; von Budingen *et al.,* 2001; Tompkins *et al.,* 2002). Cytokines are profusely secreted by myelin-reactive T cells and macrophages/microglia and kick off an inflammatory cascade which is thought to mediate tissue damage including BBB disruption, glial/neuronal toxicity or even cell death (Benveniste, 1997; Pagenstecher *et al.,* 2000; Schrœter and Jander, 2005; Sriram and Rodriguez, 1997; Koning *et al.,* 2007).

In MS, the main target of this renegade immunological and inflammatory response is the myelin sheath of long fiber tracts in the CNS (Perry and Anthony; 1999; Liblau *et al.,* 2001; Pouly *et al.,* 2000; Hemmer *et al.,* 2002a, 2002b; Totoiu *et al.,* 2004; Hafler *et al.*, 2005; Hafler *et al.*, 2005; Frohman *et al.*, 2006; Hauser and Oksenberg 2006; Koning *et al.,* 2007). The glial cells that produce the myelin in the CNS are the oligodendrocytes and it is these cells that succumb to the autoimmune attack by macrophages and T cells (both $CD4^+$ and $CD8^+$) sensitized against self-antigens contained in myelin, e.g. MBP (Hickey *et al.,* 1983; Bauer *et al.,* 1995; Goverman, 1997; Meinl *et al.,* 1997; Berger *et al.,* 1997; Owens *et al.,* 1998; Conlon *et al.,* 1999; Liblau *et al.,* 2001; Bradl *et al.,* 2005; Hauser and Oksenberg, 2006; Saikali *et al.,* 2007; Saxena *et al.,* 2008). Myelin degeneration results in demyelination, the pathophysiological hallmark in MS, conduction failure and eventually neurological deterioration (Hafler *et al.*, 2005; Frohman *et al.*, 2006). TNFα and IFNγ produced by macrophages and microglia have long been considered to be oligodendrotoxic agents in this context, although their role is now believed to be more complex (Selmaj and Raine, 1988; Hartung, 1993; Renno *et al.,* 1995; Vartanian *et al.,* 1995; Krakowski and Owens *et al.,* 1996; Merill and Benveniste, 1996; Klinkert *et al.,* 1997; Koller *et al.,* 1997; Renno *et al.,* 1998; Liu *et al.,* 1998; Allan and Rothwell, 2001; Vitarbo *et al.*, 2004;

Aktas *et al.,* 2006; Aktas *et al.,* 2007b; Koning *et al.,* 2007). Axonal degeneration secondary to demyelination has been demonstrated but direct damage on axons by immune cells or cytopathic mediators is also believed to occur (Simmons and Willenborg, 1990; Merrill and Benveniste, 1996; Koller *et al.,* 1997; Krakowski and Owens, 1997; Grigoriadis *et al*, 2004; Trapp *et al*, 1998; Anthony *et al*, 2000; Steinman, 2000, 2001a, 2001b, 2001c, 2001d; Sun *et al*, 2004; Kawakami *et al*, 2004, 2005; Raivich and Banati, 2004; Aktas *et al*, 2007a, 2007b; Koning *et al.,* 2007). Eventually, especially in *relapsing-remitting* MS, the most common clinical form of the disease, the vicious cycle of the autoimmune attack is temporarily halted by local immunoregulatory mechanisms, e.g. induction of T cell apoptosis or anergy (Hafler *et al.*, 2005; Frohman *et al.*, 2006). Recurrent episodes of such neuroinflammatory reactions eventually lead not only to severe neurological damage (*plaque burden*) but also a gradual potentiation of the autoimmune responses themselves (Hafler *et al.*, 2005; Frohman *et al.*, 2006). The progressive course of MS is explained by: 1) permanent BBB dysfunction especially in the areas of previous inflammation with facilitated access of autoreactive cells in the CNS, 2) sustained presence of autoreactive cells in the CNS because of clonal expansion, 3) sustained activation of T cells because of continued cytokine release and upregulation of MHC molecules and costimulatory molecules on T cells, resident glia or infiltrating inflammatory cells, and finally 4) generation of multiple autoreactive T cell clones to new CNS autoantigens by the mechanism of *epitope spreading* (Hickey, 1983, 2001; Lehmann *et al.*, 1992; Cross *et al.*, 1993; Claudio *et al.*, 1995; McRae *et al.*, 1995; Miller *et al.*, 1995; Irani and Griffin, 1996; Raivich *et al.*, 1998; Kielian and Hickey, 2000; Kloss *et al.*, 2001; McGovern and Truong, 2004). Logically therefore, inducing immunological tolerance towards CNS antigens would have beneficial effects on disease severity and many *tolerization* paradigms have been proposed as potential immunotherapies of MS (Neville *et al*, 2002; Conlon and Steinman, 2002; Ruiz *et al*, 2001; Steinman and Conlon, 1995, 2001; Steinman, 2000, 2001a, 2001b, 2001c, 2001d, 2004; Hohlfeld and Wekerle, 2004; Hemmer *et al*, 2006; Hemmer and Hartung, 2007).

In MS, and its experimental model of EAE, it is the proinflammatory T_H1 subset that is overactivated and supposed to inflict most damage on host tissues (Liblau *et al*,, 1995; Hemmer *et al*, 2002a, 2002b, 2006; Hemmer and Hartung, 2007; Hauser and Oksenberg 2006; Koning *et al.,* 2007). In EAE and MS, the T_H1 immune response has deleterious effects on oligodendrocytes and neurons while T_H2 seems to have anti-inflammatory and neuroprotective effects (Liblau *et al*, 1995; Adorini *et al*, 1997). In this context, T_H1 oligodendrotoxic cytokines such as TNFα and IFNγ, are thought to mediate the myelin destruction (Selmaj and Raine, 1988; Hartung, 1993; Merill and Benveniste, 1996; Hemmer *et al*, 2002a, 2002b, 2006; Hemmer and Hartung, 2007; Hauser and Oksenberg 2006). In fact, efforts to downregulate a T_H1 or enhance a regulatory T_H2 have proven beneficial in EAE models (Adorini *et al*, 1997; Rengarajan *et al*, 2000; Bettelli *et al*, 2003; Adorini, 2004). The facilitation of a buffering T_H2 response is indeed a plausible therapeutic alternative for diseases such as MS (Kappos *et al*, 2000; Garren *et al*, 2001; Steiman, 2000, 2001a, 2001b, 2001c, 2001d, 2004; Adorini, 2004; Hemmer *et al*, 2002a, 2002b, 2006; Hemmer and Hartung, 2007).

A common theme between MS or EAE and the mechanically injured spinal cord is that myelin is damaged. Chronic progressive demyelination is a central pathophysiological process in SCI and demyelinated axons can be observed even a decade after human SCI (Waxman, 1989, 1992; Hartung, 1993; Totoiu *et al.*, 2004; Totoiu and Keirstead, 2005; Buss *et al*, 2005). In SCI axonal damage is contributing to the demyelination but this is not the only mechanism, since even surviving axons may undergo significant demyelination by oligodendrocyte apoptosis as many as four segments abover or below the injury site (Casha *et al*, 2001, Crowe *et al*, 1997; Waxman, 1989, 1992; Liu *et al*, 1997; Crowe *et al*, 1997; Beattie *et al*, 2000). Indeed, the surge of local TNFα and IL1β concentrations after SCI is believed to contribute to myelin degeneration, oligodendroglial apoptosis and possibly even conduction failure (Koller *et al*, 1997; Bethea, 2000; Allan and Rothwell, 2001, 2003; Schiffenbauer *et al*, 2000; Gonzalez *et al*, 2003). In SCI of course the problem is not just the demise of oligodendroglia, since axonal die-back and abortive regeneration also determine the final outcome (Schwab and Bartholdi, 1996). Interestingly, the neurite growth MAI Nogo-A that is believed to hamper axonal regeneration in SCI has been also implicated in autoimmune-mediated demyelination (Karnezis *et al.*, 2004). Nevertheless, it is known that damaged or even severed myelinated axons require their myelin sheath for survival, residual conduction or regeneration, all the while oligodendroglia is disappearing during secondary degeneration (Casha et al., 2001; Schwab and Bartholdi, 1996; Waxman, 1989, 1992; Liu *et al.*, 1997; Crowe *et al*, 1997; Beattie *et al*, 2000; Franklin and Kotter, 2008). In the case of SCI, therefore, demyelination leads in turn to conduction failure, impaired axonal survival and regeneration failure (Cao *et al*, 2005). In conclusion, although SCI and MS have strikingly different ætiologies, they seem to share some common pathological features and demyelination is but one of them. Surprisingly, immune system dysregulation with suppression of immunological tolerance mechanisms occurs to a certain degree in SCI too. In fact, studies in experimental as well as human SCI show that purely traumatic injury of the spinal cord may result in chronic autoimmune responses, both humoral and cellular, that do not seem to target the CNS solely.

In experimental SCI in mice elevation of CNS autoantibodies in the circulation (i.e. specific for MBP, GM1, galactocerebroside, glutamate receptors) was found in more than 90% of animals (Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). Similar results have been obtained from clinical studies of chronic SCI with more than 50% of patients having elevated serum and liquor autoantibodies against MBP, galactocerebroside, and gangliosides (Taranova *et al*, 1992; Hayes *et al*, 2002; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). Indeed, the humoral autoimmune response elicited by SCI seems to be very broad since proteomic analysis showed that more than 50 different autoantigens from spinal cord homogenates were targeted by SCI autoantibodies, that not all targeted autoantigens were specific for the nervous system (e.g. actin, nucleic acids) and that some autoantibodies were polyspecific (e.g. against both NMDA receptors and DNA) (Dowler *et al*, 1997; DeGiorgio *et al*, 2001; Kowal *et al*, 2004; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010; Teeling and Perry 2009). Cellular autoimmune responses

are elicited by SCI as well. Autoreactive T cells against MBP could be isolated from secondary lymphoid organs of rats with subacute (3 days-old) SCI, that when injected into healthy rats could induce transient paralysis and neuroinflammation, i.e. a mild EAE-like illness (Popovich *et al*, 1996). Likewise, in humans with SCI the numbers of MBP-reactive T cells may reach the levels seen in MS patients (Kil *et al*, 1999). It seems though that SCI also leads to the activation of T_{REG} and not only pathogenic autoreactive lymphocytes (Lafaille *et al*, 1994; Ben Nun and Cohen, 1982a, 1982b; Brabb *et al*, 2000; Olivares-Villagomez *et al*, 1998; Zhang *et al*, 1993). Finally, it must be also said that CNS-reactive lymphocytes have also been found to be increased in other traumatic or ischaemic pathologies of the CNS, e.g. TBI and stroke, while they have even been isolated from healthy individuals but at much lower numbers and frequency (Harling-Berg *et al*, 1989; Pette *et al*, 1990; Wang *et al*, 1992; Olsson *et al*, 1992, 1993; Becker *et al*, 1997; Knopf *et al*, 1998; Jones *et al*, 2002; Fee *et al*, 2003; Gonzalez *et al*, 2003).

The most likely mechanism behind the autoimmune response after SCI is the breach in the BBB, the escape of neuroantigens into the circulation and their processing in peripheral lymphoid organs or even at the injury site by APCs, which subsequently present them to B and T lymphocytes in a classical MHC-related fashion (Harling-Berg *et al*, 1989; Popovich *et al*, 1993; Cassatella, 1995; Knopf *et al*, 1998; Schmitt *et al*, 2000; Ling *et al*, 2003; Karman *et al*, 2004). Indeed, as early as 1 day after experimental SCI in mice, lymphoid organs such as the spleen and bone marrow are enriched in activated B and T cells (Jones *et al*, 2002; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). The APCs charged with the neuroantigenic peptides probably activate not only *naïve* but also *specific memory* lymphocytes, since as mentioned above autoreactive lymphocytes against myelin-related antigens are found even in healthy individuals. But even *polyspecific* lymphocytes may be likewise reactivated after SCI given the broad autoantibody repertoire of the autoimmune response (Wilson *et al*, 1979; Freddo *et al*, 1986; Alderruccio *et al*, 1989; Levin *et al*, 1998; DeGiorgio *et al*, 2001; Hughes *et al*, 2003; Jernigan *et al*, 2003; Tejada-Simon *et al*, 2003; Bogdanos *et al*, 2005; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). Most autoreactive B and T lymphocytes then migrate from the peripheral lymphoid organs into the CNS and selectively accumulate in or near the lesion site, even up to at least 2 months postinjury, where they persist thereafter (Sroga *et al*, 2003; Kigerl *et al*, 2006; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). Thus, lymphocyte intraspinal migration continues even after the BBB integriry is restored probably due to the upregulation of the requisite adhesion molecules by endothelial cells (Popovich *et al*, 1996; McTigue *et al*, 1998; Schnell *et al*, 1999; Bilgen *et al*, 2002; Babcock *et al*, 2003; Whetstone *et al*, 2003). The lymphocytes are enriched in the lesion zone, because they are attracted by chemokine/cytokine gradients produced by inflammatory cells, activated glial cells and pioneer T lymphocytes (Popovich *et al*, 1996; McTigue *et al*, 1998; Bethea *et al*, 1999; Schnell *et al*, 1999; Ghirnikar *et al*, 2000; Babcock *et al*, 2003; Eng and Lee, 2003; Ginzalez *et al*, 2003; Bao *et al*, 2004; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). Lymphocytes remain, accumulate and may proliferate in the same area because they are reactivated *in*

situ by APCs and cytokines, which is why they are found in the vicinity of perivascular macrophages, infiltrating monocytes and microglia (Popovich *et al*, 1993, 1996, 1997; Schmitt *et al*, 2000; Sroga *et al*, 2003; Kigerl *et al*, 2006; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). Indeed, the B and T lymphocytes that accumulate intraspinally become organized in large clusters that are morphologically identical to the germinal centers and lymphoid follicles found in lymph nodes and spleen (Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). Considering that similar 'ectopic' lymphoid clusters, i.e. sites of active lymphocyte proliferation and differentiation, are found in other classical autoimmune diseases (e.g. rheumatoid arthritis in the synovium, MS in the meninges), that strengthens the hypothesis that autoreactive B and T lymphocytes are vigorously active inside the spinal cord (Schrœder *et al*, 1996; Popovich *et al*, 1996; Kim *et al*, 1999; Serafini *et al*, 2004; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010).

The pathophysiological role of these autoreactive T lymphocytes and autoantibodies produced by B cells in the setting of traumatic SCI has been the subject of some controversy lately. Nevertheless, experimental evidence is overwhelmingly in favor of their pathogenic potential. Several arguments support this tenet: 1) Autoreactive lymphocytes and antibodies, generated by similar immunological mechanisms as in SCI, are encountered in other nervous system pathologies where a pathogenic role has been established (MS, ADEM, SLE, AIDP, CIDP) as well as in CNS pathologies where neuroinflammation is believed to be detrimental (SCI, TBI, stroke) (Harling-Berg *et al.*, 1989; Pette *et al.*, 1990; Wang *et al.*, 1992; Olsson *et al.*, 1992; Becker *et al.*, 1997; Dowler *et al.*, 1997; Koller *et al.*, 1997; Knopf *et al.*, 1998; DeGiorgio *et al.*, 2001; Jones *et al.*, 2002; Fee *et al.*, 2003; Gonzalez *et al.*, 2003; Kowal *et al.*, 2004; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010; Potas *et al.*, 2006). 2) Athymic nude rats and RAG KO mice, which are devoid of T lymphocytes, had attenuated neuropathology and showed better recovery after SCI or TBI compared to controls (Fee *et al.*, 2003; Potas *et al.*, 2006; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). 3) In Lewis rats vaccinated to expand MBP-reactive T cells and in transgenic mice with a T cell repertoire biased toward recognition of MBP (mice transgenic for the MBP T cell receptor in which most of the $CD4^+$ lymphocytes are reactive with the immunodominant epitope of MBP), contusion SCI had a more severe outcome with increased lesion size, axonal injury, demyelination and functional impairment compared to controls (Jones *et al.*, 2002, 2004). 4) The repetition of the passive and active vaccination experiments using the same protocols (by MBP reactive T cells or MBP itself) that have led one Israeli group to promote a hypothesis of 'protective autoimmunity', not only did not support a beneficial role for the autoimmune response, but on the contrary confirmed its neurodestructive capacity (Jones *et al*., 2002, 2004). Thus, it was shown in rat and mouse models of both PNS or CNS injury that vaccination protocols aiming at instituting or boosting an autoimmune response directed against specific CNS antigens (MBP or *myelin oligodendrocyte glycoprotein*, MOG) but even a non-CNS antigen (*ovalbumin*) clearly and consistently exacerbated CNS or PNS histopathology (demyelination, neuron loss) and functional impairment (Wisniewski and Bloom, 1975; Sun *et al*., 2001; Munch and Robinson,

2002; Jones *et al*., 2002, 2004; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). 5) Conversely, administration of antibodies against CXCL10 in mice with SCI, resulted in reduced T cell infiltration at the injury site with significant anatomical and functional preservation (Eng and Lee, 2003; Gonzalez *et al*., 2003). 6) A strong argument for a pathogenic potential of the autoreactive lymphocytes generated after SCI is the one that reminds of the critical Koch postulate for microbial pathogenicity. In fact, T cells isolated from secondary lymphoid tissues of rats with subacute SCI and injected intravenously into naïve recipients were capable of causing a mild EAE-like pathology encompassing transient hind limb paralysis and spinal cord inflammation (Popovich *et al*., 1996). That means that the CNS-reactive T lymphocytes of the SCI donors were primed and active enough to leave the bloodstream of the nonSCI recipients, penetrate the healthy BBB and cause *de novo* neuroinflammation with clearly detrimental functional repercussions. Concerning the pathogenic potential of CNS-reactive B lymphocytes and the broad range of autoantibodies there is evidence suggesting that they too can be harmful (Dowler *et al*., 1997; DeGiorgio *et al*., 2001; Kowal *et al*., 2004; Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010). For example, mouse SCI autoantibodies induced neuroinflammation and neuronal apoptosis when injected into intact hippocampi (Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010).

The pathogenic potential of the autoreactive lymphocytes and autoantibodies generated in SCI is therefore beyond doubt, yet their actual physiological role seems to be much less pronounced with no obvious chronic autoimmune EAE- or MS-like pathology complicating experimental or clinical SCI, as is also the case in TBI or stroke (Popovich *et al*., 1996). This may be due to the following reasons. First, the autoimmune response following SCI differs in quantity and quality from the autoimmune attack in MS, which might explain the mild, self-limited and more tolerable nature of the former as opposed to the recurring and progressive course of the latter. Second, the SCI-related autoimmune response may be efficiently limited or terminated by immunoregulatory or immunosuppressive mechanisms, some of which may be inherent to the immunoprivileged status of the CNS (e.g. the FAS-FAS ligand pathway), while others are not (such as the surge in circulating glucocorticoids and catecholamines after SCI), that result in induction of T cell apoptosis or anergy (Cruse *et al*., 1993a, 1993b, 1996a, 1996b; Popovich *et al*., 1996; Kohm and Sanders, 2001; Lucin *et al*., 2001; Prass *et al*., 2003). Indeed, it has been shown that SCI results in simultaneous activation of T_{REG} that temper the effects of autoreactive T cells in neuroinflammation and gliosis and, in fact, mice deficient in T_{REG} were more susceptible to autoimmunity-mediated secondary injury following SCI (Ben Nun and Cohen, 1982a, 1982b; Zhang *et al*., 1993; Lafaille *et al*., 1994; Brabb *et al*., 2000; Jones *et al*., 2002; Shevach *et al*., 2002). Third, autoreactive T lymphocytes may even exert some beneficial actions although this argument is not well substantiated and rather contradictory. Thus, apart from macrophages, autoreactive B and T cells have been shown to express or secrete, among their rich repertoire of cytokines, bioactive neurotrophins and neurotrophin receptors such as NGF, BDNF, NT3 or NT4 both *in vitro* and probably even *in vivo* within inflammatory CNS or PNS lesions in the setting

of autoimmune disease such as EAE or MS (Ehrhard *et al.*, 1993a,1993b; Franklin *et al.*, 1995; Melamed *et al.*, 1995; Torcia *et al.*, 1996; Batchelor *et al.*, 1999, 2000, 2002a, 2002b, 2008a; Besser and Wank, 1999; Kerschensteiner *et al.*, 1999; Hohlfeld *et al.*, 2000; Moalem *et al.*, 2000; Edling *et al.*, 2004; Jones *et al.*, 2005a; Jones *et al.*, 2005b; Serpe *et al.*, 2005). Moreover, neurotrophin production *in vitro* and secretion could be triggered by antigenic activation of the MBP-reactive T cells so it was hypothesized that via this mechanism these autoreactive T lymphocytes could provide neuroprotection where most needed in a context-dependent manner, i.e. near the inflammatory lesions where the self-antigens would be exposed (Kerschensteiner *et al.*, 1999; Hohlfeld *et al.*, 2000, 2007). This would seem an intuitive mechanism to counterbalance the damaging potential of autoreactive lymphocytes in inflammatory lesions of the CNS in MS, so the finding is not entirely surprising. After all many cells with immunological functions are today known to be able to produce cytokines and growth factors with actions on neurons, e.g. most glial cells, hæmatopœitic cells and even macrophages (Hammarberg *et al.*, 2000; Moalem *et al.*, 2000; Muhallab *et al.*, 2002; Gielen *et al.*, 2003; Edling *et al.*, 2004; Jones *et al.*, 2005; Serpe *et al.*, 2005). However, others have countered that neither neurotrophin production nor neuroprotection should be exclusively attributed to the CNS-reactive T cells, since in animal models of EAE or facial nerve injury it was found that it was rather the nonspecific and non CNS-reactive B and T cells, the resident microglia and infiltrating macrophages, that were primarily responsible for the neurotrophin production and beneficial effect (Hammarberg *et al.*, 2000; Moalem *et al.*, 2000; Muhallab *et al.*, 2002; Gielen *et al.*, 2003; Franzen *et al.*, 2004; Edling *et al.*, 2004; Jones *et al.*, 2005; Serpe *et al.*, 2005). Macrophages and microglia are known to secrete growth factors with neurotrophic properties, such as NGF, BDNF and GDNF (Caroleo *et al.*, 2001; Elkabes *et al.*, 1996; Batchelor *et al.*, 1999, 2000, 2002a, 2002b, 2008a; Satake *et al.*, 2008b). Furthermore, it has not been shown that myelin-reactive T cells are a source of neurotrophins in traumatic injury of the CNS such as SCI. On the contrary, in mice transgenic for the MBP TCR in which >90% of the $CD4^+$ lymphocyte repertoire is MBP-reactive, no increase in intraspinal production of BDNF and NT3 after SCI contusion SCI could be detected (Jones *et al.*, 2002, 2004). Finally, not forgetting the fact that neuroprotective effects are not evident in all rat or mouse strains, in those EAE or SCI experiments where neuroprotection was claimed to occur, its relationship to a purported neurotrophin production by the autoreactive T cells was merely hypothetical, since causality was never addressed (Moalem *et al.*, 1999, 2000; Schwartz and Kipnis *et al.*, 2001, 2004).

In conclusion, despite the experimental evidence that autoreactive B and T lymphocytes may actually exacerbate secondary injury after SCI, whether they actually exert a deleterious effect in reality is still uncertain. Thus, the self-limited autoimmune reactions that are induced by purely traumatic SCI may simply be an epiphenomenon of the incidental abolition of the immune privilege of the CNS without pathophysiological or teleological significance. However, it is plausible and highly possible, given the experimental evidence for it, that autoreactive B and T lymphocytes exert subclinical,

yet noxious effects, that, although not discernible in the melee of secondary injury, do aggravate the already catastrophic aftermath of SCI.

NEUROIMMUNOLOGICAL ASPECTS OF FACIAL NERVE TRANSECTION

Peripheral nerve injuries are accompanied by more or less suuccesful axonal regeneration, in contrast to CNS injuries (Barron, 2004). Peripheral nerve transection leads to *Wallerian degeneration* of the distal axonal segments and their myelin sheaths but not to ascending demyelination of the proximal axonal segments (Barron, 2004). However, despite the relative robust condition of the proximal axonal stumps after axotomy of peripheral myelinated axons, peripheral nerve transection does elicit a retrograde reaction that affects the cell soma at the structural, biochemical and molecular levels (Barron, 2004). This retrograde reaction serves as the trigger for the regeneration programme that will eventually benefit the axotomized neurons but also leads to characteristic perineuronal glial events (Barron, 2004).

The facial nerve transection model in rats or mice has been extensively used to study the retrograde neuronal response to axotomy (Sendtner *et al.*, 1990, 1991, 1992, 1994, 1997; Koliatsos *et al.*, 1993; Barron, 2004; Moran and Graeber, 2004; Jones *et al.*, 2005b; Serpe *et al.*, 2005). This retrograde reaction includes morphological changes grossly characterized more than a century ago, such as swelling of the cell soma, scattering of the tigroid substance of Nissl (*chromatolysis*), loosening and detachment of synapses at the cell body surface and eventually cell soma atrophy or even cell death (Lieberman, 1971; Svensson and Aldskogius, 1992a, 1992b, 1992c; Svensson *et al.*, 1993; Kreutzberg and Raivich, 2000; Barron, 2004). Chromatolysis is a descriptive term for a subcellular process in lesioned neurons, visible under the light microscope after basophilic histological staining, first reported by Franz Nissl in 1894 that corresponds to the augmentation and regional dispersion of the rough endoplasmic reticulum cisternæ thar are closely packed under normal conditions (Lieberman, 1971; Barron, 2004). Neuronal cell body swelling and chromatolysis are associated with postinjury increase in cellular metabolism and protein synthesis (Lieberman, 1971; Kreutzberg and Raivich, 2000; Barron, 2004). As a general rule the closest the axotomy is to the *axon hillock* the more severe the consequences on the neuron itself while the peripheral neurons of adult animals are more resistant to axotomy than those of neonatal animals. Changes at the molecular level are too complex and involve shifts in the expression profile of several membrane receptors, adhesion molecules, growth-associated proteins, second messenger molecules and transcription factors that are part of a well-orchestrated injury, survival and regeneration response (Barron, 2004; Raivich *et al.*, 2004a, 2004b). Axotomy of a peripheral neuron also results in increased secretion or *de novo* expression, by the neuron itself or the surrounding glial, of growth factors, neuro/gliotransmitters or neuro/gliomodulators and diverse cytokines involved in glia activation and inflammatory cell recruitement (Raivich *et al.*, 1999, 2004a, 2004b; Barron, 2004; Jones *et al.*, 2005b).

The retrograde reaction after peripheral nerve injury involves a concomitant reaction of the glial elements surrounding the nerve cell body and its synaptic field or perineuronal net (Baron, 2004). Moreover, an inflammatory cellular reaction that may also involve the neighbouring BBB takes place. Some of the neuronal and perineuronal changes after peripheral axotomy resemble those seen in autoimmune processes, e.g. the robust upregulation of of MHC molecules on neuron and glia, the activation of surrounding glia, especially microglia with production of cytokines and cytokine receptors, and the local recruitement of immune system cells, e.g. T cells. The features of this glial/inflammatory response are not stereotypical but subject to genetic polymorphism and may covariate with susceptibility to EAE which is also strain-dependent.

The molecular signals leading to glial activation, MHC upregulation and cytokine expression after facial nerve injury are currently not fully known but IFNγ is believed to play an important role in the process. In fact, IFNγ and TNFα are involved in neuron-glia communication after facial nerve axotomy. In a mouse facial nerve lesion model, IFNγ mRNA upregulation was shown to coincide spatiotemporally with the delayed inflammatory response. IFNγ has also been shown to mediate the upregulation of MHC expression on cultured neurons, e.g. dorsal root ganglia (DRG) sensory neurons, microglia and astroglia in an autocrine and paracrine manner (Olsson *et al.*, 1989; Chung *et al.*, 1991; Neumann *et al.*, 1995, 1997; Renno *et al.*, 1995, 1998). The fact that the mRNA of these cytokines was present in and around the facial nucleus after facial nerve axotomy in SCID (*severe combined immunodeficiency*) mice, i.e. mice lacking B and T cells, suggests that infiltrating lymphocytes are not the only significant sources for IFNγ and TNFα (Raivich *et al.*, 1998).

Nevertheless, lymphocytes are very important players in the postaxotomy reaction in the facial nucleus. Several studies have shown the participation of T cells in the inflammatory response of the facial nerve nucleus after axotomy mostly in the mouse model. Peripheral nerve transection leads to rapid influx of T cells into the axotomized mouse facial motor nucleus in two waves, with a plateau reached 2-4 d after injury, and a second, much stronger increase at 14 d. These T cells frequently form aggregates around microglia removing neuronal debris. The massive influx of lymphocytes at day 14 is coincident with the upregulation of IL1β, TNFα, and IFNγ mRNA while it is not accompanied by the entry of neutrophil granulocytes, nor preceded by a BBB breach. Most of these T cells are $CD4^+$ T cells, both T_H1 and T_H2, i.e. T cells with proinflammatory and antiinflammatory actions respectively. Indeed, T cells may influence the outcome of facial nerve lesion since it has been shown that survival of axotomized motor neurons of the facial nucleus depends on the presence of anti-inflammatory $CD4^+$ T cells, i.e. of the T_H2 subtype (Serpe *et al.*, 2003).

SPECIFIC BACKGROUND:
HISTORICAL PART (PAPER VI)

Most neuroscientists are aware of the following account concerning the early days of the hypothesis of neurite growth inhibition by CNS white matter. Martin Berry clearly evoked in 1982 an inhibitory action by CNS white matter on axonal regeneration based on a synthetic evaluation of elegant experiments done by him and others scientists in the 70's (Berry, 1982; Kiernan, 1979). This is admittedly a seldom acknowledged fact (Filbin, 2003). Soon after, seminal experiments by Schwab *et al.* certified this hypothesis (Schwab and Thœnen, 1985). First, it was shown in co-culture experiments that oligodendrocytes inhibited neuronal sprouting and neurite outgrowth (Schwab and Thoœnen, 1985; Caroni and Schwab, 1988a, 1988b). It was later found that two CNS myelin components, dubbed NI-250 and NI-35, were responsible for this inhibitory action and that antibodies raised against these antigens, IN-1 and IN-2, could neutralize this inhibition *in vitro* (Caroni and Schwab, 1988a, 1988b). Moreover, it was shown that intrathecal transplantation of hybridomas producing the IN-1 antibody promoted axonal regeneration *in vivo* after SCI (Schnell and Schwab, 1990). The myelin inhibitory protein neutralized by IN-1, was finally identified as the product of the *NOGO* gene (Chen *et al.*, 2000; Prinjha *et al.*, 2000; GrandPré *et al.*, 2000). The *NOGO* gene, expressing three biologically active isoforms, Nogo-A, -B and -C was identified in rats, mice and humans and the *Nogo* glycoprotein was classified as a member of the *reticulon family*, *Reticulone 4* (RTN4) (Chen *et al.*, 2000; Prinjha *et al.*, 2000; GrandPré *et al.*, 2000; Œrtle *et al.*, 2003; Wang *et al.*, 2006). Of these three isoforms Nogo-A is the splice variant of Nogo that is blocked by IN-1 (Che *et al.*, 2000; Grandpre *et al.*, 2000; Prinjha *et al.*, 2000). The neuronal receptor of the Nogo ligand was eventually cloned (Nogo receptor, NgR), and homologous receptors were later discovered (Hu *et al.*, 2005; Venkatesh *et al.*, 2005; Schwab *et al.*, 2006). Several co-receptors to NgR have since been identified, such as *p75*, *Taj/TROY* and *LINGO* (Wang *et al.*, 2002; Wong *et al.*, 2002; Lee *et al.*, 2003; Mi *et al.*, 2004; Park *et al.*, 2005; Shao *et al.*, 2005; Schwab *et al.*, 2006).

Myelin-associated glycoprotein (MAG), is another myelin protein that inhibits neurite outgrowth in the CNS (Mukhopadhyay *et al.*, 1994; McKerracher *et al.*, 1994). The inhibitory activity of MAG has been characterized both *in vitro* and *in vivo* (Li *et al.*, 1996; Schäfer *et al.*, 1996). In one study when tested *in vitro* Nogo and MAG had equipotent growth inhibitory activity (Prinjha *et al.*, 2000). Also immunodepletion of MAG from CNS myelin reduced *in vitro* inhibition of axonal outgrowth (McKerracher *et al.*, 1994; Filbin *et al.*, 1995; Li *et al.*, 1996; Shibata *et al.*, 1998; Filbin *et al.*, 2003). However, the extent to which MAG limits outgrowth *in vivo* has been a subject of controversy (Montag *et al.*, 1994; Bartsch *et al.*, 1995; Filbin, 1996; Schäfer *et al.*, 1996; Shen *et al.*, 1998). MAG is present in both CNS and PNS but its inhibitory effect in PNS is not considerable probably due to downregulation of MAG expression in the adult PNS or due to compensation by axonal growth promoting factors such as laminin-1 (David *et al.*, 1995; Filbin, 1996). Also, regeneration in $MAG^{-/-}$ mice was poor likely due to the presence of other inhibitors such as Nogo (Montag *et al.*, 1994; Bartsch *et al.*, 1995).

Remarkably, it was found that MAG mediates inhibition via the NgR1 and even competes with Nogo, in particluar its *Nogo66* sequence, for the same binding site (Domeniconi *et al.*, 2002, 2005; Liu *et al.*, 2002).

A third white matter MAI, *oligodendrocyte myelin glycoprotein* (OMgp), a glycophosphatidylinositol glycoprotein, was subsequently discovered that like Nogo and MAG induced growth cone collapse and inhibition of axonal outgrowth (Kottis *et al.*, 2002; Wang *et al.*, 2002). Surprisingly, the inhibitory effects of OMgp are mediated by the NgR1 but through a different binding site than the one for Nogo66 (Wang *et al.*, 2002; He and Koprivica, 2005; Schwab *et al.*, 2006). Recently, *paired immunoglobulin-like receptor B* (PirB), that has been known to be implicated in CNS plasticity, was shown to be yet another high-affinity functional receptor for all three aforementioned MAIs (Atwal *et al.*, 2008; Chang *et al.*, 2010; Cafferty *et al.*, 2010; Dickson *et al.*, 2010; Raiker *et al.*, 2010; Llorens and Del Río, 2010).

Despite these advances and overwhelming evidence in favour of the *inhibitory whilte matter hypothesis*, the negative role of white matter myelin in CNS regeneration is still not unanimously accepted. Experiments where anyone and all three main *myelin-associated inhibitors* (MAI), i.e. Nogo, MAG and OMgp, were knocked out, as well as similar KO experiments of the principal MAI receptors, have given conflicting or contradictory results (Montag *et al.*, 1994; Bartsch *et al.*, 1995; Li *et al.*, 1996; Ng *et al.*,1996; Simonen *et al.*, 2003; Zheng *et al.*, 2003, 2005; Raisman, 2004; Song *et al.*, 2004; Lee *et al.*, 2009, 2010; Marklund *et al.*, 2009; Cafferty *et al.*, 2010; Omoto *et al.*, 2010; Silver, 2010). Moreover, it has been shown, albeit in microlesion experiments of the CNS, that adult CNS myelin is *not* incompatible with axonal regeneration and postulated that the fibroglial scar at the lesion site is the major factor associated with the failure of axonal regrowth (Davies *et al.*, 1997, 1998, 1999; Raisman, 2004; Silver, 2010).

As often is true in science and medicine the real roots of a concept, hypothesis or theory can be found in the past if only one searches for them. The *inhibitory white matter hypothesis* was useful in the course of experimentation for this thesis and I was intrigued to read that some neuroscientists traced this hypothesis back to Cajal (Filbin, 2003). I decided to do my own research in the old records in order to discover the *fons et origo* of this hypothesis. It was a revelation to find that a debate, similar to the current one, over the respective roles of CNS white matter and fibroglial scar in CNS regeneration failure first took place a century ago.

SPECIFIC BACKGROUND:
METHODOLOGICAL PART (PAPERS I, II, IV)

The acute, subacute and chronic stages of SCI have unique characteristics which in turn call for specific treatment approaches (Tator and Fehlings, 1991; Tator, 1992, 1995, 1996; Schwab and Bartholdi, 1996; Houlé, 1991; Houlé and Ye, 1997, 1999; Olson, 1997, 2002; Houlé and Yin, 2001; Geller and Fawcett, 2002; Baptiste and Fehlings, 2006; Moreno-Flores and Avila 2006; Cafferty *et al.*, 2008). In acute and subacute SCI, treatment strategies seek to mitigate cord degeneration, minimize tissue loss by necrosis and apoptosis, and promote axonal regeneration by blocking different steps of the secondary injury cascade or providing neurotrophic factors (Schwab and Bartholdi, 1996; Houlé, 1991; Houlé and Ye, 1997, 1999; Olson, 1997, 2002; Zhang *et al.*, 1998; Blight and Zimber, 2001; Houlé and Yin, 2001; Geller and Fawcett, 2002; Moreno-Flores and Avila 2006). Therefore, in the acute and subacute stages neuroprotection as well as regeneration are the goals of treatment. In chronic SCI, where most degenerative events have already occured, the only remaining option is to induce regeneration, tissue restitution and remnant pathway reactivation to obtain functional recovery (Houlé, 1991; Schwab and Bartholdi, 1996; Houlé and Ye, 1997, 1999; Olson, 1997, 2002; Bregman *et al.*, 1998; Blight and Zimber, 2001; Coumans *et al.*, 2001; Houlé and Yin, 2001; Ribotta *et al.*, 2001; Sofroniew *et al.*, 2001; Woerly *et al.*, 2001; Åkesson *et al.*, 2001; Schwab, 2002; Bradbury *et al.*, 2002; Geller and Fawcett, 2002; Lee *et al.*, 2002; Cafferty *et al.*, 2008). The present studies were designed to investigate the possibility of restoring sensorimotor abilities in chronically paraplegic rats at long intervals after a complete transection of their spinal cord (starting from 2 months postinjury and longer) thereby addressing the issue of whether clinical chronic spinal cord injury might be amenable to therapy.

Many animal models of SCI have been developed since Allen in 1911 began working with weight drop lesions on dogs (Allen, 1911, 1914; Dohrmann, 1972a, 1972b; Wrathall *et al.*, 1985; Hotz *et al.*, 1989; Martin *et al.*, 1994; Schwab and Bartholdi, 1996; von Euler *et al.*, 1997a, 1997b; Metz *et al.*, 2000; Spilker *et al.*, 2001; Young, 2002b; Kwon *et al.*, 2002c; Young, 2002). We chose complete spinal cord transection as our lesion model since it is the only experimental SCI model that unequivocally results in complete disruption of spinal continuity and paraplegia (Cheng *et al.*, 1995b, 1996; Fraidakis *et al.*, 1998, 2004). The complete transection model offers an unbiased baseline level of anatomical interruption of all spinal pathways with resultant paraplegia that serves as the ideal point of reference for any axonal regeneration or functional improvement as a result of treatment (Cheng *et al.*, 1995b, 1996; Fraidakis *et al.*, 1998, 2004). Moreover, were both anatomical and functional improvement present or absent simultaneously, a correlation between the two would be established (with anatomical regeneration being both sufficient and necessary for functional recovery) and a causal relation implied. The complete transection model is the only SCI model that adequately meets the scepticism that stems from the confounding role of compensatory collateral sprouting from spared axons and eludes 'spontaneous regeneration' afterthoughts

(Beattie *et al.*, 1997). It is well known that survival of less than 10% of spinal axons can lead to almost complete apparent locomotor recovery (Tator *et al.*, 1984; Blight, 1992; Schwab and Bartholdi, 1996; Beattie *et al.*, 1997; von Euler *et al.*, 2002). Moreover, apart from being parsimonious, the transection model is also the most easily reproducible and it is the only experimental model that corresponds to the clinical entity of chronic and irreversible total paraplegia, certainly the most likely candidate group for surgical treatment.

In the assessment of human SCI magnetic resonance imaging (MRI) is now the ideal method to non-invasively characterize the extent of cord trauma in the acute and chronic stages and MRI findings have been shown to correlate with neurological damage and clinical outcome after spinal cord trauma. MRI has been increasingly used also in the evaluation of experimental SCI. High resolution MR imaging studies of the gross anatomy and pathology of rat spinal cord were shown to correlate with major histopathological changes and neurological deficit in experimental SCI (Weirich *et al.*, 1990). In the early days of MRI application in experimental SCI, in order to circumvent problems arising from motion, rat spinal cord was more often excised and studied in freshly removed or fixed tissue preparations. For recordings in rat spinal cord, external surface coils or inductively coupled, implanted radiofrequency coils have been used (Bilgen, 2006a). Contrast of gray and white matter in the brain and spinal cord has been characterized for a given set of physical parameters using intermediate (1.4-1.9 Tesla) magnetic field strength for human and rat. MRI methodology for *in vitro*, *ex vivo* and *in vivo* studies has been refined during the years to the degree that even individual axons of the lamprey spinal cord can be now visualized (Fukuoka *et al.*, 1998; Takahashi *et al.*, 2002; Wright *et al.*, 2002; Bilgen *et al.*, 2006a, 2006b, 2006c). High-resolution MRI is now capable of distinguishing the neuropathological differences between rat and mice SCI, of assessing neuroprotective effects of experimental treatments in animal models and even of tracking *in vivo* specially prelabeled grafted cells in the spinal cord or brain of experimental animals with SCI (Lee *et al.*, 2004; Banasik *et al.*, 2005; Sykova and Jendelova, 2007; Byrnes *et al.*, 2010). Diffusion-weighted MRI and lately even *diffusion tensor imaging* (DTI) have been applied in experimental SCI tobetter estimate white matter damage, long tract pathology and even plasticity and they were shown to excellent correlate with histopathological and behavioral findings (Deo *et al*, 2006; Kim *et al.*, 2007; Kozlowski *et al.*, 2008; Cohen-Abad *et al.*, 2008; Herrera *et al.*, 2008; Sundberg *et al.*, 2010; Tu *et al.*, 2010). *Manganese-enhanced MRI* (MEMRI) has recently been employed to visualize the CST *in vivo* after tract specific labeling by manganese in a rat SCI model (Bilgen *et al.*, 2006; Stieltjes *et al.*, 2006). Finally functional MRI has also proven invaluable in revealing *in vivo* plasticity of cortical and spinal cord cicuitry after experimental SCI (Endo *et al.*, 2007, 2008a, 2008b).

In our studies we tried to establish a state-of-the-art protocol for MRI of experimental SCI in rat (Fraidakis *et al.*, 1998, 2004). Normal spinal cord and completely midthoracically transected cord (at various times between 2 and 6 months postinjury) were imaged with high resolution spin-echo sequences (RARE), *multislice multiecho spin echo* sequences (MSME) and inversion-recovery pulse sequences using an

experimental 4.7 Tesla MR spectrometer. The experiments were performed to assess the extent and severity of a chronic spinal cord lesion in living animals. In addition, MR images were correlated to histological investigations performed in the same animals after sacrifice. Preliminary results showed that gray and white matter in the normal and transected cord and the lesion and its extent in the experimental SCI model can be well visualized. The degeneration in chronic transected rats extended up to 1 cm both rostrally and caudally measured from the free stumps as shown by changes in intensity of MRI signal, cord diameter, dural and gray/white matter morphology, corroborated by histopathological analysis. In addition, secondary pathologies such as spinal cord instability, syringomyelia and dorsal cord compression were observed.

Previous work with acute SCI in our lab has shown that partial recovery of hindlimb function and long tract regeneration in a complete transection rat model could be achieved by implantation of multiple peripheral nerve autografts in a white-to-grey matter rerouting gap-bridging scheme with the addition of a stabilizing fibrin glue cast loaded with acidic FGF and a spinal column-immobilizing wire loop (Cheng *et al.*, 1995a, 1995b, 1996; Reuss and von Bohlen und Halbach, 2003). In this thesis work, we strove to demonstrate that the fibrin glue used in the acute-repair protocol could effectively operate as a biodegradable reservoir for slow-release of growth factors intraspinally (Cheng *et al.*, 1995a, 1995b, 1996, 1998). We then attempted to apply a similar surgical reconstructive treatment with appropriate modifications, in a chronic complete transection rat SCI model. This was dictated not only by the strong scientific challenge, but by two pragmatic reasons. First, if ever this microsurgical model would reach the clinic it would be inapplicable to patients with acute SCI since not only is it not a neuroprotective treatment but on the contrary it would necessitate additional damage to the already injured spinal cord. Therefore, only patients with ASIA-A SCI could theoretically be eligible if all ethical dilemmas were ever resolved and even then the operation would take place in a subacute to chronic, this time for strictly medical reasons pertaining to the Hippocratic credo *primum non nocere*. Second, the only SCI patient population that would be envisaged as a target group for such a surgical treatment would be those with truly chronic ASIA-A SCI of at least 1 year's duration or probably more. These patients have not much more to expect from rehabilitation and they are unlikely to receive any benefit from neuroprotective approaches since secondary injury has already occurred. Therefore, only a proregenerative treatment would have a theoretical, yet remote, chance of addressing the quagmire of chronic complete SCI. Thus, in acute SCI this treatment would carry the highest risk/benefit ratio, while in chronic complete SCI this ratio would be lowest, all the difficult ethical concerns set aside.

Using the same experimental model of paraplegia the reconstructive surgery was this time performed at intervals of 2, 4 and 8 months postinjury (Cheng *et al.*, 1996; Fraidakis *et al.*, 2004). One of the modifications to the acute repair protocol was that only half the animals were administered *acidic FGF* while all received the fibrin glue for the purpose of stabilizing the implanted autologous nerve bridges (Fraidakis *et al.*, 2004). Between injury and repair surgeries the animals underwent MRI and behavioral

analysis to document the morphological and functional totality of the lesion (Fraidakis *et al.*, 2004). The MRI protocol previouly established by us was employed to temporally evaluate the anatomical changes of the spinal cord after complete transection injury at the midthoracic level and guide the application of the surgical protocol (Fraidakis *et al.*, 1998; Fraidakis *et al.*, 2004). Finally, two novel behavioral tests designed by the author, the *Bipedal test* and the *Head-scratch test*, were also employed in the behavioral evaluation of sensorimotor recovery in addition to a standard, universally employed battery of locomotor tests (Tarlov, 1953; Tarlov *et al.*, 1954; Eidelberg, 1975; Donatelle, 1977; Rivlin and Tator, 1977; Gale *et al.*, 1985; Kunkel-Bagden *et al.*, 1992; Basso *et al.*, 1995; Metz *et al.*, 2000; Hil and Elde, 1990; Wrathall *et al.*, 1985; Basso *et al.*, 1996; Fraidakis *et al.*, 2004).

SPECIFIC BACKGROUND:
NEUROIMMUNOLOGICAL PART (PAPERS III, V)

In EAE and MS the T_H1 response is thought to mediate the myelin destruction with cytokines such as TNFα and IFNγ while T_H2 seems to have antiinflammatory and neuroprotective effects (Brosnan *et al.*, 1988; Selmaj and Raine, 1988; Liblau *et al.*, 1995; Merill and Benveniste, 1996; Adorini *et al.*, 1997; Vitkovic *et al.*, 2001). Downregulating the T_H1 response has proven beneficial in EAE models while various experimental treatment protocols for MS have the common aim of triggering or boosting the T_H2 response as a counterweight and buffer to the T_H1 blow-out (Kappos *et al.*, 2000; Garren *et al.*, 2001; Steinman, 2001b; Adorini *et al.*, 1997; Rengarajan *et al.*, 2000; Bettelli *et al.*, 2003). We clearly see here that although these T cell subsets of the adaptive immune system have evolved to meet host-defence purposes against infectious agents, they are also implicated in non-infectious, autoimmune conditions with characteristic effects (Aloisi *et al.*, 1999).

Spinal cord injury and MS share some common pathological features. One is chronic progressive demyelination. In SCI oligodendrocyte apoptosis is not just the outcome of mechanical damage, ischaemia and free radical formation since it takes place long after the BBB and circulation are restored and at significant distance rostrocaudally from the injury site. In the context of SCI oligodendrotoxic and proapoptotic cytokines such as TNFα are thought to contribute to the demise of myelin (Vitarbo *et al.*, 2004). Another surprising similarity between EAE/MS and SCI is immune system dysregulation with generation and subsequent activation of myelin reactive T_H cells *in situ* (Steinman *et al.*, 1996; Popovich *et al.*, 1996). This presupposes of course the active infiltration and participation of specific T_H and other T cell populations in the acute, subacute or chronic stages of inflammation after SCI (Popovich *et al.*, 1996; Raivich *et al.*, 1998; Kil *et al.*, 1999; Cabarrocas *et al.*, 2003; Bradl *et al.*, 2005). In fact, we now know, from studies in experimental as well as human SCI, that purely traumatic injury of the spinal cord may result in chronic autoimmune responses, both humoral and cellular. Therefore, we are faced with the remarkable fact that the adaptive immune system is not only involved in its trademark sentinel role against foreign antigens or in a renegade role against self-antigens in autoimmune diseases, but that it also participates in a preprogrammed autoimmune fashion even in purely traumatic injuries of the CNS despite the immunoprivileged status of the latter (Popovich *et al.*, 1996; Aloisi *et al.*, 1999). Although immune privilege mechanisms may actually help control the postraumatic autoimmune response targeting the CNS, there is little doubt about the neurodestructive potential of the autoreactive T cells. And although the extent of the damage mediated by myelin reactive T cells is currently unknown, one can safely assume that their presence in and around the injury site could very possible aggravate demyelination after SCI in a way similar, but less pronounced, to the EAE/MS scenario. In SCI, where axonal damage is severe, myelin destruction and demyelination not only lead to conduction failure as in EAE/MS but they also impede axonal regeneration. So theoretically, if this neurodestructive cascade could be obviated by alleviating,

modulating or reversing the posttraumatic autoimmune response there might be a possible gain in oligodendrocyte survival, remyelination and ultimately axonal regeneration.

The retrograde reaction after peripheral nerve injury involves many structural and metabolic changes at all levels of the injured neuron, i.e. cell soma, lesioned neurite and dendrites. However, axotomy results also in a concomitant reaction of the glial elements surrounding the nerve cell body. Moreover, an inflammatory cellular reaction that may also involve the neighbouring BBB takes place. Some of the neuronal and perineuronal changes after axotomy resemble those seen in autoimmune processes, e.g. the robust upregulation of of MHC molecules on neuron and glia, the activation of surrounding glia, especially microglia with production of cytokines and cytokine receptors, and the local recruitement of immune system cells, e.g. T cells. The molecular signals leading to glial activation, MHC upregulation and cytokine expression after facial nerve injury are currently not fully known but IFNγ is believed to play an important role in the process. IFNγ is involved in neuron-glia communication after axotomy. In a mouse facial nerve lesion model, IFNγ mRNA upregulation was shown to coincide spatiotemporally with the delayed inflammatory response. IFNγ is also been shown to mediate the upregulation of MHC expression on cultured neurons, e.g. DRG sensory neurons, microglia and astroglia in an autocrine and paracrine manner. However, *in vivo* data on the role of IFNγ in the retrograde response after axotomy of the facial nerve are lacking.

The participation of the innate immune system is inevitable in every process that is injurious to the human body, i.e. traumatic, infectious, neoplastic, autoimmune. The neuroinflammatory response after SCI or facial nerve transection involve not just the adaptive immune system but elicit as well an innate immune response. The only particularity is that this innate immune response takes place and affects the CNS which is otherwise immunoprivileged. TNFα is a cytokine with a key role in the innate immune responses of every kind, and therefore certainly an important role in the neuroinflammatory response after CNS injury or facial nerve injury as well (Vitarbo *et al.*, 2004). TNFα (previously a.k.a. as *cachexin*) is a potent, multitasking cytokine. In the immune system it is mainly produced by monocytes and macrophages. In the CNS it is mainly produced by the resident macrophages, i.e. microglia, but also by astroglia and even neurons. TNFα has many biological actions, such as immunomodulatory, vasoregulatory, proapoptotic and antiapoptotic, and even neurodevelopmental and neuromodulatory (Chung *et al.*, 1991; Pan *et al.*, 1997; Neumann *et al.*, 1997Liu *et al.*, 1998; Andrews *et al.*, 1998; Suganuma *et al.*, 1999; Krammer, 2000; Beattie *et al.*, 2002; Sibson *et al.*, 2002; Vitarbo *et al.*, 2004; Stellwagen *et al.*, 2005). Its role appears to be mainly proinflammatory, both in the innate immune response but also in the T_H1 acquired immune response (Renno *et al.*, 1995; Akassoglou *et al.*, 1998; Andrews *et al.*, 1998; Probert *et al.*, 2000; Raivich *et al.*, 2002; Vitarbo *et al.*, 2004). Also, its concentration and expression in the CSF and CNS increase early after stroke, brain or spinal cord injury (Bartholdi and Schwab, 1997; Klusman *et al.*, 1997; Lee *et al.*, 2000; Pan *et al.*, 1999, 2003; Schnell *et al.*, 1999; Vitarbo *et al.*, 2004). Specifically, in

autoimmune/inflammatory diseases, like MS and rheumatoid arthritis, TNFα is one of the main proinflammatory and cytotoxic (in the case of MS *oligodendrotoxic*) agents which led to the development of anti-TNFα monoclonal antibodies as therapeutics. Nevertheless, TNFα actions have been reevaluated the last decade, not only in traumatic and ischæmic pathologies but also in demyelinating autoimmune diseases where the proinflammatory role of TNFα was undisputed. It is now believed that TNFα actions are more subtle and complex than initially envisaged and it has even been proposed that TNFα acts as a regulatory cytokine in autoimmune-mediated demyelination (Xanthoulea *et al.*, 2004; Chitnis and Khoury, 2003; Liu *et al.*, 1998; Scherbel *et al.*, 1998; Marino *et al.*, 1996). Surprisingly, the TNFα KO mice created by Marino *et al.* were viable and not significantly different morphologically from the wild type mice. Nonetheless, TNFα null mice had altered immune responses in experimental conditions, such as resistance to LPS lethality, increased susceptibility to certain pathogens, and disturbed immunoregulation in the spleen (Marino *et al.*, 1996).

Using the EAE/MS immunopathogenesis paradigm as a springboard, we speculated on the potential effects of refined immunomodulation in traumatic PNS or CNS injury. Some of the inevitable questions were:

- What is the role of the above mentioned T_H cell subsets (T_H1 and T_H2) in non-infectious and non-autoimmune pathologies of the CNS or PNS, i.e. in purely traumatic pathologies, such as SCI and facial nerve transection?

- How do they affect axonal regeneration after SCI?

- How do they affect perineuronal neuroglial and inflammatory responses after facial nerve transection?

- And on the same vein how does lack of TNFα or IFNγ both central molecules of the innate and adaptive immunological responses after tissue injury, affect the same parameters in the same models of CNS and PNS injury?

We hypothesized that lack of the regulatory T_H2 response might exacerbate damage and hamper axonal regeneration, while T_H1 deficiency would tip the balance to an T_H2 response that might hold postinjury inflammation in check and benefit regeneration. It was more difficult to formulate such a hypothesis for the case of TNFα or IFNγ given their complex actions. Consequently, we designed a reductionist experiment in order to see how differential ablation of specific and nonspecific actions of the immune response would affect axonal regeneration and behavioral outcome in an experimental SCI model (*Paper V*) or glial and inflammatory reactions in the facial nucleus after facial nerve transaction (*Paper III*). For that goal we employed KO mice for molecules that participate in the innate response or govern the differential T_H cell adaptive responses of the immune system. In *Paper V* we used KO mice for STAT4, STAT6 and TNFα in a

SCI model. In *Paper III* we used KO mice for STAT4, STAT6, IFNγ, IFNγR and IRF-1 in a PNS injury model of facial nerve transection. For the specific ablation of adaptive immune responses we used the commercially available STAT4 and STAT6 KO mice (BALB/c) that lack respectively the T_H1 and T_H2 responses. The TNFα KO mice (C57BL/6J) developed by Marino *et al.* and the commercially available IFNγ, IFNγR and IRF-1 KO mice were used for the compromise of the innate immune responses (Marino *et al.*, 1997).

In order to refine our understanding of the specific role of each of the T_H subclasses *in vivo* in the inflammatory and glial response in the facial nerve transection mouse model we studied the expression of MHC class I, β2-microglobulin and GFAP in the facial nerve nucleus three weeks after axotomy in STAT4 and STAT6 KO mice, deficient in the T_H1 and T_H2 responses respectively (*Paper III*). In order to study the role of IFNγ in the above injury model *in vivo* we employed IHC and ISH to analyze the expression of MHC class I, β2-microglobulin, GFAP and GAP-43 in the facial nerve nucleus three weeks after axotomy in IFNγ, IFNγR and IRF-1 KO mice (*Paper III*).

To clarify the role of TNFα and each of the two T_H subsets *in vivo* in axonal regeneration after SCI we employed a double-labeling protocol after dorsal overhemisection injury in TNFα, STAT4 and STAT6 KO mice (*Paper V*). The dorsal hemisection lesion model was employed according to the experimental paradigm by Huang *et al.* (Huang *et al.*, 1999). The objective was to interrupt descending long-distance tracts, including the CST, which in mice runs laterally to the dorsal horns as in humans, without creating a gap between the two stumps that would render axonal regeneration almost impossible. A double-labeling protocol was then used with FluroGold application immediately after injury to be taken up by all injured axons and label their somata in the cortex and below cortex followed by FluoroRuby injections 3 weeks later, caudally to the hemisection site at a safe distance to preclude rostral diffusion and false labeling of injured axons at the injury site that had not regenerated into the distal stump. Statistical evaluation of the microscopical data would reveal possible differences in axonal regeneration between the different genotypes, $TNF\alpha^{-/-}$, $STAT4^{-/-}$ (T_H1 deficient), $STAT6^{-/-}$ (T_H2 deficient) and their wild type controls, or even between the different mouse strains, C57BL/6J and BALB/c. Thus an implcation, direct or more possibly indirect, of TNFα and the second messengers Stat4 and Stat6, in axonal injury and regeneration would be tested by this experimental design.

OBJECTIVES

- To study the usefulness of fibrin glue as a biological reservoir for the slow-release of a trophic factor intraparenchymally in the spinal cord as a preliminary to its use in a microneurosurgical repair protocol for acute and chronic SCI in rat.

- To establish a state-of-the-art MRI protocol to spatiotemporally characterize the spinal cord pathology after complete transection SCI in rat prior to application of a microsurgical repair procedure in chronic SCI in rat.

- To evaluate the benefits and risks conferred by a white-to-gray multiple autologous peripheral nerve-grafting repair protocol upon acute and chronic complete transection SCI in rat.

- To study the differential outcome of PNS injury with respect to neuroinflammation in KO mice lacking important cytokines and their receptors.

- To study the differential outcome of SCI with respect to axonal regeneration and behavioral recovery in KO mice lacking central cytokines of the innate and acquired immune responses.

- To investigate the history of neuroscience concerning the conundrum of CNS regeneration with the aim to identify the originator of the now prevalent hypothesis of white matter inhibition of CNS regeneration.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS (*I-V*)

Adult female 150 g Sprague-Dawley (SD) rats were used for the intraocular implantation experiment in paper I, and adult female 250-300 g SD rats for the intraspinal implantation experiment in paper I and all the experiments in *Paper II* and *Paper III* (B&K, Sollentuna, Sweden).

For the KO experiments C57BL/6, BALB/c and 129/Sv mice of different genotypes were used. In paper IV the following strains and genotypes, purchased from Jackson Laboratories (Bar Harbor, Maine), were used: C57BL/6 (IFN-γ null, IRF-1 null), BALB/c (STAT4 null, STAT6 null) and 129/Sv (IFN-γR null). Their respective wild type controls were age and sex-matched C57BL/6J, BALB/c and 129X1/SvJ. In paper V the following mouse strains and genotypes used were: C57BL/6 (TNFα null), BALB/c (STAT4 null, STAT6 null) and their respective age and sex-matched wild type controls C57BL/6J and BALB/c. The BALB/c STAT KOs and WT were purchased from Jackson Laboratories (Bar Harbor, Maine) while the C57BL/6 TNFα KO and WT were a generous courtesy from Dr. Marino of the Ludwig Institute at the Memorial Sloan-Kettering Cancer Center in NY.

All experimental animals were caged, with 2 rats or 4 mice per cage maximum, housed in humidity and temperature regulated animal facilities with a diurnal 12/12 h dark/light cycle, were provided for with chow and water ad lib and were cared for by specially trained animal care takers. Experiments were approved by the Animal Ethical Committee of Stockholm.

RADIOLABELING OF GDNF (*I*)

[^{125}I]-GDNF with a specific radioactivity of 2083 Ci/mmol was prepared by conjugation with [^{125}I]-labeled Bolton-Hunter reagent, purified by size-exclusion chromatography and lyophilised. The product had radiochemical purity 91% as determined by reverse-phase HPLC and was reconstituted with distilled water to a radioactive concentration of 100 μCi/ml and a final [^{125}I]-GDNF concentration of 50nmol/ml. [^{125}I]-GDNF was a gift from Amgen Inc.

FIBRIN GLUE COMPOSITION AND PREPARATION (*I, IV*)

The fibrin tissue glue used was formed by the enzymatic action of thrombin in mixture with a fibrinogen concentrate. The necessary components for the preparation of the fibrin glue (Beriplast® P, kindly provided by Behring (Behringwerke AG, Marburg,

Germany) and contained in a 1-ml Beriplast® P set were:

Fibrinogen concentrate: A vial contained 115-232 mg dry compound of a human plasma protein fraction with 65-115 mg fibrinogen and a human plasma protein fraction with factor XIII activity of 40-80 IU.

Aprotinin solution: Solution containing bovine lung aprotinin at a concentration of 1000 IU/ml.

Thrombin concentrate: A vial contained 4.9-11.1 mg dry substance containing a human plasma protein fraction with thrombin activity of 400-600 IU.

Calcium chloride solution: Calcium chloride solute in water solvent at a concentration of 40 mmol/l.

The fibrinogen and thrombin concentrates were dissolved in the aprotinin and calcium chloride solutions, respectively. The fibrin glue was formed instantaneously as a coagulate at the meeting tips of two syringes delivering fibrinogen and thrombin, respectively. In *Paper I*, 15 µl of radiolabeled GDNF (50 µg/ml, 3 µCi/µg) and an equal volume of the fibrinogen-aprotinin solution were mixed in one syringe and 15 µl of the thrombin-calcium chloride solution were drawn in the other syringe. The contents of the two syringes were emptied onto a sterile glass slide and a fibring glue mass was formed, which was then partitioned under the microscope into 1 mm^3 spherical GDNF containing fibrin glue 'balls' (16 ng GDNF, 0,05 µCi). The control group received fibring glue balls that were prepared likewise but containied Hank's buffered saline solution (HBSS) instead of GDNF. In *Paper IV*, the fibring glue cast that was used in repair or control treatment contained either acidic FGF or HBSS and was prepared in a similar fashion. Acidic FGF was mixed to a final concentration of 2,1 µg/ml.

SURGICAL PROCEDURES (*I-V*)

Using a surgical microscope animals were operated under aseptic conditions and inhalation anæsthesia. During rat surgery body temperature was monitored by a rectal probe and perioperatively adjusted at 35°-36°C with an underlying thermoregulatory heating pad. Attention was given to fluid administration in order to compensate for perioperative hæmorrhage and hypovolæmia and to prevent renal failure. Therefore, 1ml Ringer's solution was injected subcutaneously in rats preoperatively and 1ml postoperatively. A fluid regimen was continued for the first two weeks to maintain good renal and bladder function and as a preventive measure against UTIs in spinalized rats. Rats that underwent cord transection or repair were subjected to manual bladder voiding 2-4 times a day, prophylactic antibiotic treatment twice daily during the first week and prompt antibiotic treatment of any UTI (Borgal, Hoechst). In some rats bladder automatism was established within the first month. Others continued to have bladder dyreflexia and overflow voiding and manual bladder emptying was performed daily as

needed. Mouse operations were technically easier and temporally much shorter. Partial cord transection in mice did not necessitate monitoring or fluid regimen and did not lead to bladder dysreflexia or UTIs. The peroperative mortality in rat and mice operations was almost nil.

Intraocular implantation of fibrin glue balls containing GDNF or HBSS (I)

Twenty 150 g female SD rats were operated under ether anæsthesia. Before the intraocular implantation of the fibrin glue balls, 1% atropine solution was preadministered on both eyes of each animal. A corneal slit was made using a piece of a razor blade and a modified Pasteur pipette was used to deliver the fibrin glue ball into the anterior chamber of the eye (Olson *et al.*, 1983, 1985, 1988, 1990; Henschen *et al.*, 1985a, 1985b, 1986a, 1986b, 1987, 1988a, 1988b, 1989) The operated animals were then grouped randomly and sacrificed at 6 hours and at 1, 7, 14, 21 days postimplantation. Each animal received a GDNF-containing fibrin glue ball in one eye and a control fibrin glue ball in the other eye, so treatment and control groups consisted of 20 eyes each.

Intraspinal implantation of fibrin glue balls containing GDNF or HBSS (I)

Forty-eight female 250g SD rats were operated under halothane anesthæsia. After a sagittal incision of the back skin the dorsal spinal processes of vertebræ T8-9 were palpated counting backwards from the easily palpable spinous process of the T11 vertebra. The vertebral laminæ T8-9 were exposed and a laminectomy was performed with a microrongeur without inflicting any injury on the spinal cord. After a sagittal incision of the overlying dura the subarachnoid space was exposed and a sagittal 5 mm long and 2mm deep incision of the pia and dorsal cord was performed with a microknife. A fibrin glue ball was inserted intraspinally into the lesion and the dorsal surface of the cord was sealed with fibrin glue. In a randomized fashion, half of the animals received a fibrin glue ball containing [^{125}I]-labeled GDNF and the rest were controls receiving HBSS containing fibrin glue. Animals were then sacrificed 6 hours, 1, 7, 14, 21, 28 days later.

Complete transection (II, IV)

A T8-9 laminectomy was performed as described in the prevous section. With the help of microscissors the spinal cord was transected using a swift move to achieve total separation of the stumps with a single cut if possible. The totality of the lesion was meticulously ascertained. Whenever there was remaining ventral cord tissue it was cut away with microscissors and a microknife until the ventral dura was visible and the two stumps had retracted and were separated by a 0,5-1 mm gap (Fraidakis *et al.*, 1998, 2004).

Acute repair surgery (IV)

This was carried out essentially as previously described (Cheng *et al.*, 1996) with certain modifications. Firstly, electrocauterization of the dorsal spinal artery was not used. Secondly, and most importantly, the tips of the peripheral nerve grafts were inserted into the rostral and caudal gray matter instead of simply apposing them and letting them imbibe onto the stump surface. The aims were to better stabilize the grafts, minimize the risk of future detachment and minimize glial proliferation at the cord-nerve interface. After a T8-T9 laminectomy a 5-mm T8 segment of the spinal cord was resected. Prior to the spinal cord resection, ca 10 intercostal nerves had been removed from the animal and immersed in HBSS for later use as autologous grafts to reconnect the two stumps according to the 18-graft, white-to-gray, rerouting scheme. After implantation, with graft tip insertion as pinpointed above, the peripheral nerve grafts were embedded in a fibrin glue cast (Beriplast P, Behring, Germany), prepared as mentioned above, to ensure they would remain in place (Cheng et al., 1996). The fibrin glue contained acidic FGF (2.5 μg/ml) [n=3], or no added growth factors [n=3]. The T8-9 site of the vertebral column was stabilized in dorsiflexion by a surgical steel loop fastened to the spinal column with nonbiodegradable threads (Ethicon) tied around the proximal part of two ribs on each side. The duration of the whole procedure was around 3 h (Fraidakis *et al.*, 2004).

Acute sham surgery (IV)

In the acute sham procedure, everything was done as in the acute repair protocol except for the removal of intercostal nerve segments, the grafting of these segments and the spinal column stabilization (Fraidakis *et al.*, 2004).

Chronic repair surgery (IV)

The repair procedure in the chronic SCI animals was more challenging and inevitably entailed an acute-on-chronic cord lesion. The secondary degeneration of the stumps, the glial scar at the site of injury, the posttraumatic myelopathy, cavitation and cyst formation, are factors that distinguish the chronically injured spinal cord from the freshly injured one. Thus, the acute repair surgical protocol had to be further modified to accommodate the characteristics of a 2-8 months chronic SCI. After removing as long as possible intercostal nerve segments from the animal, the spinal cord lesion site was reexposed. Careful debridement of the overlying scar tissue ensued, in order to gain plain sight of the injured spinal cord. As a rule, the gap that had been formed after the complete transection had turned into a pulsating, fluid-filled cyst. Most often, the dorsal spinal artery, that had been divided at the time of the complete transection was not visible, but in some cases it appeared to have reoccupied its original track. Without the slightest damage to the cord, the laminectomy was extended for at least 1 vertebra rostrally and caudally until the cord appeared normal under the microscope. The MR

images proved to be very useful by offering accurate information on the total length of lesion, depicting rostrocaudal cyst extension and stump degeneration. Combining peroperative and MRI information, the spinal cord was retransected on each side of the lesion site and a relatively long midthoracic segment that contained the cyst at its center was resected en bloc. The length of this segment ranged from 10-15 mm. If necessary, the stumps were further trimmed until white/gray discrimination was optimal. As a rule, the distal stump appeared thinner than in the unlesioned cord. However, the grafting step was possible. Again the tips of the peripheral nerve grafts were carefully inserted into the rostral and caudal gray matter instead of simply apposing them and letting them imbibe onto the stump surface. The aims were to better stabilize the grafts, minimize the risk of future detachment and minimize glial proliferation at the cord-nerve interface. Again the autologous grafts and stumps were embedded in fibrin glue containing acidic FGF and the spinal column was fixed with a metal loop. The duration of this repair procedure was about 5 h (Fraidakis *et al.*, 2004).

Chronic sham surgery (IV)

In the chronic sham procedure, everything was done as in the chronic repair protocol, except for the removal of intercostal nerve segments, the grafting of these segments and the spinal column fixation (Fraidakis *et al.*, 2004).

Proximal cord retransection (IV)

One acutely repaired animal that exhibited locomotor recovery during extensive behavioral follow-up of until 8 months after the repair procedure, had its spinal cord retransected 5mm rostral to the engrafted area. The aim was to establish the contribution of regenerated descending pathways to the locomotor recovery. One might have transected the peripheral nerve grafts in order to prove the same point. However, the peripheral nerve grafts were more difficult to access inside the engrafted area within the scar and granulomatous tissue that had formed a cast on top of it and around the stabilizing metal loop. By transecting the proximal stump near the repair site one could directly test the role of long descending tracts in the observed functional recovery, without disturbing the repair site. If descending fibers were accountable for functional recovery they could only have accomplished that via the engrafted area and the peripheral nerve bridges, most likely, as conduits (Fraidakis *et al.*, 2004).

Facial nerve transection (III)

Mice received halothane anesthæsia. After dissection of the left facial nerve at the site of its exit from the stylomastoid foramen, a 3 mm long segment was resected and the wound was sutured. The duration of the operation was 20 min. The animals were sacrificed 3 weeks later with CO_2 and the brain stem was ecxtracted and quickly frozen

on dry ice before sectioning.

Spinal cord 'overhemisection' (V)

Mice from each genotype were chosen at random and operated on the same day by the same experimenter. Under general halothane anesthesia a lower thoracic laminectomy was performed at the level T9-T10 and the spinal cord with its meninges were lesioned by dorsal hemisection with the use of microscissors. The objective was to axotomize the corticospinal tracts without creating a wide gap between the cut surfaces. At the time of the lesion, 0.5 µl of a 5% solution of Fluorogold was injected at the injury site. The operation time was 30 min – 1 hour and the animals tolerated the operation well. Three weeks after the initial lesion, 0.2 µl of a 25% solution of Fluororuby was injected 3 mm distal to the hemisection. The Fluroruby was injected slowly into the distal cord and allowed to diffuse into the cord parenchyma. Two weeks later the mice were anesthetized and transcardially perfused with paraformaldehyde.

MRI EVALUATION (*II, IV*)

MRI recordings were performed using a Bruker Biospec Avance 47/40 spectrometer with a 4.7 Tesla field strength magnet of 40 cm bore diameter and equipped with a 12 cm inner diameter self-shielded gradient system (gradient strength 200mT/m, inductive rise time 80 µs, B-GA12). A commercially available double-tuned $^{1}H/^{31}P$ surface coil with a planar circular detection area 30 mm in diameter was used. All equipment was made by Bruker, Karlsruhe, Germany. The surface coil was fitted into a customized plexiglass rig placed in the center of the gradient system. The animals were placed on a separate rig in supine position on the top of the coil. For alignment, the rat was fixed on the rig, cranially with a teeth holder, caudally at the tail using tape and laterally using synthetic material. There was a 3x6 cm window in the rig and the rat was positioned with the thoracic spine over the window opening. Thus the back of the rat in the magnet was in direct contact with the surface coil. The rat-bearing rig could be moved over the coil for optimal positioning. Anesthesia was performed using halothane via a mask adapted to the mouth piece at the rig. To control the depth of the anesthesia, respiratory activity was monitored continuously. If necessary, halothane concentration was adjusted within the range of 1-1.5%. Body temperature was monitored and maintained at 37 ± 0.5 °C by a circulating temperature-controlled air stream around the body of the rat. For delivery of contrast agents, a 27 gauge needle was inserted into the tail vein and the needle assembly was fixed on the rig (Fraidakis *et al.*, 1998, 2004).

MRI sequences employed (II, IV)

The main sequence employed in Paper II was a standard Bruker implementation of *rapid acquisition with relaxation enhancement* imaging (RARE) (Hennig et al., 1986). Care was taken to use parameter combinations yielding short *echo times* (TE) allowing

shorter imaging time for the same *effective echo time* (TE_{eff}) by increasing the RARE-factor and use correspondingly fewer excitations. A standard Bruker implementation of the MSME experiment was used to obtain data used to calculate proton density and T2 maps of the spinal cord. Shortest possible echo time was used in the experiments to allow the acquisition of as many echoes with high signal to noise ratio as possible for best T2 calculations. Diffusion weighted images were obtained by standard multislice spin echo sequences with a pair of diffusion gradients added symmetrically around the refocusing pulse. Images were obtained with four different diffusion gradient strengths including zero gradient chosen to provide a reasonable attenuation range of the intensities in the voxels of interest. Maps of the *apparent diffusion constant* (ADC) were then calculated from the data (Stejskal and Tanner, 1965). Diffusion experiments were performed both with the diffusion gradient along (craniocaudally) and across (left-to-right) the spinal cord. T1 relaxation was estimated by correlating image contrast and intensities to different repetition times. The conclusions were also confirmed by an inversion recovery measurement performed by an inversion pulse followed by a SNAPSHOT FLASH gradient echo sequence allowing for segmented acquisition (Haase, 1990), i.e. acquisition of several phase encoding steps per magnetization inversion.

The sequence employed in *Paper IV* was as above a Bruker implementation of rapid acquisition with relaxation enhancement imaging (Hennig, 1986). Our first study showed that proton density weighted images of the rat spinal cord gave good contrast between gray and white matter and allowed the description of features of the degenerating spinal cord with considerable detail as well as the distinction of the spinal cord from the surrounding tissue (Fraidakis *et al.*, 1998). Therefore, proton density weighted images were acquired here to assess the transected spinal cords postoperatively. *Relaxation time* (TR) for these images was 2500 ms and TE was 35 ms. The RARE-factor used was eight. Generally, slice series with a slice thickness of 2 mm (interslice distance for sagittal and coronal images 2.2 – 2.5 mm and for axial images 4.5 mm) were first acquired in sagittal, coronal and axial direction for gross orientation. The average distance between two intervertebral discs at the thoracic level in rat measures 4.5 mm. Using an interslice distance of the same value, each image thus represented the spinal cord at another segment. Thereafter, serial thin sections (0.5 mm thickness, interslice distance for sagittal and coronal images = 0.65-0.8 mm, interslice distance for axial images 2.2 - 4.5 mm) placed in parallel or orthogonal to the spinal cord were produced if required to resolve more detail (Fraidakis *et al.*, 1998).

Images with contrast material (II)

0.1 ml 0.5 mmol/ml, gadolinium solution (Gadodiamid 287 mg/ml, Omniscan, Nycomed, Norway) was injected into the tail vein. Immediately after, the T1 sequence was started.

Localization of the lesion site (II, IV)

The level of the images in control rats and the level of the gap in spinal cord transected rats was localized using a RARE sequence (TR=2500 ms, TE=35 ms) to obtain serial proton density weighted 2 mm thick coronal images. After a first image taken at the lower thoracic level and identification of the most caudal rib, the rat was moved a defined distance such that the level of interest was placed into the isocenter and the level of the gap determined.

BEHAVIORAL EVALUATION (*II, IV, V*)

Functional recovery was evaluated and scored by two specially trained technicians, blinded to the animal groups, using the CBS, BBB, and a modified Tarlov scale (Tarlov, 1953; Tarlov *et al.*, 1954; Eidelberg, 1975; Gale *et al.*, 1985; Kunkel-Bagden *et al.*, 1992; Basso *et al.*, 1995; Metz *et al.*, 2000). Scorings were also made on two separate tests, the Climbing test and Gait assessment test scale (Gale *et al.*, 1985; Kunkel-Bagden *et al.*, 1992). Two novel behavioral tests, the Bipedal Test (BT) and the Head-scratch test were also developed. Long video recordings were made from the behavioral evaluations of all repaired and several control animals. Hyperreflexia and spasticity are well-known symptoms in higher motor neuron disorders, where the inhibitory input from supraspinal neurons at segmental reflex circuits is abolished, and therefore SCI leads to spastic paralysis after an intial period of flaccid paralysis. Spasticity is a confounder in behavioral evaluations of SCI animals, especially if the animals are ailing with UTIs or pressure sores (Ko *et al.*, 1999; Hiersemenzel *et al.*, 2000). In paraplegic animals spasms are sudden muscular contactions of short duration, occuring as trains of generalized spastic fits, involving the lower half of the body, with the muscular contraction progressing from more proximal to more distal muscle groups, and the hindlimbs stretching in hyperextension until the spasm runs its course and the hindlimbs return to the prespastic state of flaccid hyperextension. Care was taken to totally exclude such sources of false positive results. Spastic movements are easy to recognise and whenever they occured they were noted but not rated. In the rare cases that an UTI was spotted the affected animal was not behaviorally evaluated (Ko *et al.*, 1999; Hiersemenzel *et al.*, 2000; Fraidakis *et al.*, 1998, 2004).

Combined behavioral score (II, IV)

The CBS measures functional deficit, ranges from 100 deficit (total paraplegia) to 0 (normal), and consists of tasks testing motor, sensory and proprioceptive skills (Gale *et al.*, 1985; Kunkel-Bagden *et al.*, 1992). More specifically, the tasks are, motor score (0-45), toe spread (0-5), righting reflex (0-15), extension withdrawal (0-5), contact placing reflex (0-5), inclined plane test (0-15), and swim test (0-10). The hot plate test was not included in the employed CBS. In those tasks where the two hindlimbs should be observed separately, their average score was calculated. The inclined plane test

included in the CBS is equivalent to the Rivlin and Tator score, which records the maximum angle at which the rat could remain on an inclined plane for 5s. Two other tasks were scored separately. These were the Climbing test and the Gait assessment test. In the former, an evaluation of the use of the hindlimbs while climbing at an angle of 28° was made and each hindlimb was scored separately on the 0-5 scale used for the CBS motor score. In the latter, a gross assessment of the hindlimb locomotion and interlimb coordination on an open field was made and a single score from 0-6 was given (0-no hindlimb movements, 1-hopping or kicking with hindlimbs, no weight bearing, 2-walking with one hindlimb, 3-knee walking, 4-walking on one side or walking with heels, 5-ataxic gait, 6-normal gait).

Contact placing reflex (II, IV)

This test is included in the CBS and scores a hindlimb plantar placing reflex elicited by a light touch on the dorsal surface of the paw. It is a sensitive test of sensorimotor recovery and viewed believed to be CST dependent (Donatelle, 1977).

Basso-Beattie-Bresnahan score (II, IV, V)

In *Papers II* and *III*, the animals were scored moving spontaneously for a minimum of 5 min on an open field using the *Basso-Beattie-Bresnahan* (BBB) scale (0-21) (Basso *et al.*, 1995, 1996; Metz *et al.*, 2000). The BBB scale is commonly used in experimental rat SCI and is particularly useful for evaluating rats with contusion injuries, where a finer locomotor evaluation of animals with partial deficits is necessary. Despite the different nature of our experimental model, we nonetheless used the BBB scale to monitor transected and repaired animals in order to enable comparisons with other studies and add detail to the modified Tarlov scale (Tarlov *et al.*, 1953; Tarlov *et al.*, 1954).

The BBB scale was also applied without modification in the transgenic mouse studies (*Paper V*). Every animal was scored on the BBB scale (0-21) (Basso *et al.*, 1995, 1996) 6 times by specially trained technicians, blinded to the animal groups, within the month between the FG injection (injury time) and the FR injection, the timepoints being postinjury days 1, 2, 4, 7, 14, 21, 28. The animals were scored individually while moving spontaneously for a minimum of 5 min on an open field. The BBB scale is being increasingly used in experimentation with mice and was used in order to enable comparisons with other studies (Ma *et al.*, 2001; Steward *et al.*, 1999).

Bipedal test (IV)

This was a novel test, performed on a flat, horizontal surface. Using an index finger as weight-support under the animal's forelimbs, the tested animal was aided to an erect

position with its hindpaws in plantar contact with the horizontal surface. During testing there were no other points of contact between the examiner and the tested animal. The animals readily accepted this position. The examiner then slowly and horizontally led the animal forward and the movements of the hindlimbs and hindpaws were observed. We scored the animals on a 0 to 10 scale, with the following score representation (Fraidakis *et al.*, 2004):

0 Hindpaws dragged in hyperextension, dorsal surface of the hindpaws and knuckles in contact with ground, no weight support

1 Occasional plantar hindpaw placement/positioning, no stepping movements, no hindpaw lift-off, no weight support

2 Frequent plantar hindpaw placement/positioning, no stepping movements, no hindpaw lift-off, no weight support

3 Consistent paw positioning, no stepping movements, no hindpaw lift-off, no weight support

4 Occasional uncoordinated stepping, no hindpaw lift-off, partial weight support

5 Frequent uncoordinated stepping, no hindpaw lift-off, partial weight support

6 Occasional coordinated stepping, no hindpaw lift-off, partial weight support

7 Frequent coordinated stepping, no hindpaw lift-off, partial weight support

8 Consistent coordinated stepping, occasional lift-off of one or both hindpaws, good weight support

9 Consistent coordinated stepping, frequent hindpaw lift-off, good weight support

10 Consistent coordinated stepping, consistent hindpaw lift-off, good weight support.

Normal animals could not be tested with the Bipedal test, so the maximum score does not correspond to a normal animal but to the maximum possible score attainable by a repaired paraplegic animal in terms of locomotor capacity.

Tail motility (IV)

In some repaired animals tail lifting, flexing and whipping movements were occasionally observed, and were documented and videorecorded. The tail movements

were smooth and appeared not to be of a spastic nature. The lower trunk was not undergoing a spasm at the time, neither during the course of the behavioral testing event. During the testing event the movements were occuring for extended periods and invariably appeared simultaneously with ongoing hindlimb stepping. It is noteworthy that tail motility correlated with high overall behavioral performance among the repaired animals (Fraidakis *et al.*, 2004).

Head-scratch test (IV)

A very interesting observation was made in some repaired animals after they had reached a certain degree of motor recovery. The animals while on the usual flat surface with plantar placement of the paws and weight bearing position, were scratched gently on the head by the observer. Immediately they initiated nonspastic stepping, often alternately rythmic, bilateral hindlimb movements, with flexion and extension in all 3 major joints, and occasional tail movements. The animals remained on their stationary position during the test, being able to support their body weight while keeping their balance. There was a short lag between the initiation of head-scratching and the hindlimb movements. Sometimes the hindlimb movements persisted for a short while after the head scratching had stopped. Video recordings of this serendipitous finding were made. The test was always negative in control (transection only or sham) animals (Fraidakis *et al.*, 2004).

HISTOLOGICAL EVALUATION (*I-V*)

Detection of radioactivity and autoradiography (I)

Animals implanted intraocularly and intraspinally with radiolabeled GDNF containing fibrin glue were sacrificed with ether anesthæsia and decapitation. From the animals that received intraocular implants 10µl acqueous humor was sampled from each eyeball and the fibrin glue ball was removed and collected in a test tube. Each iris was stretch-prepared as a whole-mount and air-dried (Malmfors *et al.*, 1965). The radioactivity of the fibrin glue balls, irides and acqueous humor was measured with a mini-assay gamma counter (type 6-20, Mini-instrument). From the animals that received the intraspinal implant the spinal cord was extracted and divided into three parts, a cervical, thoracic and lumbar, which were immediately freeze-dried. Autoradiography of the dried irides and cords was evaluated with phosphoimaging (Fuji), the slides dipped in emulsion (Kodak NTB-2), and developed (Kodak D19 developer) after 6 weeks' exposure.

Spinal cord immunohistochemistry in rat (II, IV)

In *Papers II* and *III*, animals were deeply anæsthetized with sodium pentobarbital (Mebumal 40 mg/kg i.p.) and perfused via the ascending aorta with 50 ml calcium free

tyrode solution followed by 300 ml formalin-picric acid mixture (4% paraformaldehyde, 0.4% picric acid in 0.16 M phosphate buffer, pH 7.4). The CNS (spinal cord and brain) was extracted en bloc and kept in the same formalin-picric acid solution used for perfusion. After equilibration in 10% sucrose solution (changed thrice the first day and once thereafter for a week), the spinal cords were sectioned with a cryostat and processed for indirect immunohistochemistry. Because it was impossible to extract the spinal cord of repaired animals without damaging the site of repair (*Paper III*), a protocol allowing sectioning of bone was also used (Hil and Elde, 1990). After performing the above perfusion protocol, the entire spinal column was immersed in PVP solution (2.5% polyvinylpyrrolidone-10, 2.5% polyvinylpyrrolido-ne-360, 5% sucrose in 0.1 phosphate buffer) for about 6 weeks in order for the bony tissues to soften enough so that the bony canal containing the spinal cord could be sectioned *en bloc* and processed. We used antibodies against *neurofilament* (NF, Dahl and Bignami, 1:500), serotonin (5-HT, Pel-Freeze, 1:500), tyrosine hydroxylase (TH, Pel-Freeze, 1:200) and *glial fibrillary acidic protein* (GFAP, Sigma, 1:100). The secondary antibodies were labeled with FITC. Slides were examined with an epifluorescence microscope. Slides were also stained with hæmtoxylin-eosin and examined under the light microscope.

Brain stem immunohistochemistry in mice (III)

The animals were sacrificed with CO_2 3 weeks after facial nerve transection and the brain stem was extracted and quickly frozen on dry ice before sectioning. Cryostat 12 μm transverse sections were fixed in 100% acetone at –20°C for 30 seconds, transferred into ice-cold 4% phosphate-buffered paraformaldehyde for 30 seconds, washed in 0,01M PBS, preincubated with 2% donkey serum for 30 min and incubated overnight at 4°C with the primary antisera (goat anti-GFAP 1:50 Santa Cruz Biotechnology, and rat antiMHC class I 1:50, Peninsula, CA) diluted in PBS with 1% bovine serum albumin. After several rinses in PBS, the sections were incubated with Cy3-conjugated donkey anti-rat serum or biotinylated donkey anti-goat serum (Jackson Immunoresearch, Wets Grove, PA) for 30 min at 37°C. Avidin-biotin complex conjugated horseradish peroxidase (Elite PK 6100, Vector Laboratories, CA) and amino-ethyl carbazol (Sigma, St. Loius, MO) were used to visualize the biotinylated bound antibodies. Control slides were incubated with preimmune rat/goat sera, and the first antibody step was either omitted or the slides incubated with unrelated rat/goat polyclonal antisera.

In situ hybridization and image analysis (III)

Synthetic oligonucleotides complementary to rat growth associated protein-43 (GAP-43; nt 70-117, accession number M16228), mouse glial fibrillary acidic protein (GFAP; nt 7863-7910, accession mumber X02801) and mouse β2-microglobulin (β2-m; nt 304-351, X01838) mRNAs were used as described previously (Dagerlind et al., 1992). The oligoprobes were labeled at the 3'-end with [^{35}S]-α-ATP (NEN, Boston, MA) using

terminal deoxyribonucleotidyl transferase (Pharmacia, Uppsala, Sweden), left to hybridize *in situ* without pretreatment overnight at 42°C, after which the tissue sections were washed repeatedly in 1 x SCC at 58°C and dehydrated in ethanol. Radioactivity was detected either by autoradiographic film (β-max, Amersham) or emulsion dipping (NTB2 nuclear track emulsion; Kodak Rochester, NY). As a control, cold probe in 20-fold excess was added in the hybridization mixture. In tissue sections treated with this mixture only background signal was seen. A computer-based image analysis system was used to semiquantitatively measure in a blind fashion the hybridization signals from all probes employed (Lidman *et al.*, 2002). Five brain stem sections per animal from all animals were analyzed and the equipment (computer and camera) settings were identical throughout.

Single and double labeling studies (V)

Cryostat coronal sections (14 μm thick) through the brain were examined under a fluorescence microsope and counts of all labeled (single- or doublelabeled) were made from all tissue sections. The brain sections were divided into two regions for differential measurements in cortex and subcortical regions. Sagittal sections of the spinal cord were also taken from randomly selected animals to examine the Fluorogold and Fluororuby injection areas.

STATISTICAL EVALUATION (*IV, V*)

Kolmogorov-Smirnov and Spearman tests (Paper IV)

Groups (acute treatment, late treatment, sham and transection-only) were compared using the Kolmogorov-Smirnov two sample test with two-sided probabilities. Ns= non significant, * = $p<0.05$, **=$p<0.01$, ***=$p<0.001$. The mean and standard deviation of BBB in 21 control (transection-only) animals was calculated for every assessment event seperately and a critical value (CV) was calculated as mean + 3 SD (99 % confidence interval), referred to as CV_{BBB} and was: $CV_{BBB} = 0.61 \pm 0.71$, min=0, max=2.3, n=15 (n: behavioral evaluation events). The maximal critical value for BBB was selected as corrected critical value CCV_{BBB}, with >99% of the data points of the controls at every time point below that value. Scores from acute treatment or late treatment animals above the CCV were regarded as improved. BBB and Bipedal Test scores were correlated separately in acute and late treatment using the Spearman test and the Spearman correlation coefficients were 0.94 (n=16) and 0.83 (n=15), respectively.

Linear Mixed Effects Model (V)

A Linear Mixed Effects Model (LMEM) was used to evaluate if the independent variables *brain region* and *animal group* had an effect on the ratio ”sum of double-labeled neurons/sum of FG labeled (hence axotomized) neurons”. The LMEM

accommodates the *repeated measurements* by the use of a covariance/correlation matrix. The *Akaike's information criteria* suggested that a *compound symmetry* covariance structure was the most appropriate for modeling the dependence of the data (Brown *et al.*, 1999). The *compound symmetry* structure assumes that the covariance/correlation is equal for every pair of *repeated measurements* and that the variance is equal in each brain region. A *square root transformation* of the outcome variable was made to satisfy the model assumption of normally distributed *residuals*. The *Student's t-test* was performed for comparing the whole brain labeling ratios of the different genotypes. All the pair-wise comparisons were adjusted using simulated significance level corrections and p-values less than 0.05 were considered statistically significant. A LMEM was also used to assess the progression of the BBB scores over time. One great advantage with the LMEM approach is the possibility to include time as a continuous variable and fitting curvilinear relationships (Fitzmaurice *et al.*, 2004). This is done using lower order polynomials. Thus, time was treated as continuous and *goodness of fit* was evaluated by plotting the observed values against the predicted. We used an *autoregressive covariance structure* assuming that the correlation between pairs of repeated measurements decreases logarithmically with increased distance between timepoints. In other words, this means that timepoins close to each other are assumed to have a stronger dependence than timepoints further apart. Once again *Akaike's information criteria* were used to find an appropriate covariance/correlation structure. The LMEM has certain features which make it suitable for analysing data that arise from a *longitudinal*- and *repeated measurements* design. Due to its flexibility, the LMEM has in most circumstances replaced the *repeated measurements ANOVA* approach. The analyses were performed using the SAS software, version 9.1.3 (PROC mixed, SAS software, Inc., Cary, North Carolina). The Statistica software version 7.1 (Statsoft, Inc., Tulsa, USA) was used for all descriptive statistics presented in the paper.

RESULTS

[^{125}I]-GDNF CONCENTRATION IN FIBRIN GLUE (*I*)

Firstly, the relationship between different concentrations of radioactive GDNF in the fibrinogen solution and the measured radioactivity in the fibrin glue ball was evaluated. There was a strong linear correlation between the [^{125}I]-GDNF concentration (six different concentrations were tested from 200 nM to 0.004 nM -with 0 nM as control) in the fibrin glue preparation and the detected radioactivity ($r = 0.992$). Secondly, to examine the effect of rinsing during preparation of the fibrin glue balls, the variability of detected radioactivity for a single [^{125}I]-GDNF concentration, 4 nM (a concentration relevant for the *in vivo* studies), was estimated from 16 samples processed in the same manner. The mean bound radioactivity was 71262 ± 2710 CPM and accounted for 89.8% of the mean initial count of the unembedded [^{125}I]-GDNF.

INTRAOCULAR IMPLANTATION OF FIBRIN GLUE/[^{125}I]-GDNF (*I*)

The implanted fibrin glue balls could still be identified in the anterior chamber two weeks after implantation, though a gradual decrease in size occured with time. Radioactivity detected with the gamma counter was significantly higher in the group that received radiolabeled GDNF as compared to the control group up to 7 days postimplantation. However, when total radioactivity, counted from both iris and acqueous humor, was compared, a significant difference between the two groups persisted up to 14 days postimplantation. The differences in radioactivity counts from the acqueous humor of the two groups were not significant at all postimplantation time points. In phosphoimages of whole-mounted irides, radioactivity in animals that received the radiolabeled GDNF was initially concentrated at the site of implantation and with time gradually decreased in intensity and dissipated in the surrounding iris tissue but was still detectable at 3 weeks postimplantation. Under the bright field microscope the concentrated radioactivity seen initially (6 hours) had divided into discrete foci by 24 hours but was still visible as numerous radioactive spots at 21 days postimplantation with autoradiography.

INTRASPINAL IMPLANTATION OF FIBRIN GLUE/[^{125}I]-GDNF (*I*)

The radioactivity detected by the gamma counter from the thoracic segment of the spinal cord was significantly higher in the group that received the fibrin glue balls containing radiolabeled GDNF at all time points examined up to 14 days postimplantation. No statistically significant difference was detected between the radioactivity counted from the other segments (cervical, lumbar) of the radiolabeled GDNF group and the cord segments of the control group. Microscopically, the fibrin

glue balls were soon infiltrated by glial and blood-borne cells, mostly macrophages, turned into a multiseptate mass by 2 days postimplantation and gradually diminished in size by 1 and 2 weeks postimplantation. In those animals that received [^{125}I]-GDNF, radioactive foci decreased in size over time but were still visible though sparse at 14 days postimplantation at the edges of the remnant fibrin glue mass. Radioactive foci were only seen within the fibrin glue.

MRI OF NORMAL SPINAL CORD (*II*)

Gray matter and white matter were easily discerned in axial, coronal and sagittal images. An infield resolution of 156 μm per pixel was achieved using a field-of-view (FOV) of 4 cm and an imaging matrix (MTX) of 256 x 256 pixels. In 0.5 mm thick midsagittal sections the gray matter of lamina X and the central canal could be followed for several segments as a hyperintense signal in proton density weighted images. In T2 weighted images gray matter structures became hypointense. T2 weighting accentuated cerebrospinal fluid (CSF) in the subarachnoid space dorsally and ventrally. Moreover, the central canal became visible as a discrete 1 to 2 pixels thick hyperintense band. In T1 weighted images contrast between gray and white matter was weak. In proton density weighted 0.5 mm thick coronal sections gray matter of the dorsal or ventral horn gave a hyperintense signal apparent as two paracentral parallel strands that could be followed for varying distances depending on the thoracic flexure. In midsagittal coronary sections laminae VI, VII and X fused to one central though laterally accentuated hyperintense signal. The central canal in proton weighted images was not visible but became recognized in T2 weighted images as a hyperintense band where the gray matter structures gave a paracentral hypointense signal.

Calculation of proton density maps and T2 maps from data obtained by the MSME technique (TR=2124 ms, TE=8, 16, ...128 ms; FOV 4 cm, MTX 256) were employed to obtain more structural details in axial sections. This revealed that the higher proton density in gray matter greatly contributes to gray matter/white matter contrast and that T2 values in gray matter in rat spinal cord were apparently lower than those in white matter. To improve in-plane resolution, axial images were performed using a 512x512 matrix (TR = 2774 ms, TE = 62 ms or 203 ms for proton density and T2 weighting, respectively) in 3 mm thick slices. In these images, CSF in the central canal and the subarachnoid space were apparent as strong hyperintense signals both in proton density weighted and in T2 weighted images. Gray matter was clearly discerned, the H-shape of the gray matter was visualized and the region of substantia gelatinosa was defined as the most hyperintense gray matter structure in proton density weighted images.

MRI OF THE LESIONED SPINAL CORD (*II*)

Serial sagittal and coronal slices of 0.5 mm thickness were employed to describe the spinal cord at time points between 2 and 6 months after complete transection. Briefly,

the spinal cord stumps in all cases had retracted and the created gap inbetween was filled with scar tissue. The rostral and caudal stump, although to a different extent, showed tapering towards the gap, which was interpreted as a sign of cord degeneration. The rostral stump generally ended in a cyst. There was no obvious difference between animals 2 or 6 months postransection. The center of the gap was in 11 of 15 cases at the thoracic level Th 9/Th10 with a variation of 0.5-1 segments for the rest of the cases.

The rostral stump in all transected animals and to a lesser extent the caudal stump, ended in a large hyperintense area. In T2 weighted images this bright region was isointense with CSF in the subarachnoid space and became hypointense in T1 weighted images, which together with the fact that terminal cysts were found in histological slices, implied that this signal corresponded to fluid. The cyst could be in a central position as well as dorsally or ventrally in the spinal cord. Often the signal of the cyst was in continuity with the subarachnoidal CSF signal further rostrally. In histological slices it was seen that the central cyst could be continuous with the central canal. In 3 of 20 animals an intramedullary microcyst rostrally or caudally was also seen.

In all animals, scar tissue, which was heterogeneous in signal intensity, was seen extending between the rostral and caudal stump. In many cases scar tissue could be followed from the rostral to the distal stump. In T1 weighted images a rapid and strong increase of signal intensity was seen after intravenous gadolinium (Gd) injection followed by a slower washout process. The time course of Gd filling was similar to the time course of signal intensity increase in well vascularised tissues such as muscle tissue, which suggested that the scar was rich in blood vessels. Indeed this could be verified in histological sections. Within the spinal cord there was no significant increase of signal intensity following Gd injection, suggesting an intact blood brain barrier. Qualitative information of structural changes and differences between the caudal and the rostral stumps was obtained in serial axial sections taken at half-segment intervals. In the rostral stump the gray matter structure was disturbed at several levels whereas in the distal stump the gray matter contour was better preserved even relatively close to the end of the stump. As for quantitative measurements, the anterioposterior diameter of the normal spinal cord was assessed in 0.5 mm thick central sagital MR images in 6 control rats at levels between Th7 and Th11 measuring at the level of each vertebra midpoint and intervertebral disc. The mean anteroposterior diameter was found to be 2.1±0.1 mm (n=52) and appeared to be constant over this distance. To compare this value with measurements in spinal cord transected rats, the level of reference was defined as the intervertebral level Th 9/10. The average interdisc distance (distance between the centers of two consecutive intervertebral discs) in control rats at this level was 4.56 ± 0.21 mm, (n= 29). In transected cords the mean gap size defined as the distance between identifiable cord tissue was 4.9 ± 1.5 mm. To quantify stump thinning and tapering as a measure of cord degeneration, the anteroposterior diameter of the spinal cord was measured at different intervertebral and midvertebral levels and the values were given as a function of the distance from the center of the gap. In lesioned animals tapering of the stumps stretched from 2.5 to 10 mm away from the stump edge and in all cases anteroposterior diameter normalized only 1 cm away from the gap, as a

result of secondary degeneration. This information was of importance in the chronic repair SCI study.

Diffusion measurements were further utilized to characterize architectural changes seen in axial sections in transsected spinal cord, whereby the *diffusion anisotropy coefficient* $Q_{z/x}$ (=ADC_z/ADC_x) was calculated in serial axial 1 mm thick sections obtained at half-segment intervals in the rostral and caudal stump. $Q_{z/x}$ was then plotted against the distance from the gap. This revealed that changes in the diffusion anisotropy coefficient were present as far as 6.6 mm away from the gap in both directions, suggesting degeneration in fiber tracts and myelin within this region

MRI WORKUP BEFORE SPINAL CORD REPAIR (*IV*)

Magnetic resonance imaging allowed morphological assessment of the transected spinal cord in vivo. Complete transection led to the expected degenerative changes of the spinal cord stumps as previously described in *Paper II*. Thin serial MRI sections allowed identification of gray and white matter and visualization of cyst formation, information that was necessary for the upcoming repair surgery. MRI demonstrated that a gap was always seen separating the spinal cord stumps spinal and cord cysts always developed.

BEHAVIORAL EVALUATION OF RATS WITH SPINAL CORD LESIONS OR REPAIR (*II, IV*)

In all transection-only and acute sham animals, the initial injury resulted in complete hindlimb paralysis. In the acute SCI treatment group the early postrepair period of 4-6 weeks was indistinguishable from the postoperative period of the control (sham or transection-only) groups in terms of functional correlates. On the contrary, by 8 weeks postrepair, acute treatment animals had begun to exhibit signs of sensorimotor recovery in most behavioral tests. Likewise, in the chronic SCI treatment group animals showed signs of sensorimotor recovery in most behavioral tests by the 8^{th} week postrepair, while this was not the case for the control animals. At 3 months postrepair, the difference between treatment and control groups was significant (P=0.01 or P=0.001) for most of the tests. This difference was present in most tests at 8 months postrepair. There were no significant differences in behavioral performance between the acute and chronic SCI groups.

It is noteworthy that all treated animals performed better than control animals in the contact placing (CP) test. The return of this reflex is assumed to require regeneration of both ascending sensory pathways and the corticospinal tract (Donatelle, 1977; Reier and Houlé, 1988; Kalderon and Fuks, 1996). The functional improvement measured by the CP test was bilateral in 5 out of 6 acute treatment and 5 out of 6 late treatment animals. None of the control animals had a CP score above zero at any timepoint. While there

was good correlation between the CP and BBB scores in both treatment groups, animals showed signs of the CP reflex only after a BBB threshold score of 3 had been reached. This lag can be explained by the dependence of the CP reflex on both somatosensory afferent input and efferent input, probably including corticospinal tract participation. There was also good correlation between the bipedal test (BT) and BBB scores of the treatment groups. Control animals rarely achieved a BT score higher than 0.

For most of the behavioral follow-up period, all animals in the acute (irrespective of the use or not of acidic FGF in the treatment protocol) or late (irrespective of whether the repair surgery took place at 2, 4 or 8 months postinjury) treatment groups scored higher than 3 standard deviations above the mean best scores of the control animals in the BBB test. Retransection immediately abolished all evidence of regained sensorimotor capacity in one acute treatment animal (no acidic FGF) retransected at 8 months postrepair. The retransection procedure left the repair site and the distal stump unscathed, therefore the loss of sensorimotor recovery appeared directly related to the relesioning of descending pathways at the level of the proximal stump.

The average best scores of the treatment groups in each test were higher than the average best scores of the controls. The timepoints when the acute and late treatment animals reached their top scores in each behavioral task fell within the time windows 3-5 and 4-6 months, respectively.

In 2 out of 6 acute and 1 out of 6 late treatment animals, simultaneously with ongoing hindlimb stepping (and no lower trunk spasm), smooth, non-spastic tail movements (lifting, bending, whipping and circumduction) were occasionally observed. Tail motility correlated with high overall behavioral performance and was not observed in control animals. The Head-scratch test was occasionally positive in 2 out of 6 acute and 2 out of 6 late treatment animals, but always negative in control animals. It correlated with high overall performance. The BT performance of treated animals correlated positively with their respective BBB scores over time. Acute treatment and late treatment animals were tested separately and the Spearman correlation coefficients were 0.91 (n=16) and 0.83 (n=15), respectively.

BEHAVIORAL EVALUATION OF MICE WITH SPINAL CORD LESIONS (*V*)

There was no observable difference in behavior between the different mouse strains and genotypes before the operation. After the overhemisection cord injury, on postinjury day 1, all animals achieved a score between 7 and 14 as was expected due to the incompleteness of the injury. No statistically significant difference in the course and peak of behavioral recovery was found between the KO and WT animal groups of the same strain. However, a statistically significant difference ($p<0,05$) in the level of behavioral recovery over time and peak scores after the cord injury was noted between genotypes from different strains and between the two different mouse strains, C57BL/6J and BALB/c. The C57BL/6J mice had consistently higher scores on all testing events.

The course of the behavioral recovery over time for the BALB/c animals roughly paralleled that for the C56BL/6J animals.

HISTOLOGICAL EVALUATION OF RATS WITH SPINAL CORD TRANSECTION OR REPAIR (*II, IV*)

Rats that had their spinal cords transected and then underwent MRI were finally sacrifized and perfused and the spinal cord sectioned in axial, sagittal or coronal planes to confirm the anatomical findings obtained with MRI techniques. This revealed similar distributions of gray and white matter as described above as well as the observation of stump thinning and degenerative changes towards the lesion site. Motoneurons with vacuoles were numerous in both the rostral and the caudal spinal cord and seen as far as 2 cm away from the lesion. At the terminal end a large cyst which in some cases was definitely continuous with the central canal was seen. The central canal was enlarged in the rostral compared to the caudal spinal cord. The cysts were often divided by septæ. Scar tissue between the distal and proximal stumps was rich in blood vessels. Other pathologies were seen more infrequently such as vertebral dislocation (4 of 20 animals) as a sign of vertebral instability, microcysts along the central canal (3 of 20 animals) and dorsal compression of the spinal cord by granuloma tissue in one out of 20 animals.

In the animals operated with the repair protocol, in cresyl violet stained sections, stump cavitation and neovascularization of the repair site were seen. Long peripheral nerve grafts were visible spanning the gap and were richly populated with Schwann cells as long as 16 months after repair. The viability of the grafts and the presence of regenerating axons within the grafts and in the distal stump were confirmed with IHC. Even as late as 16 months postrepair the peripheral nerve grafts were densely NF-positive and contained 5HT-immunoreactive fibers. 5HT-fibers were also found in gray matter in the caudal stump of all histologically evaluated repaired animals. 5HT-fibers were observed as far as 2-3 mm (and occasionally longer) into the distal gray matter and were not encountered in white matter. In control animals 5HT-immunoreactive fibers were seen in the proximal stump but not in the distal stump.

HISTOLOGICAL EVALUATION IN MICE WITH FACIAL NERVE LESIONS (*III*)

Immunohistochemistry for MHC class I in all genotypes of C57BL/6J and BALB/c mice showed only weak staining in the unlesioned side, whereas increased labeling was present throughout the facial nerve nucleus on the side of the facial nerve transection. Thus, no intrastrain difference was evident in MHC class I immunolabeling in BALB/c and C57BL/6J mice. There was no intrastrain difference in MHC class I immunolabeling in WT 129/SvJ and its IFN-$\gamma R^{-/-}$ KO but contrariwise to the other strains (BALB/c and C57BL/6J) they both showed very weak axotomy-induced up-regulation of MHC class I antigens. A similar patttern could be seen in GFAP

immunolabeling, with increased immunopositivity in the facial nucleus of the lesioned side in all BALBc and C57BL/6J genotypes but low immunopositivity in the 129/SvJ genotypes. Therefore, an interstrain, but not intrastrain, difference was seen by IHC in MHC class I and GFAP upregulation in the facial nerve nucleus postaxotomy between BALB/c and C57BL/6J (intense MHC class I immunopositivity) and 129/SvJ (weak MHC class I immunopositivity) mice.

In situ hybridization showed highly increased GAP-43 expression in axotomized motoneurons 3 weeks postaxotomy without interstrain or intrastrain difference in GAP-43 expression. On the contrary an interstrain, but not intrastrain, difference was seen in β2-microglobulin expression in both neurons and glial cells in the lesioned side 3 weeks postaxotomy, with the BALB/c and C57BL/6J genotypes exhibiting strong induction of β2-microglobulin mRNA while the 129/SvJ showed very weak induction. Grain density, as measured by computer-based analysis, was increased 25-35 times in animals on C57BL/6J background (axotomy vs nonaxotomy side) while only 5-7 times in animals on 129/SvJ background. The unlesioned facial nucleus showed very weak β2-microglobulin expression in all animals irrespective of genotype and background. Similarly, GFAP mRNA expression was upregulated in astrocytes in axotomized facial nucleus in animals of BALB/c and C57BL/6J backround without an intrastrain difference while the 129/SvJ strains showed a much weaker response. Grain density measurements revealed a 12-16 times increase (axotomized versus nonaxotomized facial nerve nucleus) in C57BL/6J animals compared with 3-5 times in 129/SvJ animals.

HISTOLOGICAL EVALUATION IN KO MICE WITH SCI (*V*)

Very few double-labeled and FR-single-labeled cells were seen in the cortical layers that project to the spinal cord compared to the FG labeled neurons (usually less than 0.01% of the FG neurons were double-labeled). Many more double-labeled neurons were found subcortically (usually less than 0.1% of the FG neurons). Nevertheless, the presence of double-labeled neuronal bodies in the brain may signify axonal regeneration below the level of hemisection. By Student's t-test, a statistically significant difference ($p<0.05$) was found between $STAT6^{-/-}$ and its BALB/c WT ($p=0.0176$), and between C57BL/6J WT (genotype $TNF\alpha^{+/+}$) and BALB/c WT ($p=0.0155$), for whole brain measurements, i.e. when the ratio of double-labeled/FG-labeled was calculated for both cortical and subcortical neurons. There was also a non-significant difference between $TNF\alpha^{-/-}$ and its C57BL/6J WT, with the mice lacking TNFα exhibiting higher whole brain labeling ratio.

DISCUSSION:

HISTORICAL PART (PAPER VI)

The conundrum of axon regeneration failure in the CNS is one of the most fascinating in neuroscience (Zurn and Bandtlow, 2006). It has intrigued neuroscientists since the early days of neurohistology in the 19th century, in the same way the general question of tissue regeneration tantalized the first biologists, and continues to do so in the era of functional genomics well into the 21st century (Goss, 1969; Dinsmore, 1991). Dogmas that had dominated the field for decades have finally crumbled unceremoniously under the burden of technologically advanced experimentation during the 20th century, but the "old dogma of irregenerability of central paths", as Cajal liked to call it, just keeps growing older (Cajal, 1928). For example, it was long believed that, unlike most cell types in the body, neurons are not renewable before it was finally established in the 1990's that neurogenesis does occur in vertebrates thanks to a pool of adult stem cells in the CNS (Reynolds and Weiss, 1992; Eriksson *et al.*, 1998). However, the "unimpeachable dogma" of the irregenerability of central axons still remains largely unfalsifiable. Cajal and other contemporaries had studiously observed and fastidiously described the degeneration and regeneration phenomena following various lesion models of CNS axons, but despite witnessing isolated regenerative events within grey or white matter and glial or mesenchymal scar tissue, they unequivocally, inevitably and correctly concluded not only that PNS was superior to CNS in its regeneration capacity but that the CNS regeneration incapacity was also age-dependent (Cajal, 1928). Different hypotheses have been enunciated concerning the phylogenetic, ontogenetic or physiological reasons for this inability of the CNS to restitute itself, and currently the dominant trend is to combine nondivergent views into a syncretic theory. And while there has been no shortage of ætiological hypotheses since unanimity in the scientific community is seldom attainable, novel therapeutic approaches have been proposed with an extraordinary pluralism during the last 30 years.

One of the central ætiological hypotheses for the inability of the CNS neurons to regenerate their axons, that during the last 20 years has also led to exciting experimental results, is that concerning the inhibitory role of myelin components of the white matter (Xie and Zheng, 2008; Yang and Schnaar, 2008). The discovery of the inhibitory role of myelin on axonal regeneration after CNS injury had a profound effect in experimental SCI research because of its biological and methodological implications. Firstly, it provided a sound theoretical basis for the shortcomings of various experimental treatment approaches that were solely based on the *trophic deficit* hypothesis such as the many different tissue or cell transplantation or administration of growth factors protocols. For as long as the inhibitory influence of the myelin persists there is little chance for long-distance axonal regeneration in the hostile territory of white matter and thus little hope for significant functional improvement. Secondly, it led to more sophisticated experimental treatment approaches that would now have to tackle this additional obstacle. Apart from the straightforward *antimyelin approaches* that are currently in boom and aim at intercepting the signaling chain upstream or downstream

of the neuronal receptors of the various inhibitors, other strategies received broader attention thanks to this shift of focus. We will mention two in particlular that were based on a solid scientific rationale and have been met with remarkable and reproducible results in the lab. The implanation of *olfactory ensheathing cells* (OECs) and the administration of *ECM degrading enzymes*.

It was known since the 70's that the sole CNS milieu of adult mammals where regeneration occured was the olfactory bulb but it was not until the mid-90's that SCI researchers began to investigate the possible usefulness of OEC engraftment (Graziadei and Monti Graziadei, 1978). The OECs are regional neuroglial elements with the extraordinary characteristic that they allow for regeneration by providing axons of olfactory neurons remyelination *and* guidance in a manner very similar to Schwann cells in the PNS (Raisman, 2001; Murrel *et al.*, 2005). Remarkably, it even appeared as if lesioned axons that had begun regeneration in an environment of grafted OECs, were able to leave the OEC area and continue regeneration in spinal cord tissue. Many works have verified that OECs grafted in the injured spinal cord have the potential of acting as remyelinating conduits for long-tract fibers that are notoriously difficult to regenerate, such as the *corticospinal tract*, and that this regeneration can lead to a significant functional recovery (Li *et al.*, 1997; Li *et al.*, 1998; Ramon-Cueto *et al.*, 1998; Ramon-Cueto *et al.*, 2000; Barnett *et al.*, 2000; Imaizumi *et al.*, 2000; Kato *et al.*, 2000; Lu *et al.*, 2001; Lu *et al.*, 2002; Lu and Ashwell, 2002; Keyvan-Fouladi *et al.*, 2003; Li *et al.*, 2003; Plant *et al.*, 2003; Sasaki *et al.*, 2004; Boyd *et al.*, 2005; Richter *et al.*, 2005; Ruitenberg *et al.*, 2005; Sasaki, M *et al.*, 2006). The positive experimental results eventually prompted the undertaking of initial clinical trials of OECs in human SCI (Huang *et al.*, 2003; Feron *et al.*, 2005; Dobkin *et al.*, 2006).

As for the role of the glial scar, it was known since the early days of SCI research to be an insurmountable obstacle for axonal regeneration (Cajal, 1928; Jones *et al.*, 2003; Silver and Miller, 2004). Attempts to remove the fibroglial cicatrix from the spinal cord wound would achieve nothing more than expand the wound and produce fresh fibroglial scar (Weidner *et al.*, 1999; Rasouli *et al.*, 1999; Zhang *et al.*, 2004). The *inhibitory myelin hypothesis* led to a paradigm shift in the perception of the fibroglial scar as not just a physical barrier to axonal regeneration but as yet another pool of chemical inhibitors that called for disinhibition (Busch and Silver, 2007). Beginning in the 1990s, SCI researchers attempted to dissolve the scar from within by using enzymes degrading fibroglial scar or extracellular matrix components, such as collagenase or chondroitinase (Stichel *et al.*, 1999b, 1999c, 1999d; Yick *et al.*, 2000; Moon *et al.*, 2000, 2001; Bradbury *et al.*, 2002). Success was not spectacular, but it was nonetheless better than expected and for the first time it was shown that one could cut a dent through the inpenetrable fibroglial scar, enough to allow for injured axons to regenerate (Bradbury *et al.*, 2002; Caggiano *et al.*, 2005; Klapka *et al.*, 2005; Schiwy *et al.*, 2009). Naturally, several experimenters also tried to combine ECM-degrading enzyme administration with other promising treatment approaches, e.g. OEC or Schwann cell transplantation, again with encouraging results (Chau *et al.*, 2004; Fouad *et al.*, 2005).

The *inhibitory white matter hypothesis* suggested that a peripheral nerve implantation SCI repair protocol would fail unless the regenerating axons were rerouted via the nerve bridges from their white matter origin to grey matter destinations. This ‘acute SCI microsurgical repair‘ experiment was carried out for the first time in Lars Olson’s lab and led to the histological demonstration of long-tract regeneration, including the CST, with significant functional recovery becoming feasible after complete transection SCI in the adult rat (Cheng *et al.*, 1996). When I was later assigned to extend the acute repair protocol in chronic experimental SCI, the same grey-to-white rerouting principle was the centerpiece of the modified microsurgical repair procedure.

Schwab and collaborators provided definitive proof for the *inhibitory white matter hypothesis*, developed a neutralizing monoclonal antibody against purified myelin inhibitors and cloned *NOGO* (Caroni and Schwab, 1988a, 1988b; Schwab, 2006). What is a less known historical fact in the scientific community, is that before Schwab and Thœnen, Martin Berry had suggested an inhibitory role for CNS myelin (Berry, 1982). However, Mary Filbin another neuroscientist working with myelin inhibitors, especially MAG, had suggested that the historical origins of the *inhibitory white matter hypothesis* go back to Cajal [“Cajal was the first person to suggest that white matter could block regeneration in the CNS”] (Filbin, 2003). Since that was a major break from accepted knowledge and since I had not encountered any such hint from Cajal in his *Degeneration and Regeneration*, I became sceptical to Filbin’s claim and decided to further investigate the matter.

Cajal himself provides the answer to the riddle and disproves Filbin’s claim (Cajal, 1928; Filbin, 2003). Cajal was generous when discussing the ideas and contributions of his predecessors and contemporaries and indeed it seems he held Lugaro in high esteem, no less because he was an early supporter of the *neurotropic hypothesis*. Cajal discusses Lugaro’s work in several places in his classic book but argues against Lugaro’s hypothesis of *negative neurotropism*. A careful reading of Lugaro’s few papers published in his native language in his own country betrays a clarity of thought and originality of mind and a firm belief in the role of neurotropic forces in the development and regeneration of the nervous system. One might speculate on the devastating effect of Cajal’s verdict, since Lugaro’s ‘inhibitory white matter hypothesis’ was never embraced or put to the test by any of his contemporaries but went instead into oblivion. Nevertheless, Lugaro’s PNS-to-CNS implantation experiments inspired Tello’s work that was crowned with more success than Lugaro’s (Tello, 1911). Indeed, it was Lugaro’s experiments, and not Tello’s that Le Gros Clark repeated in the 1940’s and Aguayo revived in the 80’s to kickstart anew the scientific field of CNS neuroregeneration, yet there is no reference to Lugaro’s work (Sugar and Gerard, 1940; Le Gros Clark, 1943; Richardson *et al.*, 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982; Friedman and Aguayo, 1985). As for Lugaro’s original hypothesis of white matter-induced *negative neurotropism*, it was eventually reincarnated and matured into a theory in the 80’s by Berry and Schwab but, alas, again without reference to Lugaro’s precedence.

One can only regret the precocity of Lugaro's insight that did not find an ally in the histological means of his era. Lugaro's achievement was more intellectual than experimental. His '*extended* neurotropic hypothesis' surpassed Cajal's in depth and insight but unfortunately did not supersede it and did not survive. Indeed, Cajal might have come to espouse it, had he been less attached to his own views of *neurotropism* and experimental results. The possibility of symmetry operating in so many other natural forces eschewed Cajal in the question of positive and negative neurotropic forces but not Lugaro. Ironically, the man that so categorically doomed Lugaro's hypothesis one century ago was the giant on whose shoulders Lugaro stood to overtake him. And Cajal's own work is the reference measure by which we should judge the greatness of Lugaro's feat.

DISCUSSION:
METHODOLOGICAL PART (PAPERS I, II, IV)

The features of acute SCI are very different from those of SCI at the chronic stage (Tator and Fehlings, 1991; Schwab and Bartholdi, 1996; Guth *et al.*, 1999; Liu *et al.*, 1997). Acute SCI is characterized by a cataclysmic cascade of degenerative events immediately following the primary injury, while regenerative events during this phase are mostly abortive (Tator and Fehlings, 1991; Schwab and Bartholdi, 1996). After the acute injury, the spinal cord enters a subacute phase extending up to a month after the first week of spinal shock (Schwab and Bartholdi, 1996). The secondary degeneration, whose duration and extent are related to the severity of injury, is generally thought to persist for many months after the primary insult on the cord. While postinjury degeneration subsides with time, regeneration, neosynaptogenesis and other plasticity events continue to be at play and can be enhanced by rehabilitative treatment (Fouad *et al.*, 2001; Mikulis *et al.*, 2002; Schwab, 2002; Raineteau *et al.*, 2002). However, even spontaneous regeneration and plastic capacity reach a peak, so after a variable amount of time, between 6 months and 2 years, no further functional improvement is to be expected due to axonal regeneration, and that is what characterizes chronic SCI (Tator, 1998). So the term 'chronic SCI' should refer to the steady state that is achieved after secondary degeneration has ebbed, after spontaneous regeneration and compensatory sprouting has peaked and when functional deficit can safely be characterized as irreversible. By convention, a human lesion is considered to become chronic within the first year, although some sensorimotor recovery, but also myelopathy and other long term sequelæ such as chronic pain may occur beyond that timepoint (Schwartz *et al.*, 1999; Raineteau *et al.*, 2002).

We chose to work with the complete transection model, which ensures total disruption of cord continuity and a definitive state of paraplegia (Cheng *et al.*, 1996; Fraidakis *et al.*, 1998, 2004). These were *sine qua non* perequisites for our repair studies of animals with chronic SCI. The aim of using MRI was to visualize the extent and variability of the cord and spine lesions of chronically paraplegic animals (Fraidakis *et al.*, 1998, 2004). This strategy would also reflect better the clinical reality of future surgical repair of chronic SCI, where thorough preoperative MRI evaluation would be of essence to characterize the particularities of the lesion in each individual patient and help determine the optimal surgical action or preclude invasive therapy. In the complete transection SCI model in the rat, the mechanical injury is penetrating. The spinal cord continuity is interrupted, and the two stumps eventually retract, creating a 1-3 mm long gap (Fraidakis *et al.*, 1998; Cheng *et al.*, 1995; Cheng and Olson, 1995). The subarachnoid space is exposed, the blood-CSF barrier is breached, and bleeding from the ventral and dorsal spinal arteries is profuse. The cut inflicts a transient compression on the cord, which may result in œdematous and softened stumps with petechial hæmorrhages appearing on the adjacent gray matter surface within 15 minutes. However, there is instant axotomy of all ascending and descending nerve fibers, which is one major difference from contusion models. Another important difference is the nature of the local scar. Contusion leads to central cystic degeneration and the demarcation of the lesion zone by 'glia limitans', a lining formed

mainly by reactive astrocytes. On the other hand, after transection SCI, the gap between the stumps is filled with fibrous collagenous scar rich in mesenchymal tissue elements apart from the glial scar (Reier and Houlé, 1988; Schwab and Bartholdi, 1996). As transection lesions become chronic, centrifugally progressive necrosis and apoptosis ensues (Schwab and Bartholdi, 1996; Zhang *et al.*, 1997; Liu *et al.*, 1997; Casha *et al.*, 2001). After a few months the lesion can be several vertebral segments long, containing dense accumulations of collagen, large ependyma-lined cysts and cavities enveloped by astrocytic process (Schwab and Bartholdi, 1996; Fraidakis *et al.*, 1998; Guizar-Sahagún *et al.*, 1994a, 1994b). Indeed, sometimes the initial gap is replaced by a fluid-filled pulsating cisterna, which when incised yields clear fluid under pressure, similarly to when the suburachnoid space is penetrated. Histologically, some of these central cysts are multilocular, divided by septa (Fraidakis *et al.*, 1998; Guizar-Sahagún *et al.*, 1994). Moreover, in our experiments, after 4-6 months, the distal stump seemed to be thinner than the rostral, probably because of the frequently rostral expansion of the cyst and distal long-tract degeneration.

In our experimental setting, where the SCI was standardized and the animals were regularly monitored with locomotor tests, we have defined the chronic stage based on the single criterion of functional handicap. In rats, behavioral deficit after complete spinal cord transection stabilizes within the first two postinjury months and we have chosen that as the cut-off timepoint between subacute and chronic phases. Nonetheless, we are aware of the fact that axonal regeneration and neuronal circuit plasticity may occur beyond this arbitrary timepoint in the cord as well as in the brain and we didn't assume the cord's regenerative potential to be static theretofore, even despite the absence of behavioral improvement (Fouad *et al.*, 2001; Mikulis *et al.*, 2002; Schwab, 2002; Raineteau *et al.*, 2002). We therefore subcategorized our experimental chronic SCI into three degrees of chronicity, early, medium and late, commencing at postinjury time points of 2 months, 4 months and 8 months respectively.

The repair surgery in the acute SCI group was performed as previously (Cheng *et al.*, 1996; Fraidakis *et al.*, 2004) with the minor modification of inserting the tips of the peripheral nerve grafts into the rostral and caudal gray matter instead of simply apposing them and letting them imbibe onto the stump surface. The aims were to better stabilize the grafts, minimize the risk of future detachment and minimize glial proliferation at the cord-nerve interface. The repair procedure in the chronic animals was more challenging and debridement entailed an acute-on-chronic cord lesion. An acute-on-chronic lesion was therefore made on the chronically injured spinal cord before implanting the grafts. The tips of the peripheral nerve grafts were again slightly inserted in the caudal and rostral gray. An acute-on-chronic lesion differs from acute or chronic lesions (Houlé, 1991; Houlé and Ye, 1997, 1999; Olson, 1997, 2002; Ye and Houlé, 1997; Houlé and Yin, 2001; Kwon *et al.*, 2002a, 2002b). Although debridement of the chronic lesion and further trimming of the stumps as described in the methods lead to an enlarged gap between the acute-on-chronic stumps, this might actually impart a rostral and caudal advantage for the outcome of the procedure. Axotomy of adult spinal and supraspinal neurons ultimately leads to axonal retraction after an initial phase of abortive sprouting

(Blight and Decrescito, 1986; Pallini *et al.*, 1988; Schwab and Bartholdi, 1996; Houlé, 1991; Houlé and Ye, 1997, 1999; Olson, 1997, 2002; Ye and Houlé, 1997; Houlé and Yin, 2001). Although it has been shown that CST undergoes progressive retrograde degeneration for up to 8 weeks after spinal cord transaction, it was also shown that the extent of axonal die-back of three major supraspinal tracts (rubrospinal, vestibulospinal, reticulospinal) does not significantly change between 1 week and 14 weeks postinjury and that these chronically injured supraspinal axons could regrow into peripheral nerve grafts, and that axonal survival and regeneration were enhanced by pretreatment with neurotrophic factors (Tator *et al.*, 1984; Pallini *et al.*, 1988; Houlé, 1991; Houlé and Ye, 1997, 1999; Olson, 1997, 2002; Ye and Houlé, 1997; Houlé and Yin, 2001; Kwon *et al.*, 2002a, 2002b). Therefore, we hypothesized that reacutization of the chronic lesion by *en bloc* removing the glial scar occupying the lesion site, would bring the tips of the nerve grafts in contact with retracted descending axons above injury as well as in contact with retracted ascending axons below injury, and therefore facilitate ingrowth of spinal cord axons into the permissive peripheral nerve environment. To that end, nerve graft tip insertion into both stumps was performed in order to achieve better approximation, between the nerve graft tips and the retracted axon front. We also speculated that another advantage of the caudal extension of the acute-on chronic lesion would be to bring the regenerating supraspinal fibers via the nerve conduits closer to the central pattern generator upon their exit from the grafts and entry into the caudal gray matter, thus increasing their chance for successful arborization, synapse formation and stabilization (Grillner and Dubuc, 1988; Rossignol and Dubuc, 1994; Dimitrijevic *et al.*, 1998; Pinter and Dimitrijevic, 1999).

Axonal retraction as well as neuronal atrophy can be countered by local application of neurotrophic factors, with resultant enhanced neuronal survival, axonal sprouting and regeneration (Xu *et al.*, 1995a, 1995b; Sayer *et al.*, 2002b; Blesch *et al.*, 2002). The administered acidic FGF and the neurotrophins secreted by the Schwann cells of the peripheral nerve grafts can diffuse up to at least 1mm within the stump and stimulate axonal regrowth towards the CNS-PNS interface. Acidic FGF is expressed in spinal motor and sensory neurons, is released after CNS injury and exhibits neurotrophic activity in vitro (Nietro-Sampedro *et al.*, 1988; Walicke *et al.*, 1988; Bean *et al.*, 1991; Elde *et al.*, 1991; Koshinaga *et al.*, 1993; Mocchetti and Wrathall, 1995; Grothe and Wewetzer, 1996; Reuss and von Bohlen und Halbach, 2003). Schwann cells are known to secrete the neurotrophins BDNF and NGF upon injury, to express cell adhesion molecules such as L1 and N-cadherin, and produce ECM components such as laminin, all of which promote axonal regeneration (Millaruelo. *et al.*, 1988; Xu *et al.*, 1995a, 1995b, 1997; Ebadi *et al.*, 1997; Joosten, 1997; Raisman, 1997; Li *et al.*, 1999; Brook *et al.*, 2001, Nietro-Sampedro, 2002). All the repaired chronic animals received the acidic FGF containing regimen. However, in half of the acutely repaired animals acidic FGF was omitted from the treatment protocol without a resultant statistically significant difference in functional recovery compared to the group that received the growth factor (unpublished data). That might suggest that the presence of acidic FGF is not necessary for successful outcome of the procedure or that the neurotrophic factors secreted by the peripheral nerve segments are sufficient to entice an adequate minimum of axonal

regrowth to trigger the central pattern generator. The possibility remains though that acidic FGF may enhance axonal regeneration without further increase in behavioral recovery once the threshhold of excitatory input to the central pattern generator has been reached (Grillner and Dubuc, 1988; Rossignol and Dubuc, 1994; Dimitrijevic *et al.*, 1998; Pinter and Dimitrijevic, 1999). Detailed histological analysis of a larger number of animals in a simpler experimental model of spinal cord injury and/or repair would be the best way to evaluate the effects of acidic FGF on overall neuronal survival, axonal regeneration and glial reaction (Krassioukov *et al.*, 2002).

Repaired animals recovered only partially. However, all repaired animals, from the acute and chronic SCI groups, demonstrated a degree of functional recovery, while this was not the case for the controls. There was no significant difference between the locomotor performance scores of the acute and chronic repair groups. In the acute group the functional result of the immediate repair procedure as a rule became manifest during the 2^{nd} month postrepair. In the chronic group the treatment at 2, 4 or 8 month postinjury intervals resulted in partial reversal of total peraplegia. The recovery in the chronic group was observed during the 2^{nd} month postrepair. The 2 month period would be a realistic timeframe for regenerating axons to traverse a distance of about 5-10 mm in the acute and 10-20 mm in the chronic animals and establish functional synaptic connectivity with interneurons or motor neurons. The nonconservative estimates of the retraction zone-to-target gray distance for each group, were obtained by adding an extra 5mm to the lesion size to account for axonal retraction and tortuous axonal elongation due to further stump degeneration or cyst formation. Regenerating axons can reach an average growth rate of 1mm/day, according to some authors even in CNS white matter (Davies *et al.*, 1999). The fact that the first signs of behavioral recovery were seen in both acute and chronic repair animals at 2 months postrepair is not counterintuitive. In the acute repair animals, the nerve bridges were shorter but the supraspinal fibers would enter the caudal stump at a higher spinal level necessitating extensive interneuronal networking and/or longer axon growth. In the chronic repair animals, the nerve bridges were longer but supraspinal projections would enter the distal gray closer to the lumbar enlargement and the CPG, with a better chance for fast target finding.

The best scores achieved in each test in the acute repair group occured in the time window of 3-5 months postrepair while the best scores in the ‘chronic’ repair group clustered in the time window of 4-6 months postrepair. There was no significant variability between the best scores attained by the two repair groups, in other words both acute and chronic treatment resulted in the same peak performance, but in the chronic repair group this maximum recovery occured with an average delay of a month. This delay may be insignificant and attributed to normal variability and the small number of animals per group. Alternatively, it could be related to the long period of paralysis and could be partly explained by axonal changes, a slower rate of functional synaptogenesis, decreased CPG excitability to supraspinal input and prolonged muscle *disuse atrophy* in the aged animals (Nashmi *et al.*, 2000; Nashmi and Fehlings, 2001). Advanced age compounded by a long period of paralysis would certainly contribute to a delayed and slower recovery. Yet apart from this delay in peak performance compared to the acute

repair group, the recovery obtained in the chronic group was robust and consistent. Remarkably, in one third of the chronic repair animals that were allowed to be followed up until 16 months postrepair (at a time when the rats were 2 years-old) functional benefit persisted undiminished and postmortem evaluations showed rich NF immunoreactivity within the nerve bridges and 5-HT immunoreactivity in the distal stump.

Peripheral nerve graft viability with NF immunofluorescence was evident and serotoninergic fibers with their characteristic beaded appearance were present in the caudal stump of all histologically evaluated acute and chronic animals. Often the repair area was distorted by fluid filled cavities situated between or peripherally to the nerve bridges and seemed to compress/displace the bridges along the transverse plane. 5-HT fibers were also seen inside the grafts. Caudally, 5-HT fibers were seen within gray matter but not in white matter. The presence of 5-HT fibers in the repair site and in the caudal stump is a direct indication of ingrowth of regenerating fibers from above the lesion, since axotomy leads to Wallerian-type degeneration and disappearance of all 5-HT immunofluorescence below the cordotomy level. The 5-HT fibers in the spinal cord are of supraspinal origin. The thin non-myelinated 5-HT fibers have previously been shown to regenerate effectively in otherwise intact but chemically 5-HT-denervated spinal cord tissue (Nygren *et al.*, 1974). Furthermore, the role of serotonin in modulating and stimulating the CPG is well-recognized (Ribotta *et al.*, 1997, 2000; Barbeau and Rossignol, 1990; Barbeau and Rossignol, 1991; Rossignol and Barbeau, 1995; Barbeau and Rossignol, 1994; Feraboli-Lohnherr *et al.*, 1997; Hultborn *et al.*, 1998; Rossignol *et al.*, 1998; Barbeau *et al.*, 1999; Kim *et al.*, 1999; Rossignol *et al.*, 2001).

Another piece of evidence linking the observed recovery with regeneration of supraspinal axons is the immediate disappearance of hindlimb motility after proximal retransection of the spinal cord in one repaired rat. Had the observed functional improvement been irrelevant to descending pathways and was solely the result of stimulation of the spinal pattern generator by some other process at or below the repair site, e.g. inflammation or scarring, retransection of the proximal stump without disruption of the repair site or the distal stump would logically lack effect on behavior. That of course would not rule out axonal regeneration into the distal stump but it would preclude supraspinal involvement in the behavioral recovery. However, if proximal relesioning led anew to loss of function, this would link the supraspinal pathways to motor performance and would imply that somewhere between the retransection point and the distal stump in general, or the CPG in particular, functional synapsing had occured. Furthermore, this functional connectivity could only take place at the distal stump, since the nerve bridges, unlike CNS tissue grafts, cannot act as 'relay stations' for synaptic events to occur. The rythmic hindlimb stepping pattern of the repaired animals, made more visible by the Bipedal test, allude to a CPG participation in the generation of the behavioral recovery (Grillner and Dubuc, 1988; Rossignol and Dubuc, 1994; Dimitrijevic *et al.*, 1998; Pinter and Dimitrijevic, 1999; Privat *et al.*, 2000). Finally, the serendipitous finding that in treated rats head scratching could trigger hindlimb and tail movements can only be explained by the existence of rostrocaudal communication across the repair site. Taken together, the above anatomical and behavioral data support a scenario of long tract

regeneration into the caudal stump with neosynaptogenesis and segmental plasticity leading to CPG activation and increased locomotor capacity for the repaired rats. This scenario in turn implies a causal relation between regeneration and recovery.

Various descending and ascending pathways are implicated in the behavioral recovery seen in the present study (Schucht *et al.*, 2002). The CST is known to be rather resistant to regeneration after lesioning in the postnatal and adult stage (Joosten, 1997). In the adult rat when the CST is severed below its decussation it survives axotomy but undergoes a degree of die-back (Schwab and Bartholdi, 1996). Homotypic or heterotypic grafting of embryonic tissue does not promote CST regrowth (Houlé and Reier, 1988; Reier *et al.*, 1986; Bregman *et al.*, 2002; Jakeman and Reier, 1991). Earlier peripheral nerve grafting experiments in the spinal cord have failed to induce CST elongation into the implant (Richardson *et al.*, 1982; Richardson, 1984). Other groups were since able to demonstrate short- and long-distance CST regeneration in the adult rat using various lesion models and treatment approaches. CST regrowth was shown to be induced by intraspinal grafting of purified Schwann cells and olfactory ensheathing glia (Li *et al.*, 1994, 1997a, 1997b, 1998) and long-distance CST regeneration was obtained after neutralization of the myelin-associated inhibitor Nogo-A by the monoclonal antibody IN-1 or its recombinant humanized Fab fragment (Schnell *et al.*, 1993; Brösamle *et al.*, 2000). Neurotrophin-3 has also been shown to have a potent effect on CST sprouting and regeneration (Schnell *et al.*, 1994). In those studies CST regrowth was seen in gray or white matter. Our lab has previously reported CST regrowth when a surgical repair model was applied in the acute spinal cord injury adult rat model (Cheng et al 1996).

In our study the contribution of the corticospinal tract to the observed recovery was not evaluated directly but the behavioral data offer indirect evidence that the higher motor neuron has a role in the functional improvement. The contact placing reflex is believed to be an indicator of pyramidal tract integrity (Donatelle *et al.*, 1977). A functional contact placing reflex also requires an intact afferent input/loop, therefore recovery of the contact placing response is tantamount to regeneration of ascending sensory fibers. All treated rats demonstrated a partial return of the contact placing reflex (and in the case of one chronic animal treated 4 months postinjury this recovery was remarkably salient), while this was not true for any of the control animals. The postural reflexes, weight bearing capacity, rythmic stepping and interlimb coordination are known to depend heavily on the vestibulospinal, reticulospinal and propriospinal tracts, therefore the partial recovery of the aforementioned behavioral parameters in the treated animals would reflect the regeneration of fibers from some or all the relevant pathways. The Bipedal test proved helpful since it revealed hindlimb motility by relieving the tested animal's hindquarters from an overbearing body weight and the hard task of keeping its balance in a quadrupedal position. The results of this test are not to be confused with those obtained in other studies by training spinalized animals on a treadmill, since the tested animals were stepping on a firm flat surface and none of the animals had been trained on a treadmill. Moreover, the results of the bipedal test cannot be explained by mere stimulation of the CPG from intact sensory pathways, since the control animals failed even plantar paw placement and almost never scored above zero.

In conclusion, our results suggest that longstanding complete paraplegia in rats with acute or even chronic SCI can be partially reversed with a significant restoration of sensorimotor abilities. Indeed, our results have been confirmed by several labs that applied our 'microsurgical repair protocol' in adult rat models of acute complete transaction SCI, including the use of acidic FGF in fibrin glue (Lee *et al.*, 2002, 2004, 2007, 2010; Tsai *et al.*, 2005). Apart from CST regeneration by anterograde tracing with BDA, these studies also demonstrated both functional and electrophysiological improvement (evaluated by SSEP and MEP) that were all abolished after retransection of the nerve grafts (Lee *et al.*, 2002, 2004, 2007, 2010; Tsai *et al.*, 2005). The beneficial actions of acidic FGF embedded in fibrin glue on regeneration of CNS axons were further supported by peripheral nerve graft implantation studies in a rat model of cervical root transection as well as by proteomic analysis (Lee *et al.*, 2004; Huang *et al.*, 2007; Tsai *et al.*, 2008). Finally, it has been shown in several SCI models in different species (mostly incomplete, acute or chronic, mouse or rat or cat) that peripheral nerve grafting could be combined with the use of chondroitinase for a synergistic effect (Houlé *et al.*, 2006, 2009; Tom and Houlé, 2008; Tom *et al.*, 2009; Chi, 2010; Côté *et al.*, 2010; Ma *et al.*, 2010). Nevertheless, the replication of our complete repair protocol in adult rat models of chronic complete SCI has not yet been attempted.

It is unlikely that any single treatment strategy will lead to a cure. It appears increasingly likely, however, that combinations of several treatment approaches may finally lead to a repair protocol that will improve the prognosis for SCI patients. With a steady stream of important breakthroughs since the early 90's it is encouraging to know that the heyday of SCI research is yet to come, and that the SCI victims' hopes are now scientifically justifiable (Olson, 1997,2002 ; Fry, 2001; Schwab, 2002b; Behar *et al.*, 2000; Raisman, 2000, 2001; Blight and Zimber, 2001; Blesch *et al.*, 2002; Blight, 2002; Batchelor and Howells, 2003; Ferraro *et al.*, 2004; Baptiste and Fehlings, 2006; Moreno-Flores and Avila 2006; Kubo and Yamashita, 2007; Bradley, 2008; Cafferty *et al.*, 2008).

DISCUSSION:
NEUROIMMUNOLOGICAL PART (PAPERS III, V)

In *Paper III*, a facial nerve transection KO mouse model was employed to evaluate the role played by the innate and adaptive immune systems in the inflammatory process and glial reactions at the nerve cell body level in the CNS after peripheral nerve axotomy. The model of facial nerve transection has been established as the ideal PNS injury model for the study of the nerve cell body reaction to axotomy and is much preferable to the ventral root avulsion model due to its facility and standardization, so we opted for it (Kreutzberg *et al.*, 1990; Raivich *et al.*, 2004, 2006).

The retrograde reaction after peripheral nerve injury involves many structural and metabolic changes at all levels of the injured neuron, i.e. cell soma, lesioned axon and dendrites as well as the associated glial elements. Some of the neuronal and perineuronal changes after axotomy resemble those seen in autoimmune processes, e.g. the robust upregulation of MHC molecules on neuron and glia, the activation of surrounding glia, especially microglia with production of cytokines and cytokine receptors, involvement of the BBB and local recruitement of immune system cells, especially macrophages and T lymphocytes. The features of this glial/inflammatory response are not stereotypical but subject to genetic polymorphism and have been shown to covariate with susceptibility to EAE. The immunogenetic background of EAE is complex and EAE susceptibility is known to be strain-specific, with some mouse strains being more resistant than others.

The molecular signals leading to glial activation, MHC upregulation and cytokine expression after facial nerve injury are currently not fully known but IFNγ is believed to play an important role in the process. However, *in vivo* data on the role of IFNγ in the retrograde response after axotomy of the facial nerve were lacking. In order to study the role of IFNγ in the above injury model *in vivo*, we employed IHC and ISH to analyze the expression of MHC class I, β2-microglobulin and GFAP in the facial nerve nucleus three weeks after axotomy in IFNγ, IFNγR and IRF-1 KO mice.

Several studies have also shown the participation of T cells in the inflammatory response of the facial nerve nucleus after axotomy in the mouse. Most of these T cells are $CD4^+$ T cells or else known as T helper cells, of which there exists the two major subsets, T_H1 and T_H2, with proinflammatory and antiinflammatory actions, respectively. Indeed, T cells may influence the outcome of facial nerve lesion since it has beeen shown that survival of axotomized motor neurons of the facial nucleus depends on the presence of anti-inflammatory $CD4^+$ T cells, i.e. of the T_H2 subtype (Serpe *et al.*, 2003). In order to refine our understanding of the specific role of each of the T_H subclasses in the inflammatory and glial response in the facial nerve transection mouse model we studied the expression of MHC class I, β2-microglobulin and GFAP in the facial nerve nucleus three weeks after axotomy in STAT4 and STAT6 KO mice, deficient in the T_H1 and T_H2 responses respectively.

Immunohistochemistry for MHC class I in all genotypes of C57BL/6J and BALB/c mice showed only weak staining in the unlesioned side, whereas increased labeling was present throughout the facial nucleus on the side of the facial nerve transection. Thus, no intrastrain difference was evident in MHC class I immunolabeling in BALB/c and C57BL/6J mice. There was no intrastrain difference in MHC class I immunolabeling in WT 129/SvJ and its IFN-$\gamma R^{-/-}$ KO but contrariwise to the other strains (BALB/c and C57BL/6J) they showed very weak axotomy-induced up-regulation of MHC class I antigens. A similar pattern could be seen in GFAP immunolabeling, with increased immunopositivity in the facial nucleus of the lesioned side in all BALBc and C57BL/6J genotypes but low immunopositivity in the 129/SvJ genotypes. Therefore, an interstrain, but not intrastrain, difference was seen by IHC in MHC class I and GFAP upregulation in the facial nucleus postaxotomy between BALB/c and C57BL/6J (intense MHC class I immunopositivity) and 129/SvJ (weak MHC class I immunopositivity).

In situ hybridization showed highly increased GAP-43 expression in axotomized motoneurons 3 weeks postaxotomy without interstrain or intrastrain difference in GAP-43 expression. On the contrary, an interstrain, but not intrastrain, difference was seen in β2-m expression in both neurons and glial cells in the lesioned side 3 weeks postaxotomy, with the BALB/c and C57BL/6J genotypes exhibiting strong induction of β2-m mRNA while the 129/SvJ exhibited very weak induction. Grain density, as measured by computer-based analysis, was increased 25-35 times in animals on C57BL/6J background (axotomy vs nonaxotomy side) while only 5-7 times in animals on 129/SvJ background. The unlesioned facial nucleus showed very weak β2-m expression in all animals irrespective of genotype and background. Similarly, GFAP mRNA expression was upregulated in astrocytes in the axotomized facial nucleus in animals of BALB/c and C57BL/6J backround without an intrastrain difference while the 129/SvJ strains showed a much weaker response. Grain density measurements revealed an increase of 12-16 times (axotomized versus nonaxotomized facial n. nucleus) in C57BL/6J animals compared with 3-5 times in 129/SvJ animals. Therefore, an interstrain, but not intrastrain, difference was seen by ISH in β2-m and GFAP upregulation in the facial nucleus postaxotomy between BALB/c and C57BL/6J (strong induction by axotomy) and 129/SvJ (weak induction postaxotomy).

In conclusion, the results of *Paper III* do not support a non-redundant role for IFNγ, IFNγR or IRF-1, STAT4 or STAT6 in the expression of MHC class I, β2-m or GFAP in the facial nucleus after facial nerve axotomy. However, the 129/SvJ mice displayed weaker glial postaxotomy responses compared to the other strains. This testifies to a genetic polymorphism in the mouse governing glial and neuroinflammatory responses after this particular model of PNS axotomy in the mouse.

In *Paper V*, a dorsal hemisection lesion model was employed according to the experimental paradigm by Huang *et al.*, (Huang *et al.*, 1999). The objective was to interrupt descending long-distance tracts, including the CST, which in mice runs laterally to the dorsal horns as in humans, without creating a gap between the two stumps that

would render axonal regeneration almost impossible. A double-labeling protocol was then used with FG application immediately after injury to be taken up by all injured axons and label their somata in the cortex and below cortex followed by FR injections 3 weeks later, caudally to the hemisection site at a safe distance to preclude rostral diffusion and false labeling of injured axons at the injury site that had not regenerated into the distal stump. Interestingly, statistical evaluation of the microscopical data revealed a significant difference in axonal regeneration (as measured by the whole brain ratio of double-labeled/FluroGold labeled neurons) between the STAT6 KO (T_H2 deficient) and their wild type controls and between the wild types of the two strains.

These differences were not accompanied by intrastrain differences in improvement of locomotor capacity over time on the BBB scale. However, a statistically significant interstrain difference was found in the level of behavioral recovery over time and peak scores. This interstrain difference in the degree of locomotor recovery was reminiscent of the difference in neuroinflammatory parameters such as β2-m, MHC-I and GFAP upregulation between the mice strains BALB/c, C57BL/6 and 129/SvJ demonstrated in *Paper III* (Lidman *et al.*, 2002). Those results hinted at a correlation between the degree of neuroinflammatory response after facial nerve lesion and strain susceptibility to autoimmune disease (Olsson *et al.*, 2001; Lidman *et al.*, 2002). The genetics of neuroinflammation is rather complicated with polymorphisms in many loci being involved in the outcome of the few hallmark correlates of neuroinflammation chosen for study (Olsson *et al.*, 2000; Lidman *et al.*, 2003). A straightforward result that comes across though, is that genetic background is determinant of the type and degree of inflammatory changes after traumatic injury in the nervous system as well as of autoimmune susceptibility (Piehl and Lidman, 2001).

The realization of the importance of the genetic background is even more stark as regards the constitutive locomotor phenotype of different mouse strains. Interstrain differences may be preserved after nervous system injury, where strains with different habitual locomotion gradually recover their abilities up to different levels of capacity. It seems though that even strains with no visible or quantifiable difference in constitutive locomotor capacity can exhibit a postinjury difference in behavioral recovery. In that case, the postinjury difference cannot readily be attributed to different natural ability but rather to an interstrain variation in tissue response to injury, in (any or some or all of) local inflammation, extent of tissue degeneration and regeneration or other factors that determine the outcome.

The histological data were supportive of our hypothesis that a deficient T_H2 response might have a negative effect on axonal regeneration after CNS injury. A statistically significant difference in neuronal double-labeling was found between STAT6 KO and wild type but not between STAT4 and wild type or between STAT4 and STAT6. The biological significance of these results warrants further investigation. Nevertheless, interesting parallelisms exist with the findings of other studies. $STAT6^{-/-}$ mice of the same genetic background as those we used were more susceptible to EAE compared to $STAT4^{-/-}$ or wild type mice (Chitnis *et al.*, 2001). Also BALB/c $STAT6^{-/-}$ were more susceptible

to experimental myasthenia gravis (EMG) than either wild type or STAT4 KOs, though they all express the same H-2^d alleles (the resistance of the BALB/c to EMG has been attributed partly to their H-2^d haplotype) (Wang *et al.*, 2004; Œstlie *et al.*, 2003). BALB/c mice have a constitutive propensity for strong Th2 responses and weak T_H1 responses (Wang *et al.*, 2004). The T_H1 response is believed to be the culprit in autoimmune disease protocols (as in autoimmune disorders in general), so the STAT6 null mice lack the T_H2 response but not the T_H1 response, which explains their susceptibility to EAE or EMG, and implies a protective role for the T_H2 arm against autoimmune conditions. Exactly how this protection is effected by the T_H2 cytokines is not known, but their immunomodulatory and antiinflammatory actions are certainly implicated. Direct involvement of the Jak/STAT second messenger molecules in activating cytoprotective pathways may be another mechanism (Qiu *et al.*, 2005).

In our model of SCI the STAT$6^{-/-}$ (BALB/c) mice and TNF$\alpha^{+/+}$, i.e. wild type mice, (C57BL/6J) exhibited significantly less axonal regeneration than the wild type BALB/c. Thus, lack of the T_H2 response resulted in diminished axonal regeneration after mechanical CNS injury and this could be explained by the lack of the T_H2 anti-inflammatory cytokine profile with resultant enhanced demise of oligodendrocytes and even neurons. Moreover, the C57BL/6J wild type mice, that are prone to develop a T_H1 response and are susceptible to EMG (while the T_H2 prone BALB/c are EMG-resistant), exhibited also a significantly lower axonal regeneration compared to wild type BALB/c after SCI. Therefore, we arrive at the same result in two different ways. Both T_H2 deficiency and T_H1 excess seem to hamper axonal regeneration after an axotomy cord injury, which strengthens the hypothesis that the T_H1/T_H2 balance affects the course of purely raumatic, i.e. originally non-autoimmune, CNS injury.

There was a statistically non-significant tendency for a difference in axonal regeneration between the TNFα+/+ and the TNFα-/- mice, with wild type mice mice showing less regeneration. Although this difference was not large enough to be significant, it is rather consistent with previous results in experiments with TNFα KO mice. In the original description of the KO, TNFα null mice seemed to lack the acute proinflammatory actions known to be mediated by TNFα but also lacked important delayed immunoregulatory, anti-inflammatory actions that are attributed to TNFα (Marino *et al.*, 1997). In another study, TNFα deficient mice exhibited a complex biphasic phenotype in a TBI model, whereby degeneration was diminished in the acute stages after injury while permanent functional handicap was increased (Scherbel *et al.*, 1999). By analogy, in *Paper V*, the seemingly better axonal regeneration in the null genotype may be a short-term benefit since there was no significant difference in locomotor recovery between the genotypes. Indeed, TNFα has a multitude of biological actions, ostensibly antithetic, e.g. proapoptotic and antiapoptotic, trophic and toxic and as it seems even proinflammatory and anti-inflammatory or immunoregulatory, that are certainly dependent on context.

In conclusion, it is known that T cells (both $CD4^+$ and $CD8^+$) are recruited in the CNS after facial nerve axotomy and SCI and that neurons and glial cells de novo synthesize MHC molecules to enhance their capacity to present antigens or be victimized by

cytotoxicity (Bradl *et al.*, 2005; Oliveira *et al.*, 2004; Bohatschek *et al.*, 2004; Raivich *et al.*, 2003; Lidman *et al.*, 2003; Newman *et al.*, 2001; Piehl *et al.*, 1999; Raivich *et al.*, 1998; Streit *et al.*, 1998; Werner *et al.*, 1998; Perry *et al.*, 1998; Popovich *et al.*, 1993, 1996, 2001). Microglia and perivascular macrophages in the CNS are especially capable as APC to T cells, which in turn can induce microglial production of TNFα, a proinflammatory and myelinotoxic cytokine (Raivich *et al.*, 2004; Shaked *et al.*, 2004; Popovich *et al.*, 2002; Aloisi *et al.*, 1998; Chabot *et al.*, 1997). T cells reactive to myelin basic protein (MBP) are known to appear after SCI in rats, mice and humans, and have been shown to accumulate at the injury site after CNS injury (Kil *et al.*, 1999; Popovich *et al.*, 1996, 2002). Moreover, these cells are able to induce EAE in naive animals. It has finally been shown that purely traumatic SCI can lead to a detrimental autoimmune T cell response mediated by endogenous MBP-reactive T cells in MBP TCR Tg mice (transgenic mice for the MBP T cell receptor in which the majority of the $CD4^+$ lymphocyte repertoire is reactive with the immunodominant epitope of MBP) (Jones *et al.*, 2002). Therefore, intricate T cell mediated responses of the autoimmune kind, which include the participation of $CD4^+$ T helper cells, naive or neuroantigen-reactive, are likely to take place in the CNS, even after a purely traumatic SCI such as the one employed in our model (Lovett-Racke *et al.*, 2000).

These facts support a biological role for a T_H1/T_H2 balance in traumatic SCI (Bethea and Dietrich, 2002; Serpe *et al.*, 2003; Dietrich *et al.*, 2004; Pannu *et al.*, 2005; Cheng *et al.*, 2005). Indeed, our own results indicate a beneficial role for the T_H2 response in axonal survival and regeneration after SCI (Fraidakis *et al.*, 2007). Other groups have recently produced results that support this contention (Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010; Hendrix and Nitsch, 2007; Xin *et al.*, 2008). It was found therefore that adaptive immunity is biased toward the T_H1 proinflammatory phenotype after experimental SCI, which is in agreement with our hypothesis and obtained results (Ankeny *et al*, 2006, 2009; Ankeny and Popovich, 2007, 2009, 2010; Jones *et al.*, 2002, 2004). It has also been shown that survival of axotomized lower motor neurons in a model of facial nerve axotomy depends on the presence of an antiinflammatory T_H2 cells (Vitkovic *et al.*, 2001; Serpe *et al.*, 2003). Other researchers have suggested that a preferential induction of the T_H2 antiinflammatory response might prove to be neuroprotective after SCI (Hendrix and Nitsch, 2007). In the experimental PNS axotomy model it was found that both proinflammatory (Th1 or Th17) and antiinflammatory (Th2 or T_{REG}) T cells were activated and it was hypothesized that a balanced T subset activation may play a physiological role in neuroprotection (Xin *et al.*, 2008). This is not an unexpected finding since T_{REG} cells are always activated simultaneously with autoreactive T cells, even in experimental models of SCI, probably as a natural defence mechanism against the CNS autoimmune response. It is obvious that immunological (innate, adaptive and autoimmune) mechanisms in traumatic injury of the PNS and CNS should be further explored and clarified before the prospect of immunomodulatory therapies can be judiciously realized.

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